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SELECTIVE NECK DISSECTION FOR N0 NECK IN LARYNGEAL CARCINOMA

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Abstract

Objectives: *To evaluate the pattern of distribution of occult lymph node metastases, and the efficacy of selective neck dissection in N0 laryngeal carcinoma.*

Study design: *A prospective study was performed on 30 patients surgically treated for laryngeal cancer, initially classified as N0, with no detected cervical lymph node, underwent lateral neck dissection removing levels II-IV.*

Results: *Occult metastases were detected in fifteen specimens (38.5%) from eleven patients (36.7%). Involvement per neck level was: 33.3% level IIa, 6.7% IIb, 26.7% III and 3.3% IV. Lymph node involvement was significantly increased in cases of supraglottic/transglottic tumors, advanced tumor stage or presence of cartilage invasion.*

Conclusion: *This study confirmed the adequacy of SND for management of N0 laryngeal carcinoma. Level IIb and IV involvement is rare. Super-selective neck dissection (IIa, III) seems to be indicated in N0 laryngeal squamous cell carcinoma. In case of advanced tumor stage or cartilage invasion, selective neck dissection would be mandatory.*

Keywords: *Laryngeal carcinoma; occult lymph node metastases; selective neck dissection.*

Introduction

Cervical lymph nodes metastasis is one of the most significant prognostic factors in patients with laryngeal squamous cell carcinoma (SCC). Its incidence varies from 25% to 65% in different series.[1] Nodal status has a greater influence on the curability of laryngeal cancer than the status of primary tumor, and failure in the neck has been shown to be the

most likely cause of treatment failure. In treating this disease, decisions about management of the neck must be considered, both for the patient without clinical evidence of neck disease and for the patient with clinically evident cervical metastasis.[2]

Achieving earlier diagnosis of the laryngeal cancer increases the frequency of patients presenting stage N0 lymph node involvement. The conversion rate from N0 to N+ neck without treatment is approximately 25 percent and this would correlate with the incidence of pathological positive nodes in elective neck dissection specimens (around 30 percent).[3] The limitations in the identification of micrometastases and the negative impact of recurrences in the neck are still a challenge.[4]

Optimal management of N0 cases is controversial. Elective neck dissection with the surgical management of the primary tumor, elective neck irradiation or therapeutic neck dissection when the metastasis becomes evident during watchful waiting are the main treatment options of N0 neck.[5,6] The surgeon must decide whether to electively treat the neck or wait for metastases to develop and then treat the patient whenever they occur.[4] Location and stage of the tumor with the average risk of neck metastasis rates gathered from previous studies are the main factors in the selection of management protocol for N0 neck.[7]

Surgical options for management of the N0 neck vary from modified radical neck dissection to selective neck dissection. The lymphatic tumor spread to the neck follows predictable paths[8]; this evidence justified the development of selective neck dissection (SND) for removing only those levels at risk. The SND of levels II-IV is, therefore, considered adequate in patients with cN0 laryngeal cancer.[9] Furthermore, in order to minimize post-operative morbidity, it has recently been proposed by several Authors to carry out a super-selective dissection of levels IIa-III because sub-level IIb and level IV are seldom involved without involvement of sub-level IIa and level III.[10-12]

The present prospective study

includes 30 patients, operated for stage N0 laryngeal squamous cell cancer. This study assessed the incidence of occult lymph node metastasis, and sought to determine the predictive factors and impact on treatment and prognosis.

Patients and Methods

This prospective study was carried out on thirty patients at Otolaryngology head and neck surgery department at Mansoura university hospital, in a period between March 2011 and June 2013.

All patients in this study underwent surgical management for laryngeal squamous cell carcinoma, were determined preoperatively as N0 neck, the clinical N0 neck was defined as no cervical lymph nodes palpated on physical examination and no suspicious lymph nodes detected by imaging both by CT scan on neck and neck ultrasound. Inclusion criteria to define N0 neck, in laryngeal cancer patients in this series, were based upon characteristics of detectable lymph nodes at contrast CT scan, lesser diameter than 10 mm, absence of central necrosis, and absence of contrast enhancement of lymph node capsule.

Exclusion criteria for this study include prior history of head and neck cancer, palpable or radiologically demonstrable neck metastases, proven distant metastases at time of presentation, history of neck dissection, previous head and neck irradiation therapy, and non-squamous cell laryngeal cancer.

Physical examination, routine laboratory tests, chest X-ray, metastatic work up, computed tomography (CT) of the neck and neck ultrasound were carried out in all patients. Direct laryngoscopy with biopsy endoscopically assisted using 30 degree and 70 degree angled endoscopes allowing evaluation of different regions of the larynx (microlaryngoendoscopy) was performed in the preoperative evaluation of all patients. Rigid and flexible fiberoptic endoscopes were also used routinely in the physical examination.

Lateral neck dissection including dissection of levels II, III, IV (II-IV dissection) was performed in

each case either unilateral or bilateral with respect to localization of the primary tumor in the larynx. Bilateral dissection was carried out for all patients with tumors crossing midline or tumors involving midline structures (epiglottis).

Dissection specimens, removed as a single piece, were located and sent for histopathology. All nodes were examined by series of 5mm cross-sections, without immunohistochemical analysis, to determine the presence/absence, number, location of the nodes containing metastatic disease in different levels, and the presence or absence of extracapsular spread.

All patients were assessed in terms of age, gender, primary tumor site, T tumor stage based on criteria of American Joint Committee on Cancer[13], and type of surgery performed for the primary.

Postoperative complications, such as pharyngocutanous fistula, secondary hemorrhage, wound infection, chylous leakage, frozen shoulder syndrome, cervical and brachial plexus injury and phrenic nerve paralysis were evaluated.

Results

All patients in this study were males with ages ranging from 40 to 72 years (median age 55.5 years). The primary tumor was supraglottic in 11 cases, transglottic in 13 cases, and glottic in 6 cases. The T stages of these patients were as following: T2, 2; T3, 20; and T4, 8.The surgical approaches used to treat the primary tumor in this series were total laryngectomy (27 cases), supraglottic horizontal laryngectomy (2 cases) and supracricoid laryngectomy with cricohyoidoepiglottipexy (1 case).

All the thirty patients underwent lateral neck dissection (LND). Unilateral neck dissection was done in 21 patients and bilateral neck dissection in 9 patients removing levels II, III, IV.Thirty-nine specimens were obtained from the thirty patients and were pathologically analyzed.

Occult metastases were detected in fifteen specimens (38.5%) from eleven patients (36.7%). Capsule rupture was found in 2 speci-

mens (5.1%) from 2 patients (6.6%). Metastases were bilateral in 4 patients (44.4%) and otherwise ipsilateral to the tumor in 7 patients (33.3%).

The distribution of occult metastases among levels IIa, IIb, III and IV in the 328 specimens was respectively 33.3% (n= 10), 6.7% (n=2), 26.7% (n=8), 3.3% (n=1).

The distribution of occult metastases according to the location and stage of 1ry tumor are shown in table (1).

Pathological analysis revealed that cartilage involvement was present in 8 patients. From these 8 patients 6 cases showed positive occult metastases (75%) compared to 5 patients (22.7%) in absence of cartilage involvement. (Table 2) .

Statistical research for predictive factors showed that cartilage invasion, extralaryngeal extension and advanced tumor stage correlated significantly with lymph node involvement (P = 0.02, 0.04). No such correlation emerged for the histologic type of the primary tumor (differentiation) (P=0.4).

According to location of the primary tumor, occult metastases rates were significantly higher in supraglottic and transglottic tumors compared to glottic tumors. (P=0.04) (Figure 1).

Complications secondary to dissection were: spinal nerve dysfunction in 2 cases (6.7%) that were managed conservatively. lymphorrhea in 1 case (3.3%) which was ultimately controlled by conservative management.

Three patients who had underwent total laryngectomy experienced pharyngocutanous fistula. Two of those patients were managed conservatively. Only one patient with pharyngocutanous fistula required operative repair after 6 weeks of the operation after failure of conservative treatment. This patient required 2ry repair using pedicled pectoralis major myocutanous flap. Two patients experienced wound infection that underwent conservative treatment in form of antibiotic and frequent dressing.

Table (1): Distribution of occult metastases according to location and stage of 1ry tumor.

	Number	Percentage
T2 supraglottic	2	6.7%
T3 supraglottic	1	3.3%
T4 supraglottic	2	6.7%
T3 transglottic	2	6.7%
T4 transglottic	4	13.3%

Table (2): Correlation between the presence of cartilage invasion and incidence of occult metastases

Cartilage invasion	Number	Percentage	P value
Yes	5/22	22.7%	0.02
No	6/8	75%	

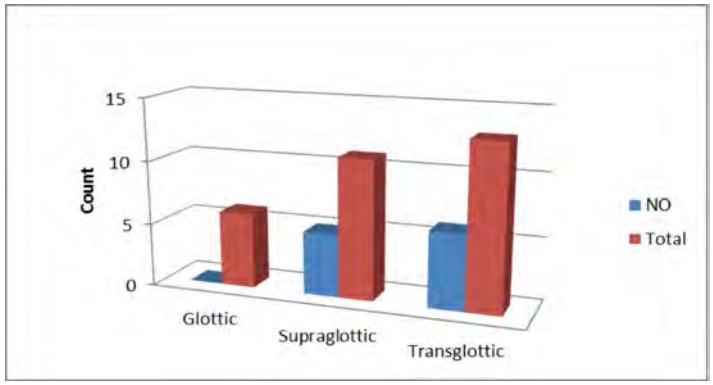

Fig. 1: Correlation between location of 1ry tumor and occult metastases.

Discussion

The management of N0 neck in patients with laryngeal squamous cell carcinoma is still a matter of controversy. The establishment of a "cutoff" point to indicate the elective treatment of the neck, and how comprehensive it must be, is still under debate. In addition, the importance of identifying occult cervical metastases for staging and prognosis, particularly for the indication of adjuvant treatment modalities, is unquestionable. Microscopic evaluation of surgical specimens after ND upstaging N status in 21.4 percent of patients confirmed the limitations for the identification of occult cervical metastases.[14]

In most institutions, a frequency rate of occult metastases exceeding 15% represents the indication for elective neck treatment.[15]

Determination of the method or type of neck dissection used in managing head and neck cancer is heavily influenced by the predictable pattern of the spread of cancer to the neck. Several authors[16-18] have demonstrated that the lymphatic drainage of the larynx occurs along predictable pathways; level II, III, and IV lymph nodes are involved more frequently, and there is an extremely low likelihood of level I or V involving lymph node metastasis. These observations form the rationale for the utilization of LND in cases of laryngeal SCC.

In the present study selective neck dissection for 30 patients with N0 laryngeal carcinoma reveals high incidence of occult metastases (36.7%). This high incidence of occult metastases in our study advocates the necessity of selective neck dissection in such patients with N0 laryngeal carcinoma.

The question arises as to whether there exist any selection criteria for patients in whom levels IIb and IV can be conserved. This requires knowing the real distribution of occult lymph node metastases in the various levels of the neck. Rinaldo et al.[19] and Bolzoni et al.[20], analyzed data from several prospective multicenter studies of N0 laryngeal cancer, reported level IIb involvement in 1.4% of cases, systematically associated with involvement of another level. Paleri et al.[21] concluded from a review of the literature that sublevel IIb was involved in only 0.4% of laryngeal squamous cell carcinomas radiologically and clinically graded as N0. All these studies agreed that level IIb dissection provides no benefit for patients free of palpable cervical adenopathy.

In the present series, IIb involvement was found in 2 cases (26.7%) systematically associated with IIa involvement. Therefore there is compelling evidence that dissecting sublevel IIb may not be indicated in patients with laryngeal cancer and clinically N0 neck. Spinal accessory nerve dysfunction can be minimized and operative time can be saved without adversely affecting the oncological outcomes.

The necessity for routine dissection of level IV has recently been questioned. The arguments for this practice are the low rates of micrometastases found at level IV, ranging from 0 to 3.5 percent, and the potential risk of complications associated with the dissection of level IV and V such as chylous fistula and phrenic nerve injury.[22] Three prospective studies[1,23-24] including 175 patients with laryngeal cancer clinically graded as N0, reported level IV involvement in 6 patients: i.e., 3.4% of cases. Khalif et al.,[25] in a retrospective study of 71 patients, reported a single case of level IV involvement, associated with level II involvement, and concluded that level IV dissection was not always required.

In our study only one patient shows occult metastases in level IV. This patient was staged as T4N0 transglottic carcinoma with subglottic involvement. It was associated with involvement of level II and level III. This study shows a rare incidence of level IV occult lymph node metastases in the treatment of cN0 laryngeal SCC. Therefore, dissection of level IV lymph node pads may be unnecessary for the treatment of laryngeal SCC patients with a clinically N0 neck. In doing so, it is hoped to limit the potential morbidity, such as chylous leakage or phrenic nerve paresis, associated with dissection of the level IV lymph node without compromising the oncological integrity of LND as a therapeutic approach in the management of laryngeal SCC.

Based on the results obtained from our study, only dissection of sublevel IIa and level III would be sufficient for elective surgical treatment of the neck in patients with N0 laryngeal carcinoma.

In the present study, advanced tumor stage and cartilage invasion emerged as significant risk factors for lymph node metastasis, whereas occult metastasis did not depend on histologic type of the primary tumor.

Conclusion

Levels IIa and IV involvement in laryngeal cancer is rare and often associated with involvement of other levels. Super-selective (IIa and III) neck dissection seems indicated, limiting morbidity without

increasing risk of failure. In cases of advanced tumor stage (T3 or T4) or supraglottic and transglottic tumors or cartilage involvement, occult lymph node invasion is more frequent and neck dissection would be mandatory.

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HEPATITIS C VIRUS INFECTION AND NON-HODGKIN'S LYMPHOMA

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Abstract

Background/aims: *This study aimed to investigate the relation between hepatitis C virus (HCV) and B-cell non Hodgkin lymphoma (NHL).*

Methods: *One hundred and seventeen cases of NHL diagnosed in Mansoura oncology center between January 2012 and February 2013 were screened for HCV infection by ELIZA and PCR testing methods. Pathology of lymphomas diagnosed in HCV sero-positive and in sero-negative patients were reviewed according to 2008 WHO classification by examination of H&E stained slides and IHC stained slides for (CD20, CD3, CD15, CD30, CD5, CD10, CD23, TdT, BCL2, BCL6, EMA, and Ki76).*

Results: *HCV sero-positivity was more frequent in B-cell NHL patients (OR, 1.06; 95%CI, 0.6-1.8). HCV sero-positive NHL patients were likely to present as primary extranodal lymphoma (P value, 0.000; OR, 4.2; 95%CI, 1.8-9.7). A highly significant association was observed with MZL (P value, 0.000; OR, 12.7; 95%CI, and 2.8-57.18).*

Conclusions: *These findings suggest that HCV may play a role in the development of B-cell NHL. Further studies are needed to more clearly understand the pathogenesis and management of HCV related NHL.*

Keywords: *Hepatitis C, Association, Non-Hodgkin's Lymphoma.*

Introduction

The number of viruses associated with lymphoma has increased over the last 20 years, and includes epstein barr virus (EBV), human T cell lymphoma virus 1 (HTLV1), human immunodeficiency virus 1 and 2 (HIV1 and 2) and human herpes virus 8 (HHV8). Some cause lymphoma by

direct oncogenesis, for example EBV and Burkitt's lymphoma (BL). Others cause lymphoma in immunosuppressed patients, for example HHV8 in primary effusion lymphoma and Castleman's syndrome[1].

Hepatitis C virus (HCV) is a global health problem and a cause of both hepatic and extrahepatic diseases. B-cell lymphoproliferative disorders (LPDs) represent the most closely related as well as the most investigated forms of extrahepatic disorders related to HCV. A pathogenetic link between HCV virus and some lymphoproliferative disorders was confirmed by their responsiveness to antiviral therapy, which is now considered the first choice treatment[2].

A possible association between HCV infection and NHL was first suggested in 1993. Overall, there was a 2-fold risk of NHL associated with HCV, but there was significant heterogeneity found between studies based on study design and HCV prevalence. Higher risks were found with case-control studies particularly those using hospital controls and in studies in high HCV prevalence areas[3].

HCV-associated lymphoid malignancies may be observed during the course of mixed cryoglobulinemia (MC) or they may be idiopathic forms. About 8%-10% of MC-II evolves into lymphoma, generally after long-lasting infection, as demonstrated also by the advanced age of patients who develop HCV-related lymphoma. In a recent survey, MC patients had a 35 times higher risk of NHL than the general population[4].

A significant association between B-cell derived NHL and HCV infection was initially reported in Italian subjects, and subsequently confirmed by a large majority of international studies[5-8].

However, discordant data appeared in northern European and North American surveys, and it is now evident that a clear south/north gradient of prevalence exists, in part reflecting different HCV infection prevalence in the general population, and suggesting the contribution of environmental and/or genetic factors[9-14].

The presentation of NHL associated with HCV differs from stan-

dard NHL. Lymphomas associated with HCV more commonly present as primary extra-nodal lymphomas, especially liver, spleen and salivary glands. Retrospective studies of patients whose infection date can be accurately determined, suggest the mean latency from acquiring the virus to presentation with lymphoma is 15 years[15].

From a histopathological point of view, although all histological types can virtually be found, B-cell derived NHL is the most common of the HCV-related lymphoid malignancies. The most prevalent forms include follicular lymphoma (FL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), lympho-plasmacytic lymphoma (LPL), and marginal zone lymphoma (MZL). Among the MZLs, a special association with HCV infection was reported for the MALT lymphoma, as well as the splenic forms, as confirmed by some reports indicating that splenic MZL regressed after antiviral therapy, inspite of previous ineffective chemotherapy[16,17].

Persistence of chronic HCV in lymphocytes along with the involvement of genetic and environmental factors have been hypothesized in the pathogenesis of HCV related NHL. Increased frequency of B-cell clonality, t (14: 18) bcl-2 and bcl-6 gene transloca¬tion, STAT3 activation, and upregulation of ERK and p38 MAPK signaling pathways is observed in NHL patients infected with HCV. Possible mechanisms for ma¬lignant transformation include clonal proliferation of B cells, inhibition of apoptotic cell death, or both[18].

Material and Methods

One hundred and seventeen cases which were recently diagnosed as lymphoma patients at Mansoura oncology center in the period between January 2012 and February 2013 were screened for HCV infection by ELIZA and PCR testing methods. Pathology of lymphomas diagnosed in HCV seropositive and in sero-negative patients were reviewed by examination of H&E stained slides and IHC stained slides for (CD20, CD 3, CD15, CD30, CD5, CD10, CD23, BCL2, BCL6, EMA, and Ki76). Classification of diagnosed types of lymphoma were done ac-

cording to 2008 WHO classification of lymphoma.

Statistical Analysis

Statistical analysis of the data was done by using Statistical Package for Social Science (SPSS) version 17.0. Data were analyzed by using simple descriptive statistical analysis (frequency distribution, cross tabulation, chi- square and Fisher's exact test). A P value <0.05 was considered statistically significant. Conditional logistic regression was used to calculate odds ratios (ORs) and corresponding 95% confidence intervals (95%CIs). The change-in-estimate criterion was used to select confounders with a 5% relative change in the OR considered important. The possible confounders examined included the matching factors (age, sex and region) factors, which may differ due to response bias (ethnicity and education level)[19].

Interactions between HCV seropositivity and the potential confounding variables were examined by entering the interaction term into the logistic regression model. Significance was based on the likelihood ratio p-value for the interaction term. Sub-group analysis of the association between HCV sero-positivity and NHL were also performed for histological subtypes. A test for heterogeneity between histologic subtypes was performed by analysis of the case group only using polychotomous logistic regression[20,21].

Results

Fifty cases out of the 117 cases of lymphoma were serologically positive for anti-HCV Abs. Age of the sero-positive lymphoma patients ranged from 20 to 80 years with mean age 56.00 (standard deviation 12.34). There was equal number of both male and female patients among the HCV seropositive patients, 25 for each. The age of sero-negative patients ranged from 14 to 84 years old with mean age 50.27 (standard deviation 14.97). There were 31 female and 36 male patients within this group.

The distribution of the anatomic sites among the fifty HCV seropositive lymphoma patients of the study was 24 out of 50 patients (48%) presented clinically with

lymphadenopathy including axillary, cervical, inguinal, porta hepatis LNs, and the remaining 26 patients (52%) were presented clinically with masses in extranodal sites including spleen, oronasopharengeal region, colon, breast, stomach, liver and buccal mucosa with the prevalence of spleen which presented 30% of the affected extranodal sites in these patients.

The anatomic distribution of the presented lesions in sero-negative lymphoma patients was 55 out of 67 (82%) of patients presented clinically with lymphadenopathy, and the remaining 12 patients (18%) presented with masses in extranodal sites including the spleen, testis, small intestine, soft tissue, liver, stomach, suprarenal gland and pelvi-abdominal region.

By comparison of the anatomic distribution of the clinically presented lesions in both HCV sero-positive and sero-negative lymphoma patients, we found that lymphoma in HCV sero-positive patients was clinically presented in extranodal sites (predominantly the spleen) in 48% of patients compared with only 18% of HCV sero-negative lymphoma patients. Statistical analysis showed a highly significant association of extranodal lymphomas with HCV sero-positivity (P value= 0.000) and detected HCV sero-positivity as a high risk factor for extranodal lymphomas (Odds Ratio (OR)= 4.2, 95%, Confidence intervals (CI)= 1.8-9.7).

On the other hand, HCV sero-positive lymphoma patients presented clinically with lymphadenopathy represented 52% of patients compared with 82% of HCV sero-negative lymphoma patients presented clinically with lymphadenopathy (table 1).

Considering the types of lymphoma diagnosed in both HCV sero-positive and sero-negative patients, there was a prevelance of NHL in both groups representing 98% of lymphomas diagnosed in HCV positive patients and 94% of lymphomas diagnosed in HCV negative patients. In HCV positive patients one case was diagnosed as HL, while fourty nine cases were diagnosed as NHL including twenty one cases of DLBCL, four

cases of FL, thirteen cases of SMZL, six cases of MALT lymphoma, two cases of SLL/CLL, one case of MCL, one case of PTCL, NOS and one case of ALCL. On the other hand, lymphomas diagnosed in HCV sero-negative patients included four cases of HL, and sixty three cases of NHL: ten cases of SLL/CLL, eight cases of FL, two cases of MCL, two cases of BL, two cases of MZL (one case of MALT lymphoma and one case of SMZL), thirty five cases of DLBCL two cases of ALCL and two cases of T-ALL/LBL (table 2).

Overall, there was an association between HCV seropositivity and NHL, with HCV seropositive subjects having an unadjusted OR of 1.04 (95% CI= 0.6-1.7) compared to subjects with negative serology. A similar risk was associated with B-cell tumors (OR 1.06, 95% CI 0.6-1.8) than T-cell lymphomas (OR 0.67, 95% CI 0.11-3.8), There was significant heterogeneity among the B-cell subgroups. A highly significant association was observed with MZL (P value= 0.000) especially SMZL (P value= 0.000) and MALT lymphoma (P value= 0.02). The largest increased risks associated with HCV were observed for MZL (OR, 12.7; 95% CI, 2.8-57.18). No significant association or increased risk was noted for HL or T- cell lymphomas in HCV patients (table 3).

Table (1): Comparison of the clinically presented anatomic sites in both HCV sero-positive and sero-negative patients.

Site	HCV positive patients	HCV negative patients	X2	P value	OR	95%CI
	NO. (%)	NO. (%)				
Extranodal	24/50 (48%)	12/67 (18%)	12.1	0.000	4.2	(1.8-9.7)
Nodal	26/50 (52%)	55/67 (82%)				

Table (2): types of lymphomas diagnosed in both HCV sero-positive and sero-negative patients.

Diagnosis		HCV negative		HCV positive	
		No.	%	No.	%
HL		4	6.0	1	2.0
All NHL		63	94.0	49	98.0
B-cell NHL		59	88.05	47	94.0
SLL/CLL		10	14.9	2	4.0
MCL		2	3.0	1	2.0
FL		8	11.9	4	8.0
BL		2	3.0	0	0
MZL	**MALT**	1	1.5	6	12.0
	SMZL	1	1.5	13	26.0
DLBCL		35	52.2	21	42.0
T-cell NHL		4	6.0	2	4.0
NHL (PTCL)		0	0	1	2.0
NHL (ALCL)		2	3.0	1	2.0
NHL (T-ALL/LBL)		2	3.0	0	0
Total		67	100	50	100

Table (3): HCV association with NHL types.

Pathologic subtype	HCV positive		HCV negative		χ^2	*P* value	OR	95%CI
	No.	%	No.	%				
HL	1	2.0	4	6	1.01	0.3	0.33	(0.03-3.09)
All NHL	49	98.0	63	94.0	0.02	0.87	1.04	(0.6-1.7)
B-cell NHL	47	94.0	59	88.05	0.05	0.8	1.06	(0.6-1.8)
SLL/CLL	2	4.0	10	14.9	3.07	0.08	0.26	(0.05-1.2)
MCL	1	2.0	2	3.0	0.1	0.7	0.67	(0.05-7.5)
FL	4	8.0	8	11.9	0.39	0.53	0.67	(0.19-2.35)
BL	0	0	2	3.0	1.4	0.22	-	-
MZL	19	38.0	2	3.0	16.2	0.000	12.7	(2.8-57.18)
MALT	6	12.0	1	1.5	4.9	0.02	8.04	(0.9-68.9)
SMZL	13	26.0	1	1.5	12.58	0.000	17.4	(2.2-137.5)
DLBCL	21	42.0	35	52.2	0.4	0.5	0.8	(0.4-1.5)
T-cell NHL	2	4.0	4	6.0	0.2	0.6	0.67	(0.11-3.8)
NHL (PTCL)	1	2.0	0	0	1.3	0.25	-	-
NHL (ALCL)	1	2.0	2	3.0	0.1	0.7	0.67	(0.05-5.6)
NHL (T-ALL/LBL)	0	0	2	3.0	1.4	0.23	-	-
Total	50	100	67	100				

Discussion

The current study provides a positive association between B-cell NHL and HCV infection (OR 1.06, 95% CI 0.6-1.8). This risk is significantly increased in old age group (above 60 years old). The risk according to NHL subgroups in our study was significantly observed in MZL (P value= 0.000) (OR 12.7, 95% CI (2.8-57.18) including SMZL and MALT lymphomas. No significant association of HCV sero-positivity with HL or T-cell NHL was observed.

Many studies reported a positive association between NHL and HCV. In Egypt, Cowgill et al.[22] observed a significant association between NHL and HCV, but they did not evaluate the impact of HCV infection on the grades and subtypes of NHL. El-Sayed et al. [23] in Egypt also reported similar results. In Saudi Arabia, Harakati et al.[24] reported that HCV-infected NHL patients were more likely to have intermediate-grade NHL than non-infected patients. Muhammad et al.[18] noted that HCV infection was strongly associated with NHL in Pakistan being more frequently observed in young and middle-aged subjects. No statistically significant impact was observed regarding HCV seropositivity on the grades and subtypes of NHL.

Researchers in Europe also documented a similar association between NHL and HCV infection, but few studies have been conducted to evaluate the impact of the grades of NHL. Mele et al.[25] documented a strong association and concluded that in Italy, 1 of 20 instances of B-NHL may be attributable to HCV infection, and thus, the patients may benefit from antiviral treatment. In Turkey, Isikdogan et al.[26] observed that HCV-seropositive NHL patients were more likely to have intermediate-grade NHL. In France, Seve et al.[27] documented a positive but non-significant trend towards an association between NHL and HCV infection (odds ratio, 1.31; 95% CI, 0.51-3.36).

Studies performed in other countries have also reported a strong association between HCV and NHL[28,29]. Spinelli et al.[3] in Canada found that the highest risk was observed in the DLBCL

and MZL lymphoma and no association with FL. Dal Maso and Franceschi[30] meta-analysis reported similar results but with an overall 2.7 fold increased risk for FL.

The finding of an association is not universal in the literature, with a large case–control study from France[14], and multiple small negative series from the UK [11], Canada[13], Germany[12], Turkey[31], Thailand[32] and the USA[33] failing to find an association. In contrast to the Japanese studies supporting the association, a further Japanese study found little evidence of a significant association[34]. Discordant results were found also by other authors who did not detect a risk for NHL in HCV sero-positive patients[35-37].

Our findings are an addition to the results observed in the former group of studies which support the association between HCV and NHL. This difference between studies probably reflects the fact that genetic, environmental and viral factors as well as the viral genotype may play a role in the complex relationship between the patient and the virus. There is, however, no direct evidence to support or refute these possibilities. From the available evidence it appears that HCV is only an important risk factor for B-cell malignancies in areas with a high prevalence of HCV carriage. Further studies with larger patient populations investigating the prognostic profile of HCV infection and the treatment response will help to clarify this issue.

Conclusion

In conclusion, HCV infection is associated with B- cell NHL. HCV infection in NHL patients was more frequently observed in old aged subjects. A statistically significant impact was observed regarding anti-HCV Abs seropositivity on the types of NHL with a significant association with MZL. On the basis of our results, larger epide¬miological studies can be conducted.

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HEPATITIS C VIRUS INFECTION AND NON-HODGKIN'S LYMPHOMA

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TRU CUT BIOPSY OF OVARIAN TUMORS: IS IT APPLICABLE?

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Abstract

Background: *ovarian cancer is the sixth most common female cancer. Many cases are diagnosed at advanced stage resulting in lesser chance for optimal cytoreduction. Recently, neoadjuvant chemotherapy has been introduced to improve cytoreduction and reduce surgery related morbidity. Consequently, preoperative pathologic diagnosis became mandatory*

Objectives: *To analyze the safety, adequacy and accuracy of tru-cut biopsy and to evaluate factors potentially affecting adequacy.*

Methods: *prospective study conducted on 50 cases of female patients presented with ovarian tumor and submitted either to obstetric and gynecology department or oncology center in Mansoura university hospital during the period between 2011 and 2013. Tru-cut biopsy was performed under ultrasound guide, using an automatic biopsy gun with disposable needle. The adequacy, accuracy, and safety were assessed. Variables potentially influencing adequacy were analyzed.*

Results: *the study conducted prospectively on 50 cases having ovarian masses. Samples containing adequate diagnostic tissue sufficient to perform IHC were available in 42 out of 50 cases (84%). The sizes of the mass and prior CT or MRI were positive predictors for adequacy. The diagnostic accuracy of tru cut biopsy is 95.7% with sensitivity is 95.5% and specificity 100%. The Positive predictive value is 100% and the Negative predictive value is 60%.*

Conclusion: *Ultrasound-guided tru-cut biopsy is an efficient, minimally invasive, accurate and safe diagnostic method in the management of advanced, and recurrent ovarian tumour.*

Introduction

Ovarian cancer is the sixth most common female cancer and the seventh most common cause of cancer death. There are about 204,000 new cases and 125,000 deaths annually (Boyle and Levin, 2008). The world rate of ovarian carcinoma is estimated to be 6.3 per 100,000, and is higher in developed countries being 9.3 per 100,000. (Rauh-Hain et al., 2011). In Egypt, according to Mahdy et al 1999, the rate per 100,000 women in Alexandria was 3.16. Data from national cancer registry, Aswan profile, revealed that ovarian cancer represented 5.6% of all female malignancies, being the second most frequent cancer among Egyptian females following breast cancer (Ibrahim and Mikhail, 2010).

The World Health Organization Histological Classification for ovarian tumors separates ovarian neoplasms according to the most probable tissue of origin: surface epithelial (65%), germ cell (15%), sex cord-stromal (10%), metastatic (5%), and miscellaneous tumors. Surface epithelial tumors are further classified by cell type into :serous, mucinous, endometrioid, etc and by degree of atypia into: benign, borderline [atypical proliferation, low malignant potential] or malignant; malignant may be invasive or non-invasive. Most malignant tumors are surface epithelial accounting for 90% of all malignant tumors. (Tavassoli and Devilee, 2003).

The problem of late presentation that characterizes any malignant tumor is more commonly encountered with ovarian tumors due to the lack of specific symptoms and the overlap with nonspecific GIT and genitourinary problems. Consequently, large numbers of cases with ovarian malignancy are diagnosed at advanced stages making optimal cytoreduction less likely which will adversely affect the prognosis with subsequently reduced survival (Schwartz and Zheng, 2003).

Spencer et al., 2006 reported that two-thirds of women with ovarian cancer present with abdominal dissemination of disease, the standard management of which comprises surgical debulking followed by chemotherapy. Un-

der such circumstances it is unlikely to achieve optimal debulking even with most experienced hands.

Introduction of neoadjuvant chemotherapy followed by interval debulking was found to improve the cytoreduction and reduce surgery related morbidity in such advanced cases. In addition, neoadjuvant chemotherapy is gaining popularity in improving physical and emotional trauma associated with intial ovarian cancer treatment. (Schwartz and Zheng, 2003).

Not only to confirm malignancy before starting chemotherapy but to confirm that a malignant tumor is an ovarian primary since management will be completely different in case of metastatic tumors. In addition, core needle biopsy is important for accurate typing and grading of the tumors that would be inaccurate if performed on postchemotherapy resection specimens (Longatto Filho et al., 1997). Finally, tru cut biopsy is a helpful diagnostic tool in patients with poor surgical performance, or patients with recurrent disease, this study evaluates the role of trucut biopsy in ovarian tumour.

Patients and Methods

This is a prospective study conducted on 50 cases of female patients presented with ovarian tumor and admitted either to obstetric and gynecology department or oncology center in Mansoura university hospital during the period between 2011 and 2013. Patients were included in the study when belonged to any of the following categories: advanced ovarian malignancy (according to clinical &/or radiological criteria), recurrent malignant ovarian tumor, ovarian tumor of doughtful origin (primary or metastatic), solid ovarian mass with an ultrasound evidence to be benign. Patients with suspicious masses appearing to be in early stage were excluded from the study to prevent possibility of tumor spillage into the peritoneal cavity and upstaging due to the procedure.

Whether completely solid or complex (partially cystic and partially solid) mass, the solid component should appear on ultrasound measuring more than 2 cm so it

can admit the tru cut needle. The INR ratio for all patients should be less than 1.4 to be candidate for the procedure. A prior CT or MRI was checked for accurate localization of the site of introduction of the needle

The skin at site of introduction of the needle was disinfected with alcohol. 2% xylocaine was injected into the skin and subcutaneous tissue as local anasthestic. The shortest puncture route is chosen. When the tip of the needle is in a desired site the pistol is retracted so that tissue is sampled then the needle is gently removed. 16-18 guage needle was used to obtain the tissue core which was immediately placed in 10% buffered formalin for fixation.

Tissue core was sent for pathology department where it was processed into a paraffin block that was cut into 5um thick sections stained with: H&E.

Each tissue slide prepared from tru cut specimen was evaluated for presence of sufficient diagnostic tissue (adequacy), and accuracy for diagnosis, tumor typing (according to the WHO classification of ovarian tumors 2003), tumor grading and availability to perform IHC staining reaction.

Data analysis was done by using Chi square test. Univariate regression analysis was done to estimate positive and negative predictors of tru cut adequacy.

Results

The study was conducted prospectively on 50 cases having ovarian masses. The age range of patients was between 11-63 years, with mean age 52.74 years. Age , size of the mass, CA125 level, presence of ascites, BMI , CT or MRI prior to the technique were studied as factors affecting the adequacy of the tru cut specimen. Samples containing diagnostic tissue sufficient to perform IHC were available in 42 out of 50 cases (84%).

In Univariate regression analysis for the studied factors only the size of the mass and prior CT or MRI were positive predictors for adequacy. BMI was a negative predictor for a adequacy but without reaching a statistically significant level.

Diagnosis in tru cut specimen was consistent with postoperative specimen in 44 out of 46 cases (diagnostic accuracy) (95.7%) with sensitivity is 95.5% and specificity 100%. The Positive predictive value is 100% and the Negative predictive value is 60% due to the presence of 2 false negative cases.

Out of 42 true positive cases, 38 cases available for typing. There were 37 cases concordant for typing (97.4%) and 1 case disconcordant.

Out of 42 cases true positive cases, 30 cases have tumor grade, 28 cases concordant (93.3%) and 2 cases disconcordant.

Diagnosis of high grade serous carcinoma was based on presence of papillary and glandular structures lined by columnar to cuboidal epithelial cells with high grade nuclei (prominent nucleoli) and frequent mitotic figures (≥10/ 10HPF). Papillae are mostly short and broad with narrow fibrovascular core(fig.1). On the other hand presence of small papillary structures lined by low grade cells and surrounded by cleft like spaces (micropapillae) was diagnostic for low grade serous carcinoma. Such micropapillae appeared in longitudinal sections as tall narrow papillae whose length is twice the width. In addition we have encountered the caput medusa like appearance of low grade serous carcinoma (fig.2).

In the current study we had 7 cases with final diagnosis of mucinous tumor one of which was mucinous cystadenoma seromucinous type based on the presence of cystic structures lined by single layer of endocervical like columnar mucin secreting epithelial cells assosciated with areas that are lined by cubical and columnar non mucinous epithelial cells some of which were ciliated (serous cells).

Such areas that are lined by serous cells were the only finding detecting in the tru cut biopsy therefore accurate typing couldn't be reached. still another case with final diagnosis of mucinous carcinoma intestinal type was non representative in the tru cut.

Of the remaining 5 cases 4

were accurately diagnosed on tru cut biopsy; 2 of which were of seromucinous type and 2 of intestinal type due to presence of goblet cells. The last one case with final diagnosis of mucinous carcinoma seromucinous type was accurately typed on trucut biopsy due to the presence of two types of cells serous like and endocervical like however, grading was inaccurate as it was diagnosed as borderline with microinvasion. This can be justified by the common presence of borderline focus adjacent to carcinomas especially the low grade ones.

The only one case diagnosed as grade II endometroid carcinoma was greatly similar to its uterine counterpart displaying glandular structures mostly with rounded lumena (in contrast to the slit like lumena of the serous glands) as well as cribriform and solid sheets representing 20% of the tumor. The cells were columnar non seceretory epithelial cells with moderate degree of nuclear pleomorphism and infrequent mitotic figures.

Cases diagnosed as undifferentiated carcinomas were entirely formed of solid sheets of epithelial cells with no identifiable papillary or glandular structures.

Diagnosis of fibroma based on the presence of bland spindle cell proliferation arranged in bundles and focal storifiorm pattern. In some cases theca cells with abundant foamy cytoplasm and rounded to polygonal outline could be identified.

Desmoplastic small round cell tumor was diagnosed in 33 years old female patient after exclusion of all other round cell tumors by IHC. The morphology was that of aggregates of dyscohesive rounded monotonous cells with moderate amount of cytoplasm and hperchromastic nuclei with occasional conspicuous nucleoli. The aggregates were separated by desmoplastic stroma through which cords of the tumor cells were present. In this case the tumor was involving the ovary secondarily to a mesentric mass.

The case that was appearing in the tru cut as dyscohesive rounded cells with moderate amount of

amphophoilic cytoplasm, vesicular nuclei and conspicuous nucleoli that are seen infiltrating singly and in dyscohesive cords and aggregates were found out to be DLBCL. A diagnosis that was in differential with sex cord stromal tumor but IHC positivity for LCA applied on cell block have solved the problem.

Table (1): predictors of tru cut Adequacy.

The variable	P value	Significance
Age	0.552	Non
Size	0.000	Significant
Ascites	0.173	Non
PRIOR CT MR	0.000	Significant
CA 125	0.475	Non
BMI	0.201	Non

Table (2): Test of validity for the tru cut biopsy.

Diagnostic accuracy	Sensitivity (95% CI)	Specificity	Negative predictive value	Positive predictive value
95.7%	95.5 (91-95.5)	100 (34.1-100)	60 (21.5-60)	100 (95.3-100)

Table (3): Flow chart for tumor typing in tru cut biopsy.

42 cases true positive cases			
2 cases Scanty tumor tissue	2 cases No typing	38 cases available for typing	
		37 cases Concordant (97.4%).	1 case Disconcordant

Table (4): Flow chart for tumor grading in tru cut biopsy.

42 cases true positive cases			
10 cases non graded tumors	2 cases scanty suspicious	30 cases tumor grade	
		28 cases concordant	2 cases disconcordant
		(93.3%)	

Table (5): Tru cut biopsy diagnosis and final histopathology diagnosis.

Case	Tru cut	Postoperative diagnosis
1	Inflammatory	Inflammatory
1	Benign cystic lesion	Complicated benign cyst
1	Benign cystic lesion	Mucinous cystadenoma
1	Borderline seromucinous tumor With microinvasion	Invasive low grade seromucinous carcinoma
1	Borderline serous tumor	Low grade serous carcinoma
2	Low grade serous carcinoma	Low grade serous carcinoma
15	High grade serous carcinoma	High grade serous carcinoma
2	Undifferentiated carcinoma	Undifferentiated carcinoma
1	Undifferentiated carcinoma	High grade papillary serous
1	Metastatic undifferentiated carcinoma	Metastatic undifferentiated carcinoma
4	Mucinous adenocarcinoma	Mucinous adenocarcinoma
1	Endometroid carcinoma	Endometroid carcinoma
1	Undifferentiated round cell tumor(lymphoma or sex cord stromal)	Diffuse large B cell lymphoma
5	Fibroma	Fibroma
1	Yolk sac	Yolk sac
2	Krukenberg	Krukenberg
1	Desmplastic small round cell tumor	Desmplastic small round cell tuomor
1	Adequate specimen negative for malignancy	High grade serous carcinoma
1	Fibroma	High grade serous carcinoma
2	Suspicious for Malignant epithelial tumor	High grade serous carcinoma
1	Suspicious for serous carcinoma	High grade serous carcinoma
4	Inadequate for diagnosis	High grade serous
		Fibroma
		Embryonal carcinoma
		Mucinous carcinoma

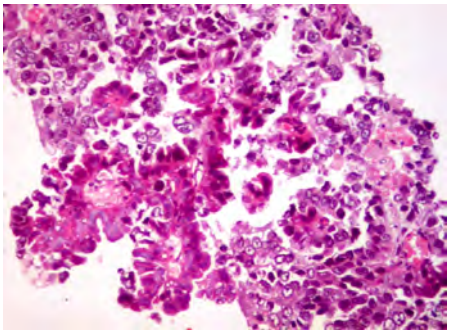

Fig. 1: high grade papillary serous carcinoma in a tru cut biopsy. (H.&E., x20 magnification).

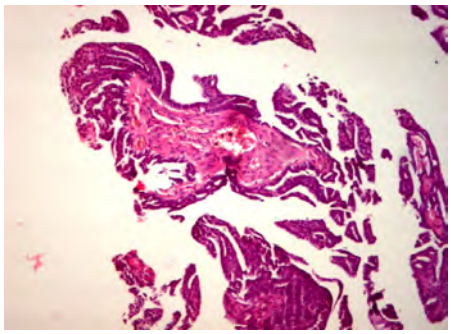

Fig. 2: Caput medusa like appearance of low grade papillary serous carcinoma (H. &E.x10 magnification).

Discussion

Pathological diagnosis of ovarian tumors has long been known to be done on post operative resection specimen while preoperative diagnosis has been reliable upon clinical and radiological findings. However, the recent introduction of neoadjuvant chemotherapy has made the preoperative pathological diagnosis mandatory. This can be achieved either by tissue biopsy or cytology. Tissue biopsy by tru cut needle was the scope of the current study.

Similar to what was reported by Zikan et al., the high reliability and safety of this minimally invasive method have been confirmed from the high diagnostic accuracy (95.7%), sensitivity (95.5%),and PPV(100%) that we have encountered in our study. In addition, our study didn't score any false positive diagnoses resulting in 100% specificity. On the other hand presence of false negative cases has resulted in NPV of 60%. Similar to the relatively low adequacy rate false negative diagnoses can be attributed to the new experience which may be the cause of non sampling of the tumor.

Larsen et al., 1993, Malmstrom 1997, & Freedman et al., 2010 were the only trials that studied the diagnostic role for both core needle biopsy and FNAC in ovarian tumors. Similar study was conducted by Stewart et al., 2002 but on intra abdominal masses without addressing the ovary specifically.

On the other hand, there has been other studies that were conducted on tru cut biopsy alone either in ovarian tumors (Zikan et al., 2010; Faulkner et al., 2005; Fischerova et al., 2008), pelvic masses (Yarram et al., 2007), or peritoneal carcinomatosis (Spencer et al., 2006; Hewitt et al., 2007).

The adequacy rate we have achieved for the tru cut biopsy (84%) was nearly similar to that reported by Faulkner et al., 2005 (85.7%), higher than that of Larsen et al., 1993(78%), but lower than that reported by Zikan et al., 2010(91.3%), Malmstorm, 1997(88%), Fischerova et al., 2008(93.02%), Yarram et al., 2007(95.2%), and Spencer et al., 2006(92%). Adding to differences in experience as well as the smaller number of cases enrolled in our study, we have adopted only the trans abdominal approach for obtaining tru cut biopsy while Zikan et al and Fischerova et al. have adopted both trans abdominal and transvaginal routes. A higher adequacy of transvaginal biopsies is probably due to the proximity of the biopsy lesion to the probe and a better capacity for guiding the biopsy probe more precisely into the vascularized, i.e. vital, parts of the tumor.

While ascites and CA 125 levels reflect large tumor loads and a larger tumor is easier to access we didn't find them to achieve a statistically significant value to be considered as positive predictors for adequacy in our study. This is despite being higher in adequate samples than scanty ones. This statistical difference can be attributed to the Small sample size in our study. On the other hand, both parameters were positive predictors for adequacy in the study conducted by Zikan et al., 2010. However, this study was conducted on 190 cases. Instead size of the mass and presence of CT or MRI prior to the technique were our positive predictors in our study. These two parameters haven't been studied in other trials. CT or MRI prior to the technique allow accurate localization of the tumor and its solid component making it easier for radiologists to introduce the needle in shorter way and more targeting.

On the other hand, BMI was a negative predictor for adequacy in our study this could be explained

by the fact that obesity can hinder the performance of tru-cut biopsy (especially by the abdominal approach). This again in contrast to the findings reported by Zikan et al., 2010.

The diagnostic accuracy for tru cut in our study was 95.7%, a figure comparable to those reported by Zikan et al., 2010 (98.3%), Fischerova et al., 2008 (97.7%), Yarram et al. 2007 (93,4%), and Freedman et al., 2010. On the other hand our diagnostic accuracy for tru cut was much higher than that reported by Malmstorm, 1997 who achieved 73% accuracy as well as Stewart et al who achieved 80.1% accuracy.

We have achieved the highest sensitivity for tru cut when compared to the reported figures by Malmstorm, 1997 (73%), Yarram et al. 2007 (91.4%) and Stewart et al. 2002. However, these studies yielded 100% specificity as ours.

The same is true regarding the PPV which was 100% in our study similar to Malmstorm , Yarram et al. and Larsen et al.

On the contrary our NPV was lower than that of Yarram et al (78.1%) and Larsen et al. (94%). A difference that can be attributed to the false negative case we have encountered.

While Zikan et al., 2010, Fischerova et al., 2008 & Hewitt et al., 2007 reported presence of complications from tru cut biopsy we didn't encounter any complication. Since the technique was a new experience in our hospital assosciated with controversial acceptance from senior staff we have been meticulous in the choice of patients that can be candidate for the procedure.

Subtyping was available in 90.3% of cases in the current study with 97.4% concordance with the final histopathological diagnosis, an issue which haven't been discussed in any study apart from that of Freedman et al., who reported less figures; 77 % subtyping with 80% concordance.

We have discussed the accuracy of tru cut biopsy for tumor grading and we have reported it to be 93.3%, again this issue wasn't addressed in any of the previously mentioned studies.

Results from the current study support the safety and beneficial use of tru cut biopsy in preoperative diagnosis of ovarian tumors whenever indicated. Tru cut has the advantage of significant predictive power for tumor typing and grading.

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TRU CUT BIOPSY OF OVARIAN TUMORS: IS IT APPLICABLE?

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EFFECT OF MALATHION ON EMBRYONIC NEURAL STEM CELL OF THE RAT

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Abstract

Developmental neurotoxicity (DNT) entails the toxic effects of various chemicals on brain during the early childhood period. As human brains are vulnerable during this period, various chemicals would have maximum effects during this early stage of development. A novel alternative method that can overcome most of the limitations of the conventional techniques is the use of 3D neurospheres system. This in-vitro system can recapitulate most of the changes during the period of brain development making it an ideal model for predicting neurotoxic effects. In the present study, we verified the possible DNT of malathion which is one of organophosphates pesticides with suggested possible neurotoxic effects on lactating children. Three doses of malathion (250nM, 1μM and 10μM) where used in cultured neurospheres for a period of 14 days. Malathion was found to affect proliferation and viability of neurospheres, these effects were positively correlated to doses and progress of time. This study confirms the DNT effects of malathion on 3D neurospheres model.

Keywords: *Malathion; Neurospheres; DNT; Proliferation; Viability.*

Introduction

Organophosphorus pesticides (OPs) are currently the most commonly utilized pesticides in the world. They combine almost 40 different chemical members registered by the US-EPA (www.epa.gov). About 70% of all insecticides used in USA are OPs, constituting about 73 million pounds in 2001[1]. Moreover, OPs problem involves both developed and developing countries[2]. The cholinesterase enzymes inhibiting effects of OPs represent the main mechanism underlying their acute

toxicity. However, other possible toxic effects has been linked to chronic exposure to OPs e.g. delayed neurotoxicity, developmental neurotoxicity and other organs toxicity[3]. Malathion (O,O-dimethyl dithiophosphate of diethyl mercaptosuccinate) is one of the OPs group with different toxic effects[4]. These findings suggest that malathion exposure during lactation induced cerebral alterations and oxidative stress in rat pups.

The most promising models for DNT testing are based on three-dimensional animal or human cell culture systems: the embryonic stem cell test (EST)[5], the whole embryo culture (WEC)[6] and human neural progenitor cells (hNPCs), grown as neurospheres[7].

The three-dimensional human neurosphere system mirrors the basic processes of brain development, namely proliferation, migration, differentiation and apoptosis. So far, however, research with neurospheres has largely focused on their application for neuroregeneration in disease states of the central nervous system[8]. Nevertheless, a few studies have also utilized neurospheres for toxicity studies in vitro by analyzing a variety of endpoints such as viability, proliferation, migration, differentiation, neurite outgrowth and apoptosis[9]. These provide support for their use in hazard identification screens for chemicals that may cause developmental neurotoxicity.

In the present study, we explored the neurotoxic effects of malathion on neurospheres, trying to extrapolate the resulting data to developmental toxic effects. Moreover, we traced the role of oxidative stress in this neurotoxic effect of malathion on cultured neurospheres with different concentrations.

Materials and Methods

All chemicals were of molecular biology grade and were obtained from Sigma-Aldrich (USA) unless otherwise stated.

Cell culture. Rat neural progenitor cells were isolated from rat embryos (E14) extracted from placental tissue. The cortices were aseptically dissected out from the

brains of the fetuses and the tissues were triturated by repeated passage through a fire-polished constricted Pasteur pipette. The dispersed tissues were allowed to settle for 3 min. The supernatant was, then, transferred to a fresh tube and centrifuged at 1,000 g for 5 min. The pellet was placed in Hank's balanced salt solution cultured as free-floating neurospheres in proliferation medium [Dulbecco's modified Eagle medium and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/mL epidermal growth factor (EGF; Biosource, Karlsruhe, Germany), 20 ng/mL recombinant human fibroblast growth factor (rhFGF; R&D Systems, Wiesbaden-Nordenstadt, Germany), and penicillin and streptomycin (1:100 vol/vol; Invitrogen) at 37°C with 7.5% CO as previously described [10]. Differentiation was initiated by growth factor withdrawal in differentiation medium [Dulbecco's modified Eagle medium and Hams F12 (3:1) supplemented with N2 (Invitrogen)] and plating onto a poly-d-lysine/ laminin matrix.

Chemical Exposure. For proliferation and viability analyses, neurospheres were treated for 2 weeks with malathion (0.25, 1, or 10 μM) in proliferation medium.

Proliferation analyses. For proliferation analyses, spheres were cultured in proliferation medium. After 0,4,5,11 and 14 days, sphere size was determined by software analyses (CellProfiler, version 2.1; Broad Institute, freely downloaded from http://www.cellprofiler.org). Diameter of each neurosphere was measured and exported to excel file further to statistical analysis.

Viability Assay. The viability of cells constituting the neurospheres was determined by analyzing the degree of deformity in sphere structure after different periods using the same image analysis software.

Results

Proliferation analysis:

As shown in table (1) and (fig. 1) control group neurospheres showed normal pattern of proliferation, where applying size analysis for the cell cultured revealed progressive increase from 317.6±61.9 in day 4 till reaching

800.6±117. Malathion exposed cell cultures, revealed arrest in proliferation which was positively correlated to the increase in malathion concentrations. Cells exposed to Malathion (250 nM) showed arrest of neurosphere diameter in day 11 and 14, so that their sizes were highly significantly lower when compared to control group (fig. 2). Cells exposed to Malathion (1 µM) showed more aggressive arrest in their proliferation as their measurements showed highly significant decrease in size as compared to controls in days 5 and 11, while on day 14 the neurospheres lost their shape. The same pattern of proliferation arrest was noticed in Malathion (10 µM).

Neurospheres Deformity:

Table (2) shows the degree of deformity in neurospheres shapes, hence the viability of cells. Based on previous reports[10], loss of the spherical shape of the 3D neurospheres structure denotes the loss of neural progenitor cells viability and ability of cohesion. As can be seen no cells in control revealed loss of shape at all test times. In group Malathion (250 nM) significant loss of sound structure was evident on 14th day. Groups Malathion (1 µM, 10 µM) revealed significant loss of continuity of cell structure from the day 5, to increase up to almost complete loss of cell continuity on day 14 (figs. 2,3).

Table (1): Proliferation analysis of different cells groups.

Days	Control	Malathion 250 nM	Malathion 1 µM	Malathion 10 µM
4±	317.6 ± 61.9	309 ± 103	342.3 ± 82	226.3 ± 30.5
5	551.3 ± 72.1	509.3 ± 50	330.6 ± 92#	318.6± 40.8#
11	892 ± 14.4	442 ± 34.6##	437.3 ± 32##	360.3 ± 3.5##
14	800.6 ± 117	411.3 ± 16##	N/A	N/A

statistically significant compared to control
Highly significant compared to control.

Table (2): Percentage of neurospheres deformity in tested groups.

Days	Malathion 250 nM	Malathion 1 µM	Malathion 10 µM
4	0	0	0
5	10	20	20
11	20	30	50
14	70	95	95

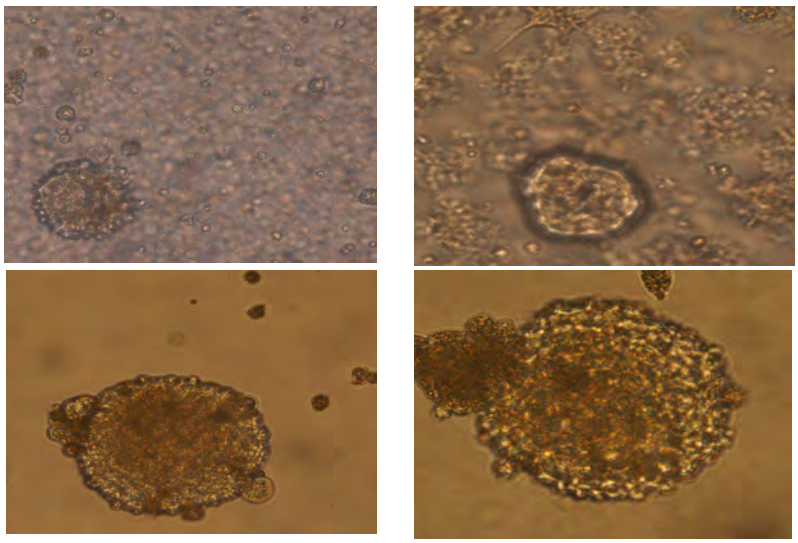

Fig. 1: Proliferative changes of neurospheres culture for control group on days 4,5,11,14 at magnifications X10.

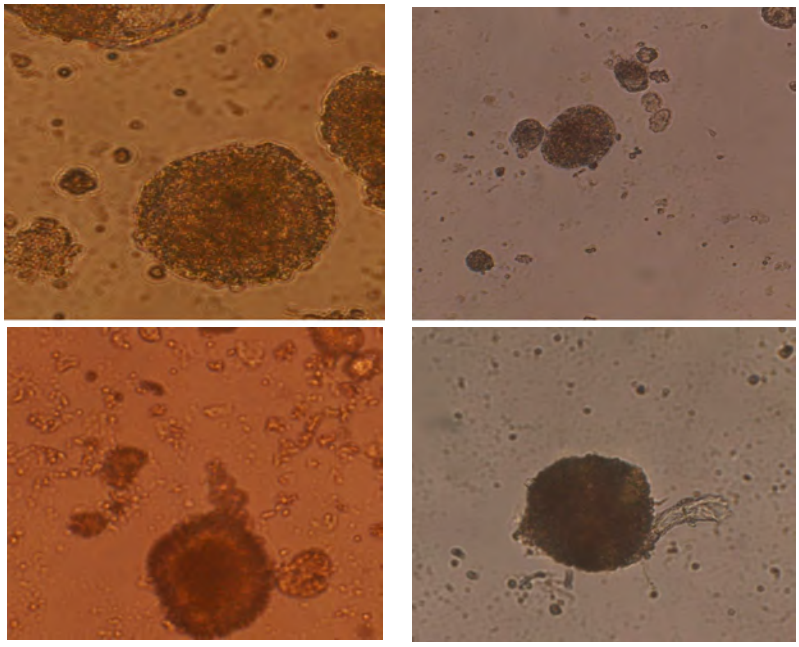

Fig. 2: Proliferative changes of neurospheres culture for Malathion (250nM) group on days 4,5,11,14 at magnifications X10.

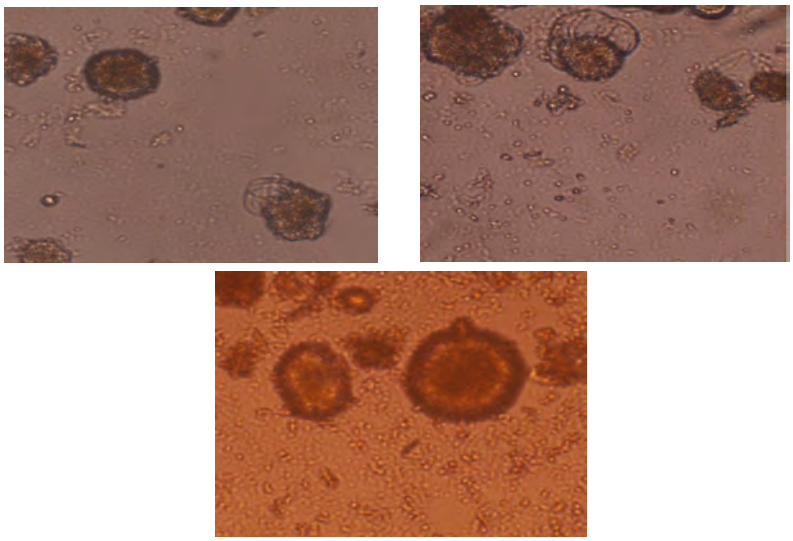

Fig. 3: Proliferative changes of neurospheres culture for Malathion (1 uM) group on days 4,5,11 [14th day was totally deformed] at magnifications X10.

Discussion

Developmental neurotoxicity (DNT) compresses a broad category of neurological disorders that may be of unknown cause. Examples include learning defects, some psychological disorders e.g. schizophrenia and autism[11]. Hence, it is of paramount importance to accurately predict the neurotoxic effects of certain compounds using more advanced systems than those regularly used[12]. In the present study, we explored the DNT of Malathion with different concentrations on 3D neurospheres system. The most valuable strength point in neurospheres is their ability to trace the predicted effects on different stages of brain development. This advantage of neurospheres in-vitro system over traditional animal models, improves our ability to verify the putative adverse effects of Malathion on children brain development in case of exposure in early life.

In our work, we used small doses (250nM, 1μM and 10 μM) of malathion to imitate the regular real life exposure, especially, through lactation. This is on contrary to most preceding studies

where much higher doses were used (1-20mM). Although previous studies showed neurotoxic effects of malathion on animal models and cell cultures, results could not be extrapolated to normal child exposure in real life due to larger doses used in these studies [13,14]. We think that the sensitivity and specificity of neurospheres system would offer a chance to show the specific toxic impact of smaller doses of malathion.

The effects of Malathion were tested regarding proliferation capacity and neurosphere shape as marker of cell viability. Moreover, the pro-oxidant effect of Malathion was verified in cell culture through measuring GSH, MDA and SOD. The effects of Malathion on proliferation were evident as noticed through the arrest of normal increase in neurospheres diameter with progress of time in a proliferation culture medium. This effect can be translated to affection of brain size in human exposure incidents which suggest possible morphological changes on exposure to Malathion during the critical period of brain development[10]. It is worth noticing that previous studies implementing neurospheres as DNT predictive system showed more effects of tested chemicals (Mercury and Polybrominated Diphenyl Ethers) on migration and differentiation than proliferation[10,15]. On the contrary, present work revealed powerful impact of Malathion on proliferation. This variation in effects denotes different mechanisms of action between different neurotoxic agents and their resultant effects on children exposure.

The findings of the present study confirm the developmental neurotoxic effects of Malathion which support previous works of[13]. These studies, however, showed the neurotoxic effects of early postnatal exposure in rodent models. Although they reflected the abnormal neurobehavioral performance of children exposed to pesticides like Malathion in early life[14], they needed to be verified by more detailed study as in neurospheres system. We believe this is the first study to confirm the DNT of Malathion on 3D neurospheres model where the mechanism of action seems through affecting proliferation hence may lead to morpholog-

ical size anomalies with absent role of oxidative stressors.

Conclusions

Malathion exposure in early childhood could lead to developmental changes on brain as evidenced from the present study. The negative effects could lead to morphological changes e.g. size diminution and focal lesions according to the affected areas.

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PERCUTANEOUS VERTEBROPLASTY FOR THORACOLUMBAR VERTEBRAL FRACTURES

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Abstract

Aim of this study: *To study the efficacy of Percutaneous Vertebroplasty (PVP) in thoraco-lumbar vertebral fractures according to the degree of pain relief and functional outcome.*

Patients and Methods: *42 vertebral bodies of 26 patients with painful vertebral compression fractures have been enrolled in a prospective study. Verbal Descriptive Scale (VDS) has been used to measure pain status; Oswestry Disability Index (ODI) was chosen to evaluate functional activity. They were done preoperative and regularly for up to 1 year.*

Results: *The mean VDS & ODI scores decreased significantly from pretreatment to posttreatment and continued through the follow up period with no significant differences between both osteoporotic and traumatic cases. There was a no significant difference in VDS improvements in relation to each amount of cement used. Cement extravasation occurred in 74% of augmented vertebrae associated with three patients (10.7%) with neurological compromise. Adjacent and non adjacent vertebral level fractures occurred in 3 cases (12%).*

Conclusion: *Vertebroplasty is effective minimally invasive procedure in markedly reducing pain and disability, and improving the quality of life in patients with vertebral compression fractures. But it may lead to serious neurological complications.*

Keywords: *Percutaneous vertebroplasty. Polymethylmethacrylate. Vertebral compression fracture. Verbal Descriptive Scale. Oswestry Disability Index. Thoraco-lumbar spine.*

Introduction

Percutaneous vertebroplasty (PVP) was first described by Galibert (1987) and performed by percutaneous injection of bone cement into one or more vertebral bodies under fluoroscopic or computed tomography control. It provides pain relief and mechanical stabilization within the vertebral body to prevent further vertebral body collapse[1,2].

It may be associated with cement leakage that can be minimized by reducing the quantity of the bone cement and/or its injection speed[3,4]. This leakage may also lead to myelopathy, radiculopathy or systemic cardiovascular or respiratory affection[5].

Many studies have been performed on vertebroplasty. And there was controversy about its efficacy in pain relief, functional outcomes, the cement amount used, its use in traumatic cases and its complications[3,4,6,7,8,9,10,11,12].

Patients and Methods

In the period between September 2009 and August 2013, Vertebroplasty was performed on 42 vertebral bodies between D6 and L5 of 26 patients complaining of back pain due to thoracolumbar vertebral compression fractures not associated with neurological affection and after at least 2 weeks from the vertebral fracture. The follow up period ranged from 3 months to 12 months with a mean of 11.5 months.

The age of patients included in the study ranged between45 and 82 years with a mean of 61.9 years (SD±9.4). There was 2 males (7.7%) and 24 females (92.3%). Osteoporotic vertebral compression fracture (VCF) in 20 patients (76.9%); (36 vertebrae) & post traumatic vertebral compression fracture (VCF) in 6 patients (23.1%); (6 vertebrae).

Patients were assessed clinically where the degree of pain and patient disability were assessed using the Verbal Descriptive Scale (VDS)[13] and the Oswestry Disability Index questionnaire (ODI) [14].

Plain radiography was done to detect the level, type and number of fractures. Also CT scan was

done to assess the integrity of the posterior wall of the vertebral body. It was done for all cases. MRI was done to differentiate between healed from non healed fractures in cases of more than 3 months duration. DEXA scanning was performed for all patients. Routine laboratory investigations and medical evaluation were ordered for all patients including: ECG, CBC, liver function, kidney function, I N R and blood glucose.

Patient Selection Criteria: The study was conducted on patients with thoracolumbar vertebral fractures.

A. Inclusion Criteria:

• Patients with painful vertebral compression fractures with collapse of the vertebral body more than 20% and less than 80%.

• Patients with vertebral fractures not involving the posterior wall of the body.

B. Exclusion criteria:

• Non symptomatic vertebral compression fracture.

• Patients with vertebral fractures involving the posterior wall of the body.

• Vertebral fractures associated with neurological deficit.

• New vertebral fracture after previous percutaneous vertebroplasty for the same vertebra.

• Systemic contraindications e.g. bleeding disorder, systemic infection.

• Local infection.

• Medical problem preventing the positioning of the patient in prone position for about one hour, which is nearly required for the procedure e.g. uncontrolled cardiac diseases, chest diseases or large abdominal hernia.

• Patient's disagreement for percutaneous Vertebroplasty.

• Absence of bone marrow oedema in STIR or fat suppression window MRI for the cases with history of back pain more than 3 months which indicates a healed fracture.

Vertebroplasty procedure:

Vertebroplasty was performed in the operating room with radiolucent table using a uniplanar fluoroscopy device whose position was changed between the AP and lateral directions as needed. IV Antibiotic prophylaxis was given for all patients preoperatively with 1 gram cefotaxime (3rd generation cephalos-

porin). All patients were operated under local infiltration anaesthesia (Lidocaine 2% 4.5 mg/kg/dose) and intravenous conscious sedation with IV fentanyl, if needed.

All patients were injected using the percutaneous transpedicular technique. The unipedicular technique was used in 17 vertebrae (40.5%) while the bipedicular technique was used in 25 vertebrae (59.5%). An 11 gauge needle (cannula & trocar) was used.

In this study, self curing poly methylmethacrylate (PMMA) cement of an appropriate viscosity with Barium sulphate of about 28% was used. The cement package was kept in the refrigerator for at least 30 minutes before use (Precooling method). This aimed at prolonging the setting time. The cement was injected in the toothpaste state and continued under continuous fluoroscopic guidance until adequate vertebral body filling has occurred, or appearance of cement leakage specially vascular, canal or foraminal leakages (fig.1). The amount of bone cement injected ranged from 3cm^3 to 12 cm^3 with a mean of 5.35 cm^3 (SD±2.47 cm^3).

The patients were evaluated clinically with neurological examination, VDS & ODI and radiologically with plain radiographs postoperatively and at each follow up. CT was done for all cases in the 1st post operative day. Follow up was carried out at regular intervals; 1.5 months, 3 months, 6 months and 12 months. The minimum follow up period was 3 months (Fig. 2 a, b, c, d).

Results

1. Pain subsidence score (using the VDS): According to the preoperative VDS; 73.1% of our patients were presented with very severe pain, 26.9% with worst pain but there were no patients with mild, moderate, severe or no pain. According to the postoperative VDS; 53.9% of the patients were presented with mild pain, 42.3% with moderate pain, 3.8% with very severe pain but no patients presented with severe, worst or no pain.

At 1.5 months 77% of the patients were presented with mild pain, 11.5% with moderate pain

and 11.5% with no pain but there were no patients with severe, very severe or worst pain. At 3 months; 61.5% of our patients were presented with mild pain, 30.8% with no pain and 7.7% with moderate pain but no patients presented with severe, very severe or worst pain. At 6 months 58.3% of our patients were presented with mild pain, 33.3% with no pain, 4.2% moderate pain and 4.2% very severe pain but no patients presented with severe or worst pain. At 12 months 60.9% of the patients were presented with mild pain, 34.8% with no pain and 4.3% with moderate pain but no patients presented with severe, very severe or worst pain. The improvement outcome of these differences compared with preoperative VDS was found to be statistically highly significant (P<0.001) (fig.3).

There was a no statistically significant difference (P value > 0.05%) in VDS improvements at any time between patients with osteoporotic VCFs or traumatic vertebral fractures. Through all the follow up period there was a no statistically significant difference in VDS improvements in relation to each amount of cement used (P value > 0.05%).

2. Oswestry Disability Index (ODI): The mean preoperative ODI was 91.7 points (SD±5.9). It was not done post operative as most of the sections of ODI questionnaire cannot be completed at this time as the questions about sexual life, social life and travelling. At 1.5 months the mean ODI was 37.3 points (SD±20). At 3 months the mean ODI was 29.8 points (SD±17.64). At 6 months the mean ODI was 25.7points (SD±23.2). At 12 the mean months ODI was 21.9 points (SD±19.2). The improvement outcome of all of these different values compared with preoperative ODI was found to be statistically highly significant (P<0.001). (Fig.4)

As regard to the effect of the type of fracture to ODI changes we found that there was no statistically significant difference (P value > 0.05) in ODI improvement between patients with osteoporotic or traumatic VCFs at any time in the study period.

Complications:

A- Cement leakage: The ce-

ment leakage occurs when the cement present at any site outside the vertebral body. It was assessed by x-ray and CT and it was classified in to 5 types Intradiscal, Vascular, Perivertebral, Canal and Foraminal leakages. The total incidence of cement leakage was 74% was found to be statistically highly significant (P<0.001). Single type of leakage was encountered in 25 vertebrae while mixed was encountered in 17 vertebrae (Table 1).

The incidence of cement leakage in osteoporotic injected vertebrae was 70% which was statistically highly significant (P<0.001). Also the incidence of cement leakage in traumatic injected vertebrae was 100% which was also statistically highly significant (P<0.001). The incidence of different types of cement leakage in osteoporotic in relation to traumatic injected vertebrae wasn't statistically significant (P>0.05) except vascular leakage which was significant in traumatic in relation to osteoporotic injected vertebrae (P<0.05).

There were statistically highly significant (P<0.001) difference between the cement amount in bipedicular (mean value was 6.34±2.54 cm^3) & the unipedicular (mean value was 3.88±1.45 cm3) approaches. But there was no statistically significant difference in any type of cement leakage with the approach used (P > 0.05). The incidence of perivertebral cement leakage increase with increasing the amount of cement used which was found to be statistically significant (P<0.05). In contrast to the other types of leakage they were not statistically significant (P>0.05) in relation to amount of cement used.

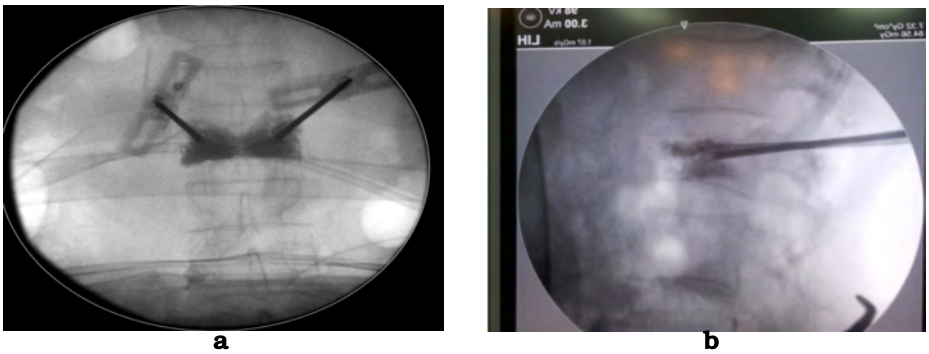
B- Neurological compromise: We had three patients (10.7%) with neurological compromise in our results. Canal and foraminal leakage had occurred in all of them while vascular leakage in one patient and perivertebral leakage in another patient. All of them were with osteoporotic vertebral fractures. Although it was not statistically significant (P>0.05) this leakage led to incomplete paraplegia in one patient and peripheral radiculopathy in two patients.

C- Adjacent and non adjacent

vertebral level fractures: Adjacent and non adjacent vertebral level fractures occurred in 3 cases (12%) and it was not statistically significant (P>0.05). Adjacent level fractures occurred in 2 patients (8%) while the non adjacent level fractures occurred in one patient (4%) in this study. None of the patients suffered any intraoperative complications of anaesthesia, positioning or any Infection in the post operative or thought the follow up period.

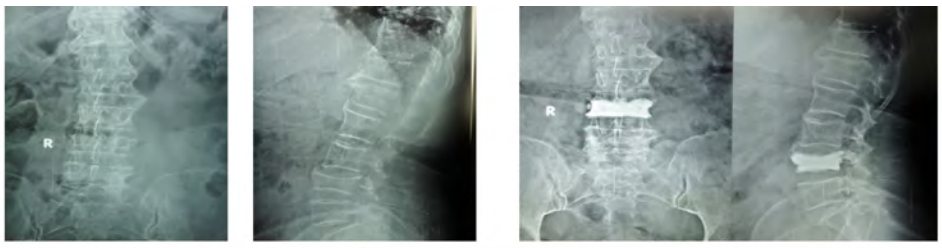
Table (1): The incidence of different types of cement leakage.

	No	%	P value
Perivertebral	11	26.2	< 0.001
Intradiscal	7	16.7	0.006
Vascular	3	7.1	0.078
Canal	22	52.4	< 0.001
Foramen	7	16.7	0.006



a **b**

Fig. 1: Injection of 1st lumbar vertebra under continuous fluoroscopic guidance; **a-** A p view, **b-** lat. View.



a b c d

Fig. 2: Female patient 72 years with OCF of the 4th lumbar vertebra with very severe pain, ODI 97.80%. Bipedicular approach with 12 cm cement were used. The post operative was VDS mild, ODI 6%at 1.5 months. a- Pre AP view. b- Pre lat. view. c- Post AP view. d- Post lat. view.

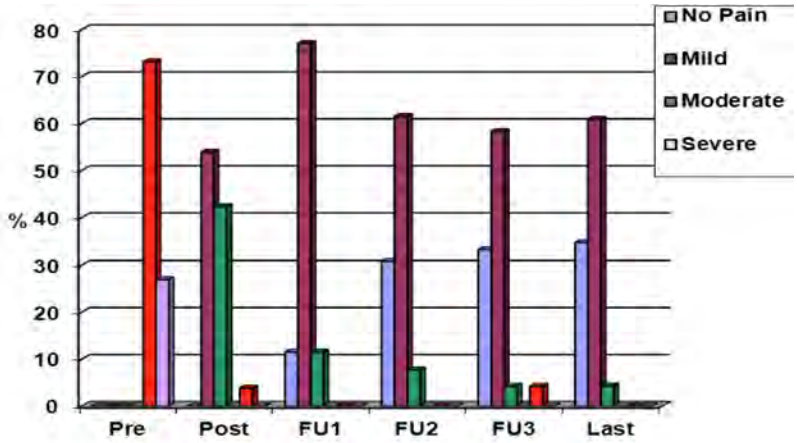


Fig. 3: Comparison between preoperative & postoperative, follow up one, two, three& last VDS Outcomes.

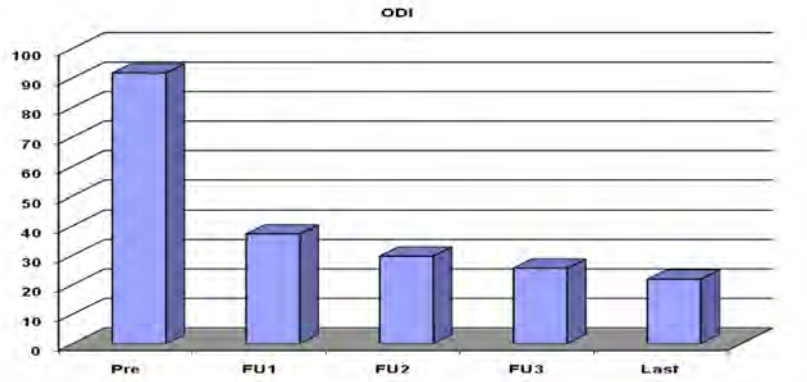


Fig. 4: Comparison between preoperative, postoperative, & follow up one, two, three& last ODI Outcomes.

Discussion

The results of this study showed that vertebroplasty was effective in markedly reducing pain and disability, and improving the quality of life in the majority of patients within a very short period of time.

The improvement outcome of these different values compared with preoperative VDS was found to be statistically highly significant. Pain relief was rapid and marked especially at 1.5 months with continued improvement and didn't decline with time. Pain relief

was obviously reflected on the activity level of the patient and his/her resumption of social activities which were obvious in ODI improvements. There were no significant differences in pain and disability Improvements due to the fracture etiology or the amount of cement used. As regard to pain improvement there is agreement with the results obtained by Heini et al (2000)[15], Carlier et al (2004) [10], Neto et al (2004)[8], Kobayashi et al (2005)[4] and Yehya & El-Nekady (2010)[9]. According to their studies the median scores of pain intensity were reduced significantly following vertebroplasty. Also they found this effect was long lasting.

There is disagreement with the results obtained by Buchbinder et al (2009)[11]. As they reported that there is no beneficial effect of vertebroplasty compared with a sham procedure in patients with painful osteoporotic vertebral fractures. Their results are challenged by many factors firstly; they reported that they used the analgesia in both groups without identifying the amount used in both of them as one of the most important aims of vertebroplasty is to eliminate or decrease the amount of analgesia due its effects on pain relief which may be higher in the group with sham procedure. Also their follow up was only up to 6 months so the long term effects of vertebroplasty cannot be assessed including the mechanical effects, improvement of sagittal balance through variable degrees of RVH and wedge angle restoration and decreasing the risk of adjacent fractures.

Although most PMMA augmented vertebrae have shown one type or more of cement leakage, the majority have been asymptomatic. The symptomatic ones were usually of neural origin. These types include; intradiscal, vascular, perivertebral, canal & foraminal leakage.

This high incidence of cement leakage was 74% may be explained by using C.T. in leakages detection and not only by using plain x-ray.

The incidence of cement leakage in osteoporotic injected vertebrae was 70% while in traumatic injected vertebrae it was 100%. The higher incidence of cement leakage in traumatic fractured

vertebrae may be explained by the presence of cracks in the vertebral body that was not healed or concealed with fracture hematoma and not apparent in preoperative x-ray films or the C.T. cuts. The incidence of different types of cement leakage in osteoporotic in relation to traumatic injected vertebrae wasn't statistically significant (P>0.05) except vascular leakage which was significant in traumatic in relation to osteoporotic injected vertebrae (P<0.05).

There was significant incidence of intradiscal cement leakage which was 16.7% between augmented vertebrae. It occurred due to presence of a communication between the vertebral body and the disc space. This communication is possibly due to either an end-plate fracture coinciding with the primary vertebral body fracture, or could be a result of end-plate fracture by the introduced needle used for injection.

Although the vascular cement leakage was not significant as it was occurred only in7.1% of augmented vertebrae, it was present in one case with neurological complication in association with canal and foraminal leakage. Although the canal cement leakage was statistically highly significant as it was occurred in 52.4% of augmented vertebrae it was associated with only three cases of neurological compromise. Also there was a significant foraminal cement leakage as it was occurred in 16.7% of augmented vertebrae and it was present in the three cases of neurological compromise.

There was highly significant incidence of perivertebral cement leakage as it was occurred in 26.2% of augmented vertebrae. In contrast to all other types of leakage it increases significantly with increasing the amount of cement used. Truumees et al (2004)[16] in their review of the literature stated that there was a mean rate of cement leakage ranging from 3% to 65% in different studies. Hulme et al (2006)[17] reviewed the literature and showed a mean rate of cement leakages occurred in 41%. In a review of 30 PVP studies, they reported the distribution of leakage was 32% canal, 32.5% paraspinal, 30.5% intradiscal, 3.3% foraminal, and 1.7% pulmonary in

PVP. It was noted that pulmonary complications occurred at a rate of 0.6% and neurologic deficits occurred in 0.6% per level treated. Although most cement leakages are asymptomatic, the long-term effects are unknown.

This study agree with the results obtained by Kobayashi et al (2005)[4] in their study as they found that the incidence of cement leakage was 75.6%. But despite this high incidence of leakage, they reported no clinical symptoms were seen. No major complications such as pulmonary embolism, osteomyelitis or radicular neuritis were observed. There was no significant change in blood pressure, heart rate or blood oxygen saturation. In our results the same occurred but we had 3 cases with neurological affection. Also in the study of Chen et al (2004)[18] on 6 patients they found that four vertebrae (66.7%) revealed evidence of PMMA leakage into the disc space and the paravertebral space, without any evident clinical symptoms. No intracanal leakage occurred; no patient needed a secondary surgical intervention. But the low incidence of complications may be attributed to the low number of cases in their study.

The incidence of cement leakage in this study is higher than that of Heini et al (2000)[15] in their study as they reported that the extraosseous cement leakage was noted in eight vertebral bodies (20%), five times (12.5%) into the paravertebral soft tissues, twice (5%) into the spinal canal and once (2.5%) into a segmental vein. also they reported that all of these cement extrusions remained without clinical sequelae.

Although leakage of cement into the spinal canal and neural foramen was well tolerated in most cases, as the neurological compromise associated with cement leakage was not statistically significant, it led to serious neurological complications. Intraforaminal leakage was found to be more harmful as its incidence was less than that of the canal leakage and both of them were present in the only three cases of neurological compromise. Although there were three cases of neurological compromise in this study, Neto et al (2004)[8] & Yehya & El-Nekady

(2010)[9] found no cases with neurological deficit in their series. In the other way there were many cases in the literature with varying degrees of neurological affection associated with PVP[19,20,21,22].

In this study adjacent and non adjacent vertebral level fractures were not statistically significant as it was occurred in 3 cases (12%). The incidence of developing adjacent vertebral level fractures was greater than that of non adjacent vertebral levels.

There is agreement with Trout et al. (2006)[22] as they reported that the relative risk of developing a new VCF at a level adjacent to a VP level is 4.62 times greater than at a nonadjacent level. It is still controversial whether adjacent level compression fractures after VP are a consequence of rigidity caused by augmentation with bone cement or simply due to the natural progression of osteoporosis. Studies of the natural history of VCF have reported a four times greater risk of developing additional VCFs after the initial one than in patients without VCF[23,24]. According to Hadjipavlou et al (2005)[25], a kyphotic deformity moves the centre of gravity forwards resulting in an increased forward bending moment which increases the load within the kyphotic angle and predisposes adjacent vertebrae to suffer secondary fractures.

There is disagreement with Lin et al (2004)[26] & Chen et al (2010)[27] as they identified that there were a statistically significant increase in the incidence of new fractures of adjacent vertebral bodies in patients with cement leakage into the intervertebral disc compared with that in patients without leakage. But in the present study the reported cases with adjacent or non adjacent vertebral fractures, there were no associated Intradiscal cement leakage. Also in the cases with Intradiscal leakage there were no associated adjacent or non adjacent vertebral fractures.

According to the results of this study there was no significant relationship between the amount of cement used and the clinical outcomes. Belkoff et al. (2001)[28] reported that only 2 cm3 of injected cement during vertebroplasty is

needed to begin restoring height of a collapsed vertebral body. They also showed that up to 8 cm^3 of cement might be needed to restore stiffness in the thoracolumbar region. According to Liebschner et al. (2001)[29] there are larger differences in individual vertebral body volumes. Different vertebral augmentation techniques allow injection up to 12 cm^3 of cement, and until now, there has been no data on how much a vertebra could take physiologically. Furthermore, surgeons usually subjectively estimate how much cement one can put in a vertebral body. Also Liebschner et al. (2001)[29] reported that only 14% (3.5 cm^3) cement- filled vertebral body was necessary to restore stiffness to its initial value. But different regions of the spine needed different amounts of cement. More cement in the vertebral body increased both strength and stiffness of the vertebra.

There is agreement with Kaufmann et al (2006)[30] as they demonstrated that there is no significant relationship between volume of cement injected in a compressed vertebral body and clinical outcomes of percutaneous vertebroplasty. they also claimed that vertebroplasty operators need not feel compelled to achieve particular volumes of cement injected but should be guided by their clinical sense of what constitutes an adequate and safe fill of a compressed vertebral body. Also Hadjipavlou et al (2005)[25] reported that the ideal amount of cement necessary to restore a desirable degree of local strength and stiffness has not been established.

Conclusion

Vertebroplasty is effective minimally invasive procedure in markedly and rapidly reducing pain and disability, and improving the quality of life in patients with osteoporotic and traumatic vertebral compression fractures. But it may lead to serious neurological complications.

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BENHA MEDICAL JOURNAL

PERCUTANEOUS VERTEBROPLASTY FOR THORACOLUMBAR VERTEBRAL FRACTURES

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VALUE OF CD56 AND Bcl-2 IMMUNOHISTOCHEMISTRY IN THE DIAGNOSIS OF HIRSCHSPRUNG'S DISEASE

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Abstract

Purpose: *The aim of this study was to investigate the expression of CD56 and bcl-2 in paraffin-embedded sections of rectal punch biopsy from patients suspected clinically to have Hirschsprung's disease.*

Patients and Methods: *Immunohistochemical (IHC) study for CD56 and bcl-2 was performed on archival material of 71 patients diagnosed with Hirschsprung's disease between January and December 2011.*

Results: *Seventy-one cases were included in the study. Based on H and E stained section prepared from paraffin blocks belonging to these patients; 10 cases showed identifiable ganglion cells in submucosa, between the two muscle layers or in both sites. In 54 cases, no ganglion cells could be identified. Seven equivocal cases were diagnosed as suspicious for the presence of ganglion cells. All cases demonstrating ganglion cells on H and E-stained sections showed positive membranous reaction for CD56 and positive cytoplasmic reaction for bcl-2 antibodies. In 8 cases (15%) with no detected ganglion cells by H and E staining, a positive reaction was shown in CD56-stained sections. Three cases (5%), as well, showed positive cytoplasmic reaction for bcl-2. Immunoreactivity for CD56 and bcl-2 had higher sensitivity in identifying ganglion cells when compared to routine stains. In 7 equivocal cases, a definite diagnosis could be reached.*

Conclusion: *Ganglion cells are identified more readily by IHC stains than by routine H and E stains. Immunohistochemical staining for CD56 and bcl-2 is a reliable diagnostic tool in cases of Hirschsprung's disease especially when ganglion cells are undetected or equivocal with routine stain.*

Introduction

Chronic constipation is a common and persistent problem in childhood, accounting for approximately 3-5% of visits to pediatric outpatient clinics and 10-20% of visits to pediatric gastroenterology clinics. Organic causes for constipation are anatomical such as colonic stenosis and duplication or neurological such as Hirschsprung's disease (HD) and intestinal neuronal dysplasia. Endocrine and metabolic causes should be considered as well. Hirschsprung's disease should be suspected in any newborn who fails to pass meconium within 24-48 hours after birth. The diagnosis of HD is made by means of rectal punch biopsy[1].

Congenital intestinal aganglionosis, or Hirschsprung's disease, is a malformation of the enteric nervous system, in which the obligate diagnostic feature is absence of intrinsic ganglion cells from the distal rectum and a variable length of contiguous bowel. Enteric neurons are derived from the neural crest and migrate caudally with the vagal nerve fibers along the intestine. These ganglion cells arrive in the proximal colon by 8 weeks of gestation and in the rectum by 12 weeks' gestation[2]. Multiple loci appear to be involved, including chromosomes 13q22, 21q22, and 10q. Arrest in migration leads to an aganglionic segment. This results in Hirschsprung's disease, which affects estimated 1:5,000 live births, with a male-to-female ratio of approximately 4:1[3]. Dysmotility occurs due to constriction of the aganglionic segment, which requires intrinsic innervations to relax[4,5].

The presence of hypertrophic nerve fibers in the myenteric and submucosal plexuses is a diagnostically useful finding. In 90% of rectal biopsies from patients with Hirschsprung's disease, nerves with diameters of 40 μm or greater can be detected[6,7,8]. These hypertrophic nerves represent extrinsic fibers, primarily from pelvic autonomic ganglia. In aganglionic bowel, small branches from hypertrophic nerves distribute to the muscularis propria and mucosa, but do not express synaptic proteins or other antigens that characterize the normal intrinsic innervation of these targets[4,9].

Hematoxilin and Eosin (H and E) staining and acetylcholinesterase staining (AChE) are commonly used in the diagnosis of Hirschprung's disease (HD). However, H and E stain is not always sufficient for accurate diagnosis, because it has limitations in the diagnosis of immature ganglion cells in neonates[10]. In these instances, the ganglion cells are few in number (3-5 cells per ganglion); small and irregularly distributed and so their identification is difficult. Also, immature ganglion cells may be unipolar or bipolar and can be mistaken for stromal cells [11].

Although acetyl choline esterase (AChE) activity is a diagnostically useful, it is not sufficient. AChE stains the parasympathetic nerve fibers and trunks of fibers that increase dramatically in the lamina propria of the mucosa and sub mucous layer, but is not a specific marker for ganglion cell [12]. One of the problems is the interference of red blood cell (RBC) acetyl cholinesterase due to hemorrhage in the lamina propria. Technical difficulties in addition to reports of false positive and false negative results have compromised the utility of this maneuver [12,13]. For this reason immunohistochemistry was studied and found to be a very helpful diagnostic adjunct in the diagnosis of HD and it helps to detect ganglion cells using CD56, bcl-2, Synaptophysin, and S-100 protein [15,16,17].

CD56 is a glycoprotein of the Ig-super family. Its antibody targets an isoform of the neural cell adhesion molecule (NCAM). It is believed to regulate cell motility, binding to heparin/heparin sulphate and collagens, stimulation of neurite outgrowth and fasciculation via the fibroblast growth factor receptor. CD56 encoding gene is located at 11q23-24 chromosome. It is expressed normally in NK cells, activated T cells, large granular lymphocytes, specific endocrine, and brain tissue, including neurons and glia cells, but not choroid plexus. It is also expressed in thin nerve fibers, fine varicose and sensory nerve endings, cell membranes of ganglion cells and fetal striated muscle cells. On the other hand, thick nerve fibers, perikarya of ganglion

cells and adult striated muscle fibers are CD56 negative. It was also found in the base of crypt cells in a bowel tissue, Meissner's corpuscles, axons of Pacinian corpuscles, various epithelia (enterocytes and newly formed bile ductular cells), ovarian stromal cells, uterine smooth muscle cells and osteoblasts[18,19,20,21]. Memarzadeh et al[11] reported that CD56 improved the accuracy of diagnosis of HD when used in combination with H and E especially in negative and suspicious cases.

Human bcl-2 protein is a 26 kD anti-apoptotic, membrane-associated oncoprotein. The gene encoding bcl-2 is located on chromosome 18. The bcl-2 protein can promote cell survival through protein-protein interactions with other bcl-2 related family members. The anti-apoptotic function of bcl-2 can also be regulated through proteolytic processing and phosphorylation. Bcl-2 may promote cell survival by interfering with the activation of the cytochrome c/Apaf-1 pathway through stabilization of the mitochondrial membrane. Mutations in the bcl-2 gene can contribute to cancers where normal physiological cell death mechanisms are compromised by deregulation of the anti-apoptotic influence of bcl-2[22,23,24].

uring the development of the normal nervous system, cell death takes place, resulting in a loss of 20% to 80% of all neurons. The bcl-2 protein is widely expressed in the central nervous system during development. On the other hand, bcl-2 expression is selectively retained in the adult peripheral nervous system, suggesting a role in neuronal survival. It is hypothesized that aganglionosis occurs as a result of apoptosis of neuroblasts that results either from intrinsic abnormalities within these cells or from deficient signaling by mesenchymal cells along their migratory pathway[25].

Expression of bcl-2 in enteric ganglion cells of the myenteric and submucous plexuses is displayed in the fetus and during childhood and is also retained in adult bowel. Consequently, immunohistochemical analysis of bcl-2 provides a good marker for identification of ganglion cells in Hirschsprung's disease and may also be valuable

for the diagnosis of disorders characterized by hypoganglionosis or hyperganglionosis[25,26].

In this study, we compare IHC results using CD56 and bcl-2 with H and E staining to find out the best diagnostic panel for HD.

Materials and Methods

This is a retrospective study. The database of pathology laboratory in Mansoura University Children Hospital (MUCH), Mansoura, Egypt, was searched and all cases diagnosed clinically with Hirschsprung's disease or suspected to have the disease and for whom a rectal punch biopsy was received in pathology laboratory, during the period from January to December, 2011 were retrieved from the archives.

Inclusion criteria were cases that underwent rectal punch biopsy in MUCH during this period with complete medical report, available pathology report, paraffin blocks, and lastly biopsy should be representative formed of mucosa, subucosa +/- snips of muscle layer. Cases with superficial biopsies that do not contain submucosal tissue to assess ganglion in meissner's plexus were excluded from the study.

For each of these cases, the pathology report was revised to obtain, demographic and clinical data. Original H and E sections were retrieved and reassessed for adequacy of the specimen, presence of ganglia, and hypertrophied nerve bundles. Cases were then categorized into three diagnostic groups ganglionic, aganglionic and suspicious for the presence of ganglion cells. Thereafter, the paraffin blocks of all cases included in the study were retrieved from the archive and recut at the thickness of 3-4 u on coated slides. Slides were then submitted for immunohistochemistry for CD56 and bcl-2 for the detection of ganglion cells. Results were compared with the results H and E stains.

Immunohistochemistry:

Immunohistochemistry was performed using the avidin-biotin-peroxidase complex method. Antibodies against the following antigens were used: CD56 mouse, monoclonal antibody, clone:

123C3.D5, 7ml prediluted, Cell Marque (cat NO 156M-88). Antigen retrieval was done in EDTA boiling in microwave at Ph 9, incubation 60 minutes at room temperature. Positive external control is neuroblastoma. Bcl-2, rabbit monoclonal antibody, clone ID: E17; Epitomics (Cat NO: 1017-1), dilution 1:100-1:200. Antigen retrieval was done in citrate buffer boiling in microwave at pH 6.0. incubation 60 minutes at room temperature. Positive control is tonsil tissue.

After antigen retrieval, 3% H2O2 and pure methanol were added for five minutes sections were washed with distilled water. Next primary antibodies were added for the recommended time for each. Biotinylated Secondary antibody was added for 10 minutes and washed. Streptavidin was added for 10 minutes and washed with phosphate buffered saline (PBS). After adding substrate chromogen for 10 minutes, the slides were counterstained and coverslipped with the mounting media. Slides were scanned for the positive brown reaction in ganglion cells.

CD56 immunostaining was considered positive if characteristic ganglion cell demonstrated strong complete membranous staining. While positive bcl-2 was identified by characteristic cytoplasmic staining of the ganglion cell.

Statistical analysis:

Data analysis was performed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, US). Data were shown as mean, standard deviation or number of cases and percentages. Diagnostic performances (e.g. sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) of IHC staining results for each marker versus H and E stains (i.e. gold standard) were also calculated.

Results

Seventy-one consecutive rectal punch biopsies were enrolled in the study. Patients ranged in age from 1 month to 11 years (mean 22 m + 25.2 months). In the aganglionic group more than half of patients (63%) were in their first year of life. To the contrary most of the ganglionic group patients (80%) were older than 1 year. Table 1 il-

lustrates age distribution of the three categories.

Forty-seven cases (66%) were males and 24 (34%) were females. As regards gender distribution within different groups, of the 10 cases with detected ganglion cells, 4 were males and 6 were females. In the group designated as aganglionic, 39 (72%) were males and 15 (28%) were females, consistent with the male predominance of Hirschsprung's disease. Seven cases were diagnosed as suspected for the presence of ganglion cells, 4 (57%) of these were males and 3 (43%) were females.

Presenting symptoms in patients were constipation in 34 cases (48%), abdominal distention in 10 cases (14%), and meconium plug syndrome in 5 cases (7%), bowel obstruction in 2 cases (3 %), and one patient (1.4%) was presented with fecal incontinene. However, in the remaining 19 cases (26.6%), no definite clinical complaint was reported and Hirchsprung disease was suspected by the physician upon clinical examination. All patients underwent rectal biopsies for detection of ganglion cells.

Microscopic study of H and E-stained sections of these 71 cases revealed absence of ganglion in 54 cases (76%), presence of ganglion cells in 10 cases (14%) and suspected presence of ganglion cells in 7 cases (10%). Thorough examination of serial H and E-stained sections revealed presence of hypertrophied nerve bundles in 6 cases (87%) of the equivocal group and in 26 cases (48%) of the aganglionic one.

Immunohistochemistry results:

Immunohistochemical staining for CD56 applied to cases with definite ganglion cells, detected in H and E-stained slides, revealed membranous staining of ganglion cells in all cases. On the other hand 8 (15%) cases with no detected ganglion cells on H and E stain revealed positive membranous reaction that highlighted ganglion cells (figure 1&2). In the group diagnosed as suspected presence of ganglion cells, 3 (42%) cases were positive for CD56.

Cases diagnosed as ganglionic

on the basis of H and E were confirmed by positive cytoplasmic reaction for bcl-2. Of the 54 cases reported aganglionic through H and E staining, 51 (95%) were negative and three (5%) were positive (figure 3&4). Out of seven cases suspected of having ganglion cells on H and E staining, two cases (28%) turned to be ganglionic by demonstrating positive cytoplasmic reaction for bcl-2, while in the remaining 5 (72%) cases, IHC for bcl-2 revealed negative results and the final diagnosis was aganglionic. Table 2 illustrates comparison of H and E and IHC results.

Validation and agreement of immunohistochemical staining versus H and E staining results, which is the standard method in our laboratory (Table 3), revealed a higher sensitivity and NPV (negative predictive value) in CD56 stain (100% for both) compared to bcl-2 (94% and 77% respectively). On the other hand, specificity and PPV (positive predictive value) were higher in bcl-2 than CD56 (67% and 90% versus 56% and 85%, respectively).

Table (1): Age distribution of the three diagnostic categories.

Age	With ganglion cells (n=10)	Without ganglion cells (n=54)	Suspected ganglionic (n=7)
< 1year	2 (20 %)	34 (63 %)	1 (14%)
1year	8 (80 %)	20 (37 %)	6 (86%)

Table (2): Comparison between H and E results and IHC results (CD56, bcl-2).

	Ganglionic (n=10)	Aganglionic (n=54)	Suspicious (n=7)
CD56 +ve	10 (100%)	8 (15%)	3 (42 %)
CD56 -ve	0	46 (85%)	4 (58 %)
bcl-2 +ve	10 (100%)	3 (5%)	2 (28 %)
bcl-2 –ve	0	51 (95%)	5 (72 %)

Table (3): Validation and agreement of IHC markers against H and E results.

IHC markers	Sensitivity [95% CI]	Specificity [95% CI]	PPV [95% CI]	NPV [95% CI]
CD56	100% [92.2% -100%]	56% [30.8 % - 78.4 %]	85% [72.9 %- 93.4 %]	100% [69 % - 100.00 %]
bcl-2	94% [83.1 % -98.6 %]	67% [38.4 % -88.1 %]	90% [78.6 % - 96.7 %]	77 % [46.2 % - 94.7 %]

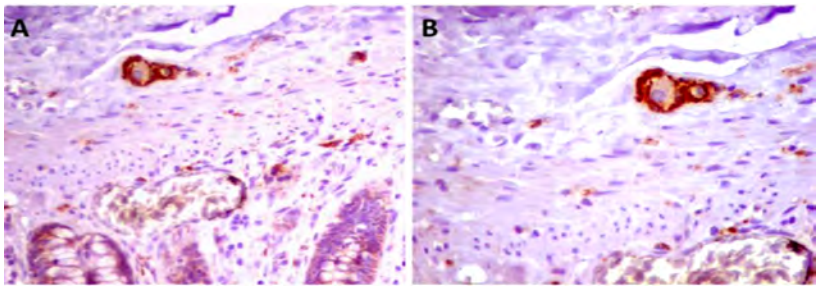

Fig. 1: Immunohistochemistry for CD56 on rectal punch biopsy: showing large cells exhibiting positive membranous reaction easily detectable at low magnification in submucosa. This pattern excludes HD. **A)** (original magnification X200). **B)** (original magnification X400).

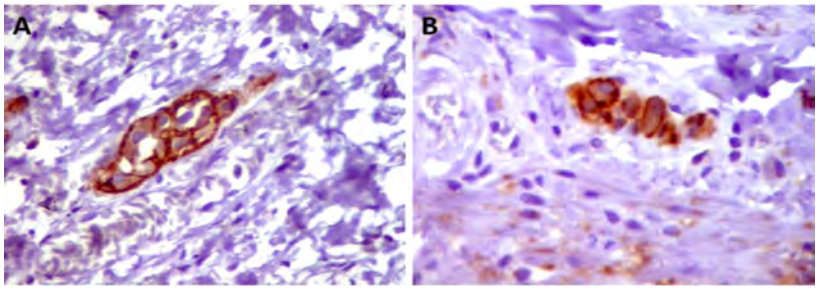

Fig. 2: (A&B) Immunohistochemistry for CD56 on rectal punch biopsy two suspicious cases for HD, showing with large cells exhibiting positive membranous reaction in the submucosa. This pattern excludes HD (original magnification X400).

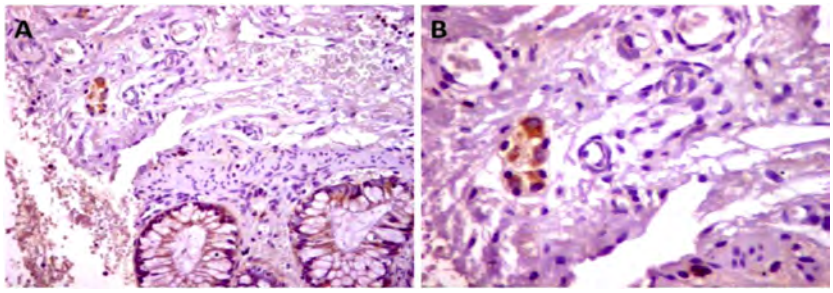

Fig. 3: Immunohistochemistry for bcl-2 in rectal punch biopsy for suspicion of HD showing typical cytoplasmic reaction for bcl-2 in submucosal ganglia **A)** (original magnification X200). **B)** (original magnification X400).

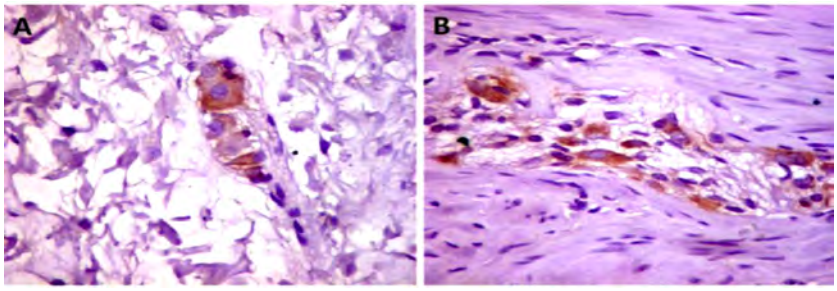

Fig. 4: Immunohistochemistry for bcl-2 in rectal punch biopsy for suspicion of HD. **A)** Typical cytoplasmic reaction for bcl-2 in large cells within submucosa. **B)** Typical cytoplasmic reaction for bcl-2 in large cells in the inter muscular space. This pattern excludes HD (original magnification X400).

Discussion

Hirschsprung's disease (HD) is the most important type of intestinal pseudo-obstruction in neonates. Detection of ganglion cells in H and E-stained sections may be difficult because the ganglion cells of young infants, the population most often investigated for HD, are morphologically immature and can be readily confused with endothelial cells and plasma cells [13]. This difficulty in interpretation may result in inadequate resection of bowel segments or resection of unnecessarily long segments of bowel[2].

As a consequence, the need for a more reliable method for identification of ganglion cells has stemmed. Researchers have concentrated on immunohistochemical analysis because of its worldwide availability[13]. A large number of immunohistochemical staining protocols with diverse antibodies have been proposed to assist in the identification of ganglion cells. These concluded that ganglion cells were easier to identify immunohistochemically rather than with H and E staining alone [17]. In this work, the role of immunohistochemical staining for CD56 and bcl-2 in diagnosis of HD was evaluated.

This retrospective study was performed on archival material of patients for whom rectal punch biopsies were received at the pathology laboratory of Mansoura University Children Hospital during

the year 2011. Statistical analysis was performed to explore diagnostic performances of the immunohistochemical results versus H and E.

In the current study, patients ranged in age from 1 month to 11 years (mean 22 m + 25.2 months). These data are not far from those obtained by Guinard-Samuel et al [10], whose series had a lower mean age (15 month). Difference may be explained by the fact that MUCH is a referral centre and most of these patients had been managed or treated in other primary health care centers before being investigated or biopsied in MUCH. To the contrary in Kacar et al[13], their patients had a slightly higher mean age (27.8±34.1) months.

In the aganglionic group more than half of patients (63%) were in their first year of life. These findings are not far from those were reported by Kannaiyan and coworkers[14], as 83.3% of their cases had the same age. Difference may be explained by different sample size and the relative early seek of medical care in developed countries.

In the two groups designated as negative or suspicious for the presence of ganglion cells male gender predominated female one (72% & 57% versus 28% & 43% respectively) and the reverse occurred in the group which was diagnosed as positive for the ganglion cells based on H and E stain. This distribution was consistent with the male predominance of Hirschsprung's disease as reported in literature[3]. kacar et al[13], as well as Holland et al[17], found similar results.

Clinically, Hirschsprung's disease should be suspected in any newborn that presents with constipation and/or failure to pass meconium within 48 hours of life. In the current patient cohort, the leading presenting symptoms were constipation, abdominal distension and meconium plug syndrome accounting for (48%, 14% and 7% of cases respectively). Meanwhile bowel obstruction and fecal incontinence were reported in (3% and 1.4% of patients respectively). However, in the remaining 19 cases (26.6%) no definite clinical complaint was reported in the pathology report

and Hirchsprung disease was suspected by the physician upon clinical examination. Similar results were reported by Holland and associates[17]. In their retrospective study, they found that constipation, abdominal distension and meconium plug syndrome were the main presenting symptom accounting for 42%, 19% and 16% of patients, respectively.

The neural hypertrophy in the rectal submucosa is associated with aganglionosis and is a surrogate marker for the disease[8]. Monforte-Munoz et al[6] found that submucosal nerve trunks that are 40 micron or greater in diameter strongly correlate with abnormal innervation/ aganglionosis. In the present work, thorough examination of multiple sections of rectal punch biopsies, revealed hypertrophied nerve bundles in 26 out of 54 cases (48%) of the aganglionic group. This figure is quite lower than that reported in other studies performed by Monforte-Munoz et al [6], Sangkhathat et al[7] and Holland et al[17]. Hypertrophied nerve fibers (larger than 40 microns in diameter) were found in 81%, 90% and 92% of their series respectively. The marked difference between their results and ours can be explained by the fact that all of the previous studied used an objective methods namely digital imaging and image analysis software and morphometry which are not available at our lab at the time of the study. As an alternative, the presence of hypertrophied nerve bundles was determined on a subjective basis with comparison to sections from normal ganglionic colonic biopsies.

Although H and E staining remains the gold standard method for identifying ganglion cells, H and E analysis has some limitations, especially in infants who are the majority of Hirschsprung's patients. In these instances, ganglion cells are small, immature and few in number. As a result, these cells can be easily overlooked on routine stain[17].

CD56 immunohistochemistry holds several advantages, such as: it is carried out on a formalin-embedded rectal biopsy, its staining pattern is simple and distinct, and it is either positive or nega-

tive. It is expressed in the ganglia of normal colon and in the ganglionic colonic segments of patients with Hirschsprung's disease. Ganglion cells are immunoreactive for CD56[15]. However, thick peripheral nerve elements are nonimmunoreactive, as are endothelial cells and fibroblasts, leading to readily identifiable ganglion cells[11,18].

In the current work, microscopic study of H and E-stained sections of 71 specimen revealed absence of ganglion in 54 cases (76%), presence of ganglion cells in 10 cases (14%) and suspected presence of ganglion cells in 7 cases (10%). On the other hand evaluation of specimens through IHC for CD56 revealed that 8 (15%) cases with no detected ganglion cells on H and E stain exhibited positive membranous reaction that highlighted ganglion cells (figure 1&2). In the group diagnosed as suspected presence of ganglion cells, 3 (42%) cases showed positive reaction for CD56. These findings are close to that reported by Memarzadeh and co-workers[11]. In their cases, 6.7% of the group classified as aganglionic based on H and E stain revealed the presence of ganglion cells which could be detected after examination of sections submitted for IHC for CD56. Seven cases of their cohort were diagnosed as suspicious for the presence of ganglion cells on routine stain basis. After IHC, 5 cases (71.4%) of demonstrated ganglion cells highlighted by a membranous reaction for CD56.

Validation and agreement of IHC for CD56 versus H and E stain which is the standard method used in our laboratory to detect ganglion cells reveals that CD56 has 100% sensitivity. However the superiority of the latter stems from its discriminative ability in suspicious cases which could save the patient an equivocal diagnosis or the risk of another repeat biopsy. Moreover, 8 cases were initially diagnosed as aganglionic, after IHC for CD56 proved to contain ganglion cells. In these instances IHC for CD56 could save the patient mis-diagnosis and consequently mismanagement. These findings were similar to that reported by Memarzadeh and associates[11], as they concluded that CD56 is the best marker for detection of ganglion cells especially in

negative or suspicious biopsies.

Cases diagnosed as ganglionic on the basis of H and E were confirmed by positive cytoplasmic reaction for bcl-2. Of the 54 cases reported aganglionic through H and E staining, 51 (95%) were negative for bcl-2. However in 3 cases (5%), bcl-2 immunohistochemistry clearly disclosed the enteric neurons of the myenteric and submucous plexuses showed positive reaction for bcl-2 and the final diagnosis turned to be ganglionic biopsy (figure 3&4).

Out of seven cases suspected of having ganglion cells on H and E staining, two cases (28%) turned to be ganglionic by demonstrating positive cytoplasmic reaction for bcl-2, while in the remaining 5 (72%) cases, IHC for bcl- 2 revealed negative results and the final diagnosis was aganglionic. These results are not far from those reported by Wester et al[25] and Song et al[26]. As bcl-2 positively stained ganglion cells in the ganglionic segment in both myenteric and meissner's plexuses. Meanwhile it was not detected in the aganglionic segment. However their specimens were all of colonic resection after confirmed diagnosis and they did not include any rectal punch biopsies and no case was primarily diagnosed as suspicious in their studied patients. The researchers concluded that Expression of bcl-2 in enteric ganglia of the adult bowel indicated that bcl-2 is involved in promoting survival of enteric neurons throughout life. In patients with Hirschsprung's disease, bcl-2 immunohistochemistry may serve as an additional marker for this diagnosis[25,26].

Validation and agreement of immunohistochemical staining results for bcl-2 versus H and E results, which is the standard method in our laboratory, shown in table 3, revealed that bcl-2 had a lower sensitivity and NPV than CD56 (94% and 77% versus 100% and 100 % respectively). Hence, it can be used as an adjunct for diagnosis with another marker for ganglion cells. In the research undergone by Park et al, similar results were found. Hirschsprung's disease was diagnosed in 77% of pediatric intestinal pseudo obstruction cases with the help of

bcl-2 and other IHC marker. They concluded that among different IHC markers, bcl-2 was the most helpful diagnostic adjunct to detect immature neurons[15].

Conclusion

Based on sensitivity indices, our results suggest that a combination of routine H and E-stained sections with intervening sections submitted for immunohistochemical staining using either CD56 +/- bcl-2 is an optimal method for evaluating rectal punch biopsies for Hirschspung's disease. A more cost-effective method is to reserve unstained sections for future immunohistochemical analysis until the initial evaluation of H and E-stained sections. If the initial examination identifies ganglion cells or deems a biopsy specimen insufficient, the unstained slides are not subjected to immunohistochemical evaluation. However, if the specimen is adequate and no ganglion cells are found by H and E examination, the intervening unstained slides are evaluated for CD56, bcl-2 or a combination of both markers by immunohistochemical analysis. The diagnostic value of CD56 and bcl-2 in aganglionic and equivocal cases mandates that no case should be diagnosed as such based on H and E stain alone, without IHC testing for at least one of these markers.

Noteworthy this technique could be applied on samples taken for frozen sectioning during intraoperative consultation and may help to take an accurate and beneficial decision. This point of research may be recommended for future studies.

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VALUE OF CD56 AND Bcl-2 IMMUNOHISTOCHEMISTRY IN THE DIAGNOSIS OF HIRSCHSPRUNG'S DISEASE

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ROLE OF IMAGING IN PRE-OPERATIVE ASSESSMENT OF COCHLEAR IMPLANT CANDIDATES

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Abstract

Objective: *To assess role of CT and MRI in preoperative evaluation of cochlear implant candidates as regard candidacy for operation and selection of the side of implantation.*

Patients and methods: *This study included 46 patients. For preoperative assessment, 45 patients were evaluated by high resolution CT (HRCT) of petrous bone, contrast material was used only in one patient. Forty three patients were evaluated by MRI using mainly 3D fast imaging enabling steady-state acquisition (FIESTA) technique, also contrast material was used in one patient only.*

Results: *This study enrolled 46 patients with severe to profound sensory neural hearing loss (SNHL), 23 male and 23 female with their ages ranged from 1 to 40 years. The mean age was 7.47 year (5.8 for males and 6.4 for female). Forty five patients underwent non contrast CT examination, while 43 patients underwent non-contrast MRI. Contrast media was administrated in two patients (one in CT and the other in MRI examination). Data from the preoperative CT and MRI examinations were collected and reviewed as regarde definite analytic parameters. Radiological data were confirmed by audiologic, otologic and behavioral tests.*

Conclusion: *Results of this study suggested that CT and MRI has a complementary role in preoperative evaluation of cochlear implantation candidates.*

Key words: *Cochlear implantation; multislice CT; 3D FIESTA; cochlear congenital malformation.*

Introduction

SNHL is a major cause of childhood disability worldwide, with an estimated prevalence of 1 in 2000 neonates and 6 in 1000 children by 18 years of age. Early diagnosis and treatment of SNHL especially in children is critical because it is well recognized that a delay in identification of hearing impairment can adversely affect speech and language development[1].

Cochlear implantation has become an accepted treatment for severe to profound deafness in patients who derive only minimal benefit from conventional amplification. It is important to be familiar with the various available imaging options and with findings that could significantly impact or even preclude implantation[2].

High-resolution computed tomography (HRCT) and MRI of the temporal bones provide vital information; these are baseline investigations and are necessary in all patients posted for cochlear implant surgery[3]. Computed tomography and MRI provide different, but complementary information. CT is excellent for demonstrating bony details of the temporal bone, mastoid pneumatization and cochlear patency. However, CT is inadequate for visualizing inner ear neural structures, fluid or fibrosis. MRI is superior to CT in demonstrating inner auditory canal nerves, retro cochlear diseases and membranous alterations of the inner ear. However, it fails to provide information about bone structures and is more costly[4].

Patients and Methods

Patients: A total number of 46 patients (23 males and 23 females) of severe to profound sensorineural hearing loss were enrolled in this study from December 2009 to October 2013 in Mansoura University Hospital, Department of Radiodiagnosis. All patients were referred from ENT department and out patients clininc. They failed to make appropriate progress during hearing aid trials. Their ages ranges from one to 40 years old with mean age of 7.47 (8.5 for males and 6.4 for females). The majority of patients (40 patients representing 89%) were of pediatric age group. A complete review of hearing history was the first step in evaluating a

cochlear implant candidate. All patients then underwent a series of hearing tests to qualify for a cochlear implant. A complete neurologic, head and neck examination was also performed. Particular attention was paid to evidence of subtle otologic malformations and syndromic features.

Our patients were subjected to the following radiological examinations; high resolution CT scan was done in 45 patients, while 43 patients were imaged by non- contrast MRI with contrast material was only used in two patients (one in CT and one in MRI).

Surgical planning was done after analysis of the radiological findings with considering audiological and behavioral data. Thirty five patients underwent unilateral cochlear implantation by the otorhinolaryngolists in Mansoura University Hospital.

Methods:

1-High resolution CT:

Forty five were imaged by a standardized protocol of temporal bone HRCT scanning. CT was done by a 64 row multi-slice volume scanner (Brilliance 64 CT scanner, Philips Healthcare, Best Netherlands). Forty four patients were scanned without contrast administration in the axial plane (parallel to the orbitomeatal line). The remaining patient had CT finding of bilateral destructive lesion in both petrous bones so, needed administration of intravenous contrast by injection of non-ionic contrast material (Omnipaque 350 mg/ml) at a dose of 1-2 ml/kg and infusion rate of (3 ml/ sec).

Image parameters:

The images were obtained in 1mm slice thickness, 0.5 second rotation time, o.75 pitch factor, 140 KV tube voltage, 160 mA tube current, a 200 mm scan field of view (FOV), 512x512 matrix, window level=600 and window width= 4000. The image is reconstructed with slice thickness=0.6mm.

Post processing:

This includes generation of 2D reformations and 3 D reconstruction as follows:

• 2D reformats in the coronal and sagittal planes.

• Multiplanner reformatted images (MPR) in:

- Single oblique sagittal long and short axes of the cochlea for depiction of anatomy of cochlear turns, cochlear aperture and modiolus.
- Single oblique sagittal in long axis of both round window and vestibular aqueduct.
- Double oblique sagittal of facial nerve canal

• 3D reconstructions were made by using maximum intensity projection (MIP) for visualization of cochlear turns and measuring cochlear distance.

Image analysis:

All scans were analyzed on a viewing workstation using a modified guidelines for the preoperative check list of cochlear implant candidates, as published by Chaturvedi et al.,[5] so as to ensure consistency of reporting and not to miss any finding. The most important presurgical implantation CT check list includes cochlear presence, morphology and patency, status of modiolar, vestibular and SCC morphology, size of vestibular aqueduct, bony cochlear nerve canal (BCNC), cochlear aqueduct and IAC {with comparison to indices reported by Joshi et al.,[6]}, course of facial nerve, patency of middle ear pathway, and other incidental findings (bone thickness, jugular bulb, sigmoid sinus, carotid canal and emissary veins).

2- MRI examination:

MRI was done in 43 patient using 1.5 T MRI scanner (SIGNA HDe 1.5-T GEHC, USA). The examination was carried out after screening for contraindication to MRI examination. Children patients (40 patients) were sedated before examination by chloral hydrate at a dose of 0.5 ml/kg. Older patients were instructed to prevent movement of head during scanning to overcome motion artifacts. The examination was done using standard head coil.

Patients underwent non-contrast MRI examination of both brain and temporal bone imaging. Temporal bone MRI examination includes conventional turbo spin echo sequences and high resolution 3D-FIESTA (fast imaging enabling steady-state acquisition) sequence in axial and sagittal oblique planes. Contrast administration was done in one female (14 years) presented by postlingual

SNHL (sensorineural hearing loss), with MRI findings of NF2 (neurofibromatosis type II). She was injected with 10 ml of gadolinium-based contrast material (Magnevist).

Image parameters:

- 2D TSE of temporal bone: T1: TE=11, TR=420, T2: TE=107, TR= 4320, echoes=1, slice thickness=2mm, FOV=15cm, matrix 320x129, Nex=2.

- 3D gradient echo of temporal bone (FIESTA: fast imaging enabling steady-state acquisition): TE=3.3/Fr, TR=9, FOV=18x16, slice thickness=1.0 mm, matrix 320x320, Nex=2, scan time=4 minutes, reconstruction of image was done in sagittal oblique plane for visualization of IAC contents of nerves.

Image analysis:

The presurgical implantation checklist includes assessment of labyrinthine morphology and patency, modiolar deficiency, size of endolymphatic duct and sac, cochlear nerve integrity and intra-axial lesions.

Statistical analysis:

Statistical analysis of the data was done by using Statistical Package for Social Science (SPSS) version 17.0. Data were expressed as frequencies and percents. Chi-square test was used for comparisons of categorical data. Significance was considered when P value <0.05. All graphic representations of the data were performed with MicrosoftO Excel for windows (Microsoft Inc., USA).

Results

This study enrolled 46 patients with severe to profound SNHL, 23 male and 23 female with their ages ranged from 1 to 40 years. The mean age was 7.47 year (5.8 for males and 6.4 for female). Forty five patients underwent non contrast CT examination, while 43 patients underwent MRI; contrast material was injected in only two patients. Data from the preoperative CT and MRI examinations were collected and reviewed as regard previously described analytic parameters. Radiological data were confirmed by audiologic, otologic and behavioral tests.

The patients were divided according to the onset of hearing loss into three main categories: peri-natal, pre-lingual (deafness before patient began to speak),

and post-lingual (deafness after acquisition of speech) hearing loss. Only four patients presented with perinatal onset SNHL (8.7%). Thirty five patients presented with post-natal pre-lingual SNHL (76.1%); while seven patients (15.2%) presented with post-lingual SNHL.

The patients were distributed according to wide spectrum of etiological factors (Table 1). The most common etiological factor was congenital SNHL (35 patients representing 76% of the total number of the patients) with none of them showed syndromic manifestation, however, sex patients (representing 13%) had a positive family history. The second commonest cause was perinatal factors (8.7%); two females had history of prematurity, one had perinatal hypoxia and the other had history of congenital infection.

Seven patients (15.3%) were presented with SNHL due to various non-congenital causes. One of them had a tympanogenic labyrinthitis ossificans. Another one had bilateral symmetrical otospongiosis. Trauma was the cause in only one patient while neoplastic lesions were the cause in two patients. Two patient had idiopathic SNHL with no significant history could be obtained.

As regard the patients with congenital sensorineural hearing loss, 11 patients (23.8%) were found to have congenital malformation of the inner ear. The first had bilateral cochlear nerve aplasia with absent bony cochlear nerve canal (BCNC). He had associated common cavity malformation on the right side and cochlear aplasia on the left side with bilateral narrowing of IAC. This was supported by behavioral and functional data in the form of ABR (auditory brain stem response) test.

Bilateral incomplete partition type I malformation (IP-I) (figure 1) was found in three patients with dilated vestibule and dysplastic SCC in two of them. One of them showed normal BCNC and cochlear nerve, while the other two showed stenotic right BCNC with absent cochlear nerve on the same side.

Two patients had unilateral incomplete partition type II malformation (IP-II) consisting of cochlear dysplasia, modiolus deficiency yet dilated vestibule and vestibu-

lar aqueduct were seen only in one patient with normal IACs and cochlear nerves in both of them.

One patient had normal morphology of inner ear with a stenotic left BCNC and deficient cochlear nerve, yet size of IAC was normal.

Four patients had dilated vestibular aqueduct (VA) (figure 2) in only 7 ears, three of them showed isolated enlargement of the duct, while in the remaining patient the dilatation was a part of IP-II.

Out of 4 patients with perinatal-caused SNHL, one patient showed unilateral labyrinthitis ossificans after history of congenital infection. The other 3 patients had unremarkable radiological studies.

As regard the non-congenital causes of the SNHL, one female patient had cochlear ossification. She had a history of labyrinthine fistula after recurrent cholesteatoma (figure 3&4). Both CT and MRI showed subsequent calcification with virtually no significant lumen available for implantation.

One patient had otosclerosis (figure 5&6) and one had a history of left petrous fracture not intersecting cochlea or IAC suggesting cochlear concussion.

Two patients showed neoplastic lesions, one of them showed bilateral jugulo-tympanic glomus with destruction of the IAC and basal turn of the cochlea only on the left side, the other patient was a teenager with SNHL who showed NF2 with deep intracanalicular extention of the vestibular schwannomas.

Imaging findings determined the side selected for implantation as follows:

a) Two out of four patients with high jugular bulbs had unilateral high bulb which altered the surgeon to approach the opposite side for a safe surgery.

b) In three patients there was a unilateral large emissary veins in retromastoid portion, this altered the surgeon to choose the opposite side.

c) In two patients with thickened temporal bone on one side, the other side was selected for more easy and time saving procedure.

d) In patients with unilateral stenotic BCNC (three), the other side was selected for successful operative outcome.

e) One patient with unilateral dilated vestibular aqueduct the other side was chosen for avoiding CSF gusher.

We compared between HRCT and MRI effectiveness in imaging of anatomical details of the ear (figure 7), and found that sex patients of congenital malformation of the cochlea had CT and MRI equally described the morphological abnormality in contour and internal architecture, however in one other patient CT showed globular appearance of the cochlea simulating that in IP-II malformation, however, when the patient underwent MRI there was a clear separation of complete cochlear turns with preserved sufficient scalar and modiolar anatomy. MRI significantly clarified the details of scalar and modiolar than CT did. In addition, MRI permitted subjective comparison between both moduli in the same patient which confirm diagnosis in cases of IP-II. No significant difference between the two modalities in assessment of both vestibule and SCCs. Visibility of vestibule and SCC was nearly the same in all patients apart from two patients. The first had otospongiosis with CT efficiently demonstrated osteolysis of the otic capsule with patent vestibule and SCC. However MRI was more sensitive in demonstration of luminal occlusion by fibrovascular tissue .In the second patients of tympanogenic labyrinthitis ossificans, both CT and MRI detected calcification, however, in the case of MRI revealed the extensive affection of labyrinthine structures much more better than CT did.

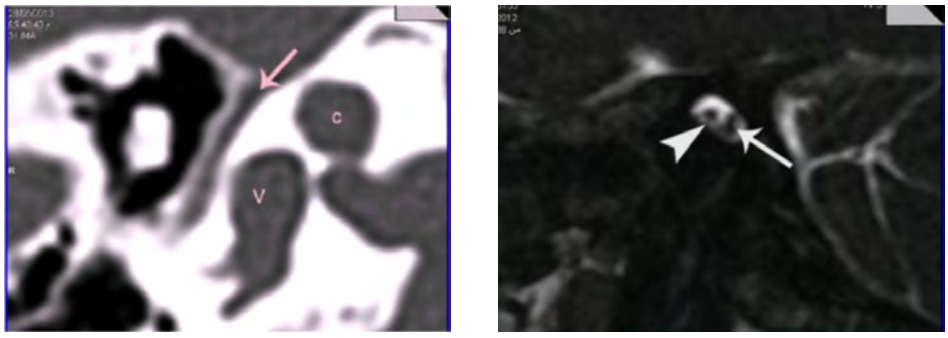
Bony cochlear and vestibular aqueducts could only be visualized by HRCT. Vestibular aqueduct was seen along its whole length in 40 (88.8%) out of 45 patients who underwent CT scan, while it was hardly/non-visualized in 5 patients (11.2%). It was dilated in 4 patients; three of them showed corresponding dilated endolymphatic ducts (ELDs) on MRI. On MRI, ELD was not visualized in 2 patients (3 temporal bones) in spite of normally visualized vestibular aqueduct on HRCT scans.

Cochlear nerve could be assessed only by using MRI. It was seen in 40 patients, while was absent in three patients. Out of those patients with absent cochlear nerve on MRI, one showed stenotic IAC while all the three showed stenosis of their cochlear aperature on CT scan. Another patient with bilateral stenotic IAC and absent cochlear aperture whose MRI study was not available, yet absence of cochlear nerve was confirmed by audiometric and behavioral tests. Computed tomography was much more accurate in measuring diameter of bony cochlear aperture.

Opacification of middle ear was visualized efficiently by both CT and MRI, however, CT was superior to MRI in detection of bony complication in two patients of cholesteatoma. Detection of opacification of mastoid air cells was better with MRI as it detected subtle changes. Also, CT was superior MRI in detection of abnormalities of facial nerve canal. CT detected emissary veins and asymmetric thickness of reteromastoid bone.

Table (1): Patients distribution according to various etiological factors.

Etiology		No. of pt.		Total
Congenital	Congenital non familial	♂	15	29
		♀	14	
	Familial & syndromatic	♂	0	0
		♀	0	
	Familial& Non-syndromatic	♂	5	6
		♀	1	
Prenatal and Perinatal factors	Congenital infection	♂	0	1
		♀	1	
	Perinatal Hypoxia	♂	0	1
		♀	1	
	Prematurity	♂	0	2
		♀	2	
Idiopathic		♂	1	2
		♀	1	
Inflammatory		♂	0	1
		♀	1	
Trauma		♂	1	1
		♀	0	
Noise exposure		♂	0	0
		♀	0	
otosclerosis		♂	0	1
		♀	1	
Neoplastic lesions		♂	1	2
		♀	1	

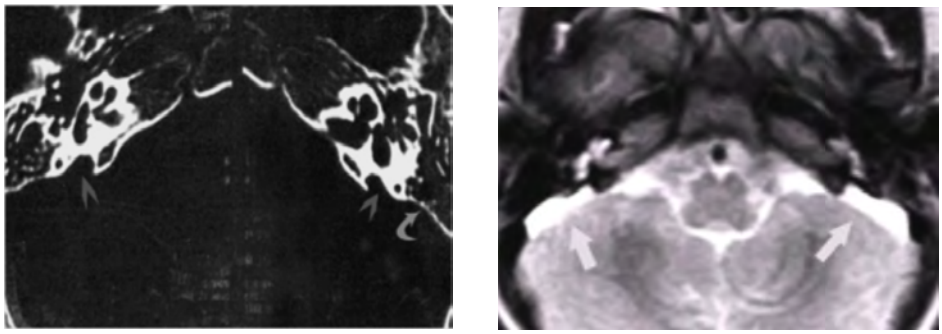


(A) (B)

Fig.1: incomplete partition type-I malformation.

A) Normal tympanic segment of facial nerve with intact bony canal with exposed geniculate ganglion (arrow), c: ccochlea showing cystic configuration with loss of internal architicture, V: vestibule showing dilatation with short truncated lateral SCC.

B) FIESTA MRI with sagittal oblique view through the Rt. IAC showing presence of only three nerves; facial (arrow head) and two branches of vestibular (arrow) with absent cochlear nerve.



(A) (B)

Fig.2: Dilated vestibular aqueduct.

A) HRCT axial image showing dilatation of the vestibular aqueduct bilaterally (arrow heads) in comparison to adjacent posterior SCC. Note the scalloping of the medial aspect of the mastoid bone (curved arrow) suggesting dilated endolymphatic sac.

B) Axial T2WI MRI showing bilateral enlargement of endolymphatic ducts and sacs (solid arrows).

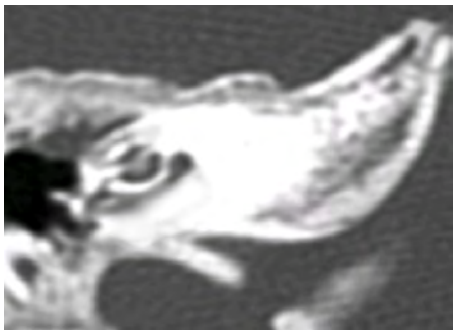


Fig.3: Labyrinthitis ossificans Axial Ct scan showing increased attenuation of the Rt. Cochlea (mainly basal turn) with rarefaction of otic capsule ovelying right cochlea and middle ear opacity of cholesteatoma.

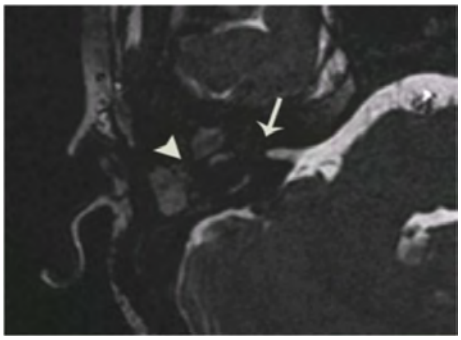


Fig.4: Labyrinthitis ossificans Axial FIESTA showing intermediate signal in right middle ear and mastoid air cells (arrow head) with no detected signal in the inner ear (arrow) due to calcification.

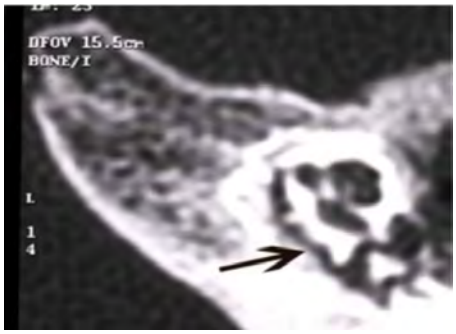


Fig.5: CT of otospongiosis axial images showing pericochlear peri vestibular and perimeatal osteolysis (double ring sign).

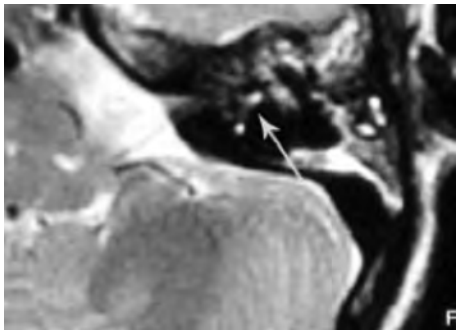


Fig.6: MRI of otospongiosis axial T2 showing intermediate signal in the left inner ear (arrow) with loss of fluid signal of membranous labyrinth (suggesting luminal obliteration) with areas of high signal is seen in mastoid air cells.

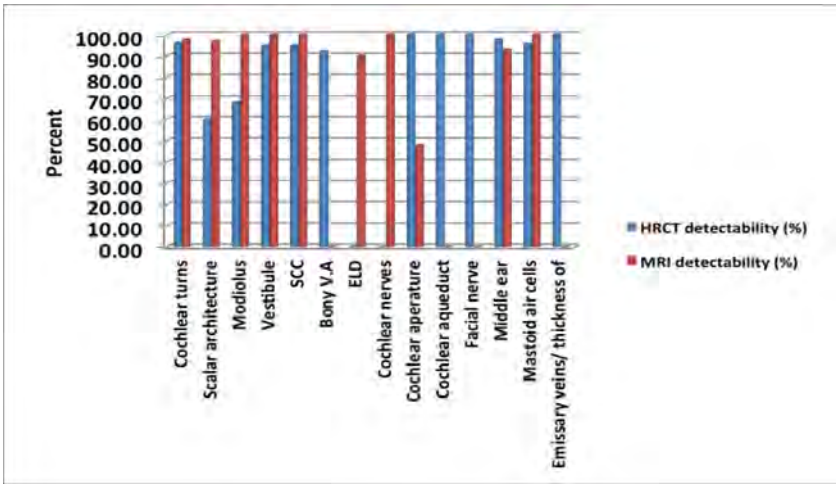


Fig.7: bar chart demonstrating comparison between CT and MRI in detection of various anatomical structure of inner ear.

Discussion

The efficacy of cochlear implantation for the treatment of pre- and post-lingually deaf children and post-lingually deaf adults is well established[7]. Accordingly, imaging the auditory pathway of the implant candidate is necessary to screen for morphologic conditions that will preclude or complicate the implantation process[8].

The aims of preoperative Imaging are to answer the following questions: 1. Are there cochleo - vestibular anomalies? 2. Is there evidence of luminal obstruction? 3. Are there additional findings that can complicate surgery or post-operative recovery, such as middle ear disease and anatomic variation?[5]. A crucial part of determining suitability for implantation and for choosing the side for implantation is preoperative radiologic imaging[7].

High-resolution computed tomography (CT) and magnetic resonance (MR) imaging provide excellent delineation of the anatomical details of the inner ear: CT depicts the minute details of osseous structures, and MR imaging allows visualization of the fluid-filled spaces and the vestibulocochlear nerve. Together, these

complementary modalities can aid decision making about the best management strategy by facilitating the identification and characterization of inner ear malformations and any associated neurologic abnormalities[6].

Out of our 35 patients with congenital SNHL, only 11 patients (23.8 %) showed congenital malformation of their inner ears ranged from cochlear aplasia to isolated dilated vestibular aqueducts. This percentage matched the results of Gupta et al.,[3] who reported that congenital malformations of the inner ear are rare anomalies; they can be identified on imaging with HRCT and/or MRI in about 20% of patients with congenital sensorineural hearing loss.

In the current study, various cochlear malformation were presented. Only one case showed unilateral cochlear aplasia with ipsilateral vestibular dysplasia and contralateral common cavity malformation. This was associated with bilateral stenotic IACs and absent cochlear aperature suggesting cochlear nerve aplasia. This agreed with Shama.,[9] who found that out of 7 cases of cochlear aplasia in his study, 6 of them had unilateral involvement and all the 7 cases had absent cochlear nereves. Also, he found that all cases of unilateral aplasia were associated with dysplasia in the other ear.

In the present study, three cases were presented with IP-I anomaly with cystic cochlea absent modiolus, dilated vestibule and dysplastic SCC. Similar findings were described by Sennaroglu et al.,[10]. Only one of our patients with IP-I anomaly had a dilated IAC but with no fundic defect. This was inconsistent with results of Swartz & Mukhreji.,[11] and Joshi et al.,[6] who reported that the cribriform area between the cochlea and IAC is often defective, and all patients of IP-I have a large IAC, predisposing them to increased risks for meningitis and for a perilymphatic gusher in the event of surgery.

In this study, two patients had main CT and MRI criteria of IP-II anomaly with 1.5 turn of cochlea instead of 2.5 (due to fusion of

middle and apical turns). Both of them had deficient modiolus. Only one of them showed associated dilated vestibule and enlarged vestibular aqueduct. This was inconsistent with Sennaroglu et al.,[10] who found that all cases of IP-II had an enlarged vestibular aqueduct. However, Aygun et al.,[12] described milder form of cochlear dysplasia which is due to incomplete development of apical turn of cochlea which occur at week 8 of gestation resulting in modiolus deficiency with mild asymmetry of scala vestibule and tympani. It is not always associated with enlarged vestibular aqueduct which agreed with our study.

In the present study, four cases (three males and one female) with 7 petrous bones (36.3% of congenital malformation in this study) had dilated vestibular aqueduct on HRCT, three of them were males. It was bilateral in 85%. This is agreed with study of Joshi et al.,[6] who mentioned that enlarged vestibular aqueduct is bilateral in 90%, may be asymmetric, however they found this abnormality is slightly more in females.

In the early era of cochlear implantation, cochleovestibular malformations were considered a contraindication to implantation due to concerns about proper electrode insertion, array stability, absent or dysfunctional neurons that might preclude significant auditory perception, and the increased risk of complications such as facial nerve injury and cerebrospinal fluid leak[1].

According to the classification of cochleovestibular malformation which was first proposed by Jackler et al.,[13], complete absence of cochlea or cochlear nerves bilaterally were considered the traditional contraindication for implantation because of their poor postoperative outcome[14].

Thus, we had only one of our patients who was absolutely contraindicated for the surgery, as he had bilateral cochlear nerve aplasia with associated common cavity anomaly on the right side and cochlear aplasia on the left side, bilateral narrow IAC and absent BCNC (by HRCT). His MRI examination was unavailable, so the diagnosis of absent nerve was sup-

ported by audiologic tests. Absent nerve was seen in other three cases unilaterally; two had bilateral IP-I anomaly with a stenotic right BCNC, the third had normal inner ear structures with narrowed left BCNC. MRI findings suggesting CND. This agrees with Hang et al., [1] who reported that the diagnosis of cochlear nerve deficiency (CND) is based on both high-resolution CT and MRI findings. They concluded that on HRCT, a bony cochlear nerve canal (BCNC) of less than 1.3-1.4mm or internal auditory canal (IAC) of less than 3mm is suggestive of CND. A closed BCNC confirms the diagnosis. Roche et al.,[15] reported that, in 38% of CND cases on MRI there was normal-sized IAC and BCNC on HRCT, and in order to avoid a missed diagnosis of CND, MRI has been suggested as the first-line imaging modality over HRCT. The pitfall of MRI may be in the case of a narrow IAC in which resolution may be insufficient to identify separate nerves within the canal. In such cases the study of Kutz et al.,[16] found that intact auditory nerve fibers may be present but not bundled as a separate nerve, making detection by MRI difficult.

In the past, the diagnosis of CND was a contraindication for cochlear implantation; however, Vlastarkos et al.,[17] found that there was evidence that these patients may still benefit from cochlear implantation despite imaging findings on the basis of possibility of presence of small auditory fibers beyond resolution of imaging modalities.

These wide variations in inner ear malformations may influence the surgeon's technique for electrode placement. In addition, the surgeon must consider the associated facial nerve abnormalities and increased risk of perilymphatic/cerebrospinal fluid (CSF) leakage when planning the surgical approach[18].

In the present study, two female patients had cochlear calcification. The first one was 8 years old and had a long history of recurrent cholasteatoma and mastoidectomy. HRCT and MRI revealed complete ossification of membranous labyrinth with no residual patent lumen which contraindicate implantation. The second case shows brain parenchymal

and partial cochlear calcifications. Chaturvedi et al.,[5] reported that cochlear implant is contraindicated only if there is total or near total bilateral cochlear ossification. Fortunately, in most cases the ossification is partial and imaging can guide the surgeon to the less affected side. Witte et al.,[2] reported that cochlear ossification makes the cochleostomy more challenging and often results in choosing an implant with a shorter electrode array.

In the present study, both CT and MRI cochlear picks up calcification but MRI was more sensitive in detection of extensive labyrinthine affection. Abdullah et al.,[19] and Chaturvedi et al.,[5] found that MRI was superior to HRCT in assessment of cochlear patency. This was probably because early fibro-ossific changes do not manifest on CT and can be missed.

In our study one case was presented with bilateral symmetrical reterofenestral otosclerosis with pericochlear osteolysis on CT and luminal fibrosis on MRI. Gomes et al.,[20] reported that fenestral otosclerosis is most frequently associated with conductive hearing loss, while cochlear otosclerosis induces sensorineural deafness due to involvement of the basilar membrane. Witte et al.,[2], found that retrofenestral otosclerosis is not a contraindication for cochlear implantation. However, facial nerve stimulation following transplantation is more common in patients with otosclerosis and is likely related to conduction of current through otospongiotic bone. This problem is usually corrected by programming out the electrodes causing the stimulation.

In the current study, one female (14 years) presented by bilateral SNHL. On MRI, the diagnosis of NF2 was established. Celis-Aguilar et al.,[21] reported that bilateral vestibular schwannomas in a NF2 patient can invade and grow within the cochlear nerve, while unilateral sporadic vestibular schwannoma (VS) only compresses it. Cochlear nerve function is not always preserved in spite of anatomic preservation of the cochlear nerve. An intact nerve does not necessarily mean a normal nerve histologically. Sennaroglu and Ziyal.,[22] concluded that bi-

lateral hearing loss due to neurofibromatosis Type 2 (NF2) was the initial indication of auditory brainstem implantation.

The present study revealed that HRCT remains superior to MRI in assessment of bony structures, facial nerve (in all patient imaged by CT scan and vascular variants; 11 patients had anteriorly protruded sigmoid sinus and 6 patients had jugular bulb abnormalities. In addition, status of middle and external ears could be efficiently assessed by CT scan particularly in the patients with cholesteatoma (3 patients); CT detected bony erosions in two of them. Conversely, MRI was much better than CT in imaging of cochlear nerve integrity (in all patients imaged by MRI), cochlear patency (two patients) as well as brain and CPA. Adunka et al,[23] and Joshi et al.,[6] who agreed that MRI is more sensitive than CT in evaluation of critical conditions for the surgery and mentioned that the consequences of missing cochlear nerve deficiency or brain pathology might result in inappropriate treatment of the patient. However, Vlastarakos et al.,[17] found that that the dual modality imaging is essential in the assessment of cochlear implant candidates which was consistent with our results.

Conclusion

Both CT scan and MRI provide vital information in the preoperative evaluation of the cochlear implant candidates as regard the etiology of SNHL, fitness of patient for implantation, and the selection of appropriate side for implantation.

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ROLE OF IMAGING IN PRE-OPERATIVE ASSESSMENT OF COCHLEAR IMPLANT CANDIDATES

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EARLY INITIATION OF GnRH ANTAGONIST VERSUS LONG AGONIST PROTOCOLS IN PCOS PATIENTS UNDERGOING ICSI CYCLES

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Abstract

Purpose: *To compare the efficacy of early initiation of GnRH antagonist in comparison with the GnRH agonist protocol in OCP pretreated PCOS patients undergoing ICSI cycles.*

Materials and methods: *Prospective randomized controlled trial. University-based fertility center. Seventy infertile PCOS patients under 35 years of age coming for the first trial IVF/ICSI treatment at the infertility care unit were included in the study. They were randomized to an ovarian stimulation protocol consisting of either GnRH agonist long protocol (control group A) or early initiation of GnRh antagonist (study group B) after pretreatment with OCP. Ovulation was triggered with hCG when at least three mature follicles 18 mm were detected. All patients were scheduled for ICSI. The primary outcome measures were the number of gonadotrophins ampoules used per each cycle, the duration of stimulation, number and degree of maturity of oocytes retrieved, rate of fertilization and available embryo for transfer. While secondary outcome measures were implantation rate, incidence of OHSS.*

Results: *Age, body mass index, duration of infertility, basal FSH,LH, E2 at the first day of the cycle, the number of oocytes retrived, the total number of embryos, chemical, clinical pregnancy and implantation rates were comparable in both groups and there was no statistically significant difference. The stimulation period was shorter in group B than group A and number of ampoules of HMG and Serum E2 level on the day of hCG administration were significantly lower in group B than that*

of the group A with statistically significant differences. Fertilization rate and the number of grade A embryos was higher in group B compared to in group A and this difference between both groups was statistically significant. Moderate OHSS was documented in 4 cycles of group A but was not in any cycle of group B and this difference was statistically significant. No cases of severe OHSS were documented in both groups.

Conclusions: *This novel antagonist protocol may be a safe and efficient treatment for PCOS patients undergoing ICSI cycles with comparable results to the standard long GnRH agonist protocol.*

Introduction

Polycystic ovary syndrome (PCOS) is the most common cause of anovulatory infertility, affecting approximately 5%-10% of reproductive-age women. Approximately 85% of these women have elevated levels of Luetinizing hormone (LH) resulting in the arrest of ovarian follicular growth[1]. The optimal infertility treatment for PCOS women is still a matter of controversy. In 2008, a consensus was reached on treatment for PCOS patients that includes the use of clomiphene citrate, exogenous gonadotrophins, laparoscopic ovarian surgery and in vitro fertilization (IVF)[2]. A proportion of PCOS women do not respond to the conventional treatments and ultimately require assisted reproductive technology (ART). Ovarian stimulation in women with PCOS poses a particular challenge, as many of these women exhibit exaggerated response[3].

Since the early 1980s, the GnRH agonists in ovarian stimulation were used to reduce the incidence of premature LH surges by suppressing gonadotrophin release via pituitary desensitization following an initial short period of gonadotrophin hypersecretion. Since 1999, GnRH antagonists with high potency and fewer side effects have been introduced into IVF and have emerged as an alternative in preventing premature LH surges[4].

GnRH agonists and GnRH antagonist were used in PCOS patients undergoing IVF/ICSI for controlled ovarian stimulations with variable results. But none of these until now is ideal for these patients. Most of the randomized control trials (RCT) comparing GnRH antagonists versus agonists have employed either

a fixed (starting on day 6) or a flexible dose (starting when the leading follicle reaches 14-15 mm)[5].

One of the currently debatable issues regarding the use of GnRH antagonists refers to the timing of GnRH antagonist initiation. Research for alternative GnRH antagonist protocols regarding the timing of its initiation has shifted towards earlier antagonist initiation than Day 6 of stimulation[6].

There is a theoretical benefit in the addition of GnRH antagonists to controlled ovarin hyperstimulation (COH) protocols, especially in patients with PCOS with high LH levels, during the follicular phase. But profound inhibition of endogenous LH at early stage may result in faulty folliculogenesis[7]. We performed this prospective randomized controlled trial to compare the early initiation antagonist protocol with the standard GnRH agonist long protocol for PCOS patients undergoing ICSI treatment.

Materials and Methods

Study design

The present study is a prospective randomized controlled study conducted at Mansoura University Hospital, Fertility Care Unit during the period from October 2009 to December 2012. The patients were randomly allocated into two groups by using computer-generated randomization: group A (agonist group) and group B (antagonist group). All Participants signed an informed consent after explaining the two protocols of controlled ovarian hyperstimulation to them.

Patient population

Seventy Patients with PCOS (based on the Rotterdam consensus criteria) who have PCO by Ultrsound, under 35 years of age coming for the first trial IVF/ICSI treatment at the infertility care unit were included in the study. Both groups (group A and B) were matched as regard age, body mass index (BMI) and duration of infertility. Additional inclusion criteria are: basal serum FSH of <10 mIU/ ml, uterine cavity without abnormalities and normal semenogram according to the WHO criteria (WHO, 2010). Patients with known previous poor ovarian response were excluded.

Ovarian stimulation and Monitoring of the cycle:

All patients received oral con-

traceptive pill (OCP) contained 30 µg ethinyl E2 and 75 µg gestodene (Gynera, Bayer Schering, Germany), starting on Day 2 of Spontaneous or induced menses of the cycle prior to the treatment cycle, after Blood test confirmed the presence of a baseline hormone profile.

Patients in GnRH agonist group (group A) received the standard long GnRH agonist protocol. Triptorelin (Decapeptyl@, Ferring, Germany) 100 µg was given via subcutaneous injection starting 3 days before discontinuation of the oral contraceptive. On day 2 of menstrual cycle (day 1 of stimulation), a daily injection of HMG (Merional@, IBSA, swizerland) was administrated at 225 IU IM After documentation of pituitary desensitization (absence of ovarian follicles of >4m and the absence of a thick endometrium on transvaginal ultrasound examination and serum estradiol <50 pg/ml) The administration of Triptorelin (Decapeptyl@, Ferring, Germany) was continued until the day of HCG administration.

In the GnRH antagonist group (group B), patients started daily HMG (Merional@, IBSA, swizerland) at 225 IU IM on Day 1 of stimulation. Subcutaneous cetrorelix (Cetrotide@, Serono) 0.125 mg (0.5 ampoule) was given daily concomitantly with HMG from the Day 1 of stimulation up to the leading follicle reaches 14mm. At this time subcutaneous cetrorelix (Cetrotide@, Serono) 0.25 mg (1ampoule) was given daily until the day of hCG administration.

In both groups, serial transvaginal ultrasound examinations using vaginal probe were performed to monitor the follicular growth. The starting dose of HMG was then adjusted individually depending on the ovarian response, as assessed by E2 levels and ultrasound. A step-down protocol was used, if necessary. Endometrial thickness was also assessed on the day of HCG administration.

Ovulation was triggered with 10 000 IU of IM hCG (Choriomon@, IBSA, swizerland) when at least three mature follicles 18 mm were detected on ultrasound scan. Transvaginal ultrasound guided oocyte retrieval was done. All pa-

tients were scheduled for ICSI. Embryo transfer procedure was arranged 48-72 hours after oocyte retrieval. Grade A and B embryos were transferred under transabdominal ultrasound guidance. All patients received luteal phase support with I.M. progesterone 100 mg (Prontogest@, IBSA, AMSA, Italy) daily starting on the day of oocyte retrieval.

Outcome measures

The primary outcome measures were the number of gonadotrophins ampoules used per each cycle, the duration of stimulation, number and degree of maturity of oocytes retrieved, rate of fertilization and available embryo for transfer. While secondary outcome measures were implantation rate, incidence of OHSS.

Statistical analysis

Statistical analysis was carried out via both Statistical Package for Social Science (SPSS) version 17 on windows XP. Qualitative data were represented in the form of number and frequency, while quantitative data were represented in the form of mean ± standard deviation (mean±SD). Kolmogrov-smirnov test was used to test normality of quantitative data, and some data were non-normally distributed. Student's t test, Mann-Whitney and chi square tests were used to compare groups. The difference is considered significant if P value is less than or equal 0.05.

Results

Seventy patients were initially randomized but three patients in group A and four patients in group B were lost follow up. There were four patients in group A and two patients in group B cancelled early due to exaggerated ovarian response and did not complete the follow up after counseling the couple regarding the risk of life-threatinig OHSS.

There were no statistically significant differences between two groups as regard age, body mass index (BMI), basal FSH, LH, serum E_2 at the first day of cycle and type and duration of infertility. Most of the patients were cases of primary infertility in both groups.

There were statistically significant differences between both groups as regard the stimulation

period, number of ampoules of HMG and Serum E2 level on the day of hCG administration while there were no statistically significant difference between the two groups as regard endometrial thickness at the day of hCG administration.

No statistically significant difference was detected in the total number of oocytes and degree of maturity of retrieved oocytes in the two groups.

Fertilization rate was higher in group B compared to in group A and the difference between both groups was statistically significant. There was no statistically significant difference implantation rate in the two groups.

There was no statistically significant difference in the total number of embryos in the two groups. The number of grade A embryos was higher in group B than in group A and this difference was statistically significant. There was no statistically significant difference in the number of Grade B embryos beteen the two groups.

There was no statistically significant difference in Chemical, Clinical pregnancy rates in the two groups.

Moderate OHSS was documented in 4 cycles of group A (14.29%) but was not documented in any cycle of group B (0%). This difference was statistically significant (p=0.035).

Table (1): Baseline characteristics in the two groups.

	Agonist group (A) N=28	**Antagonist group (B) N=29**	**p**
Age (years) Range **Mean ± SD**	21- 35 28.04±4.43	22- 34 27.45±3.66	0.587
BMI(kg/m2)	31.03± 2.34	30.12±1.25	0.071
Duration of infertility (years)	7.2 ± 3.01	7.31 ± 3.36	0.893
Type of infertility • **Primary** • **Secondary**	 85.7% 14.3%	 89.7% 10.3%	0.650

Table (2): Duration of stimulation, number of HMG ampoules, E_2 and endometrial thickness at hCG day in the two groups.

	Agonist group N=28	Antagonist group N=29	p
Duration of stimulation (days) Mean ± SD	11.25±1.69	9.48± 0.83	0.000
Number of ampoules of HMG	32.46±7,34	26.59± 2.67	0.000
Serum E2 (pg/ml) Mean ± SD	3012.57±845.31	2441.55±493.30	0.003
Endometrial thickness (mm) Mean ± SD	10.44±.90	10.42±0.90	0.967

Table (3): Number and degree of maturity of retrieved oocytes in the two groups.

	Agonist group (A) N=28 Mean ± SD	Antagonist group (B) N=29 Mean ± SD	p
Total number of oocytes	10.18±3.9	10.55±2.92	0.683
number of GV oocytes	1.71±1.61	1.55±1.24	0.888
number of MI oocytes	.75 ± 1.3	.55±.91	0.985
number of MII oocytes	7.71± 2.79	8.85±2.8	0.326

Table (4): Fertilization rate in the two groups.

	Agonist group N=28	Antagonist group N=29	p
Fertilization rate	77.49%	87.89%	0.004
Implantation rate	14.99%	15.94 %	0.96

Table (5): Number of embryos and their grades in the two group.

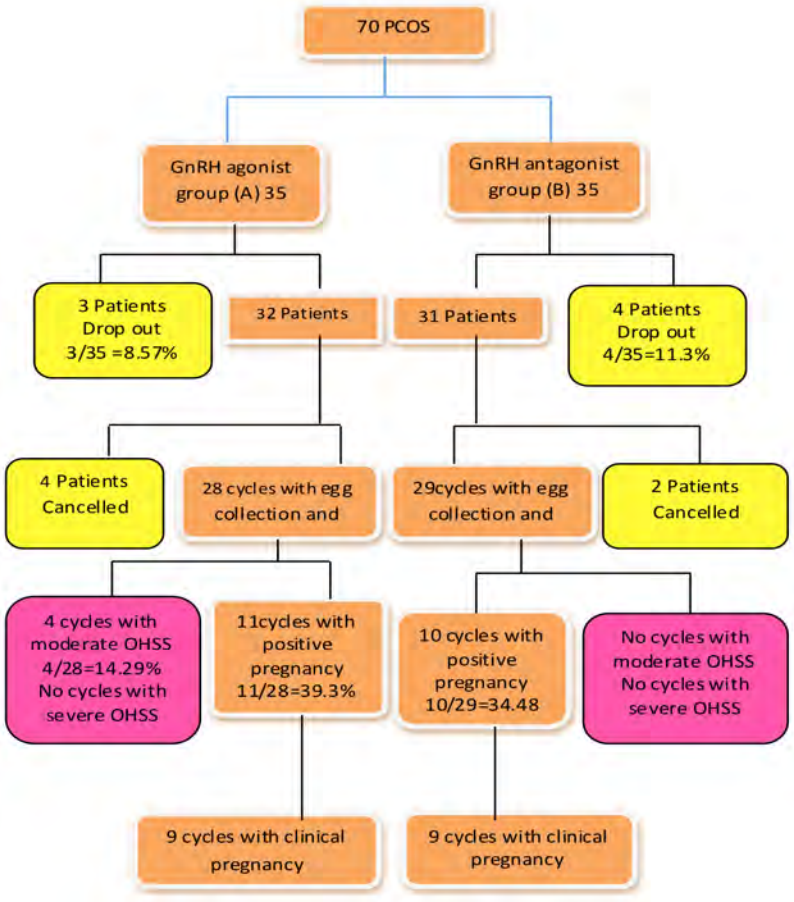
	Agonist group N=28 Mean ± SD	Antagonist group N=29 Mean ± SD	p
Total number of Embryos	6.12±2.64	7.38± 2.58	0.072
Grade A Embryos	4.57±2.17	6.38± 2.31	0.004
Grade B Embryos	1.50±1.75	0.97± 0.73	0.947

Table (6): Pregnancy rates in both groups

	Agonist group N=28	Antagonist group N=29	p
Chemical pregnancy rate	11/28=39.3%	10/29=34.48%	0.71
Clinical pregnancy rate	9/28=32.1%	9/29=31.00%	0.928

Table (7): Moderate OHSS in both groups

	Agonist group N=28	Antagonist group N=29	p
Moderate OHSS	4/28=14.29%	0/29=0%	0.035



Flow chart for the study

Discussion

Many RCTs have compared the GnRH agonist long luteal protocol to the GnRH antagonist protocol. The antagonist based protocols allowed to reduce the dose of gonadotrophins used and the duration of the stimulation regimens. Besides, the antagonist granted a more physiological pattern of follicular recruitment, with fewer small growing follicles and lower E2 levels, reducing the risk of severe ovarian hyperstimulation syndrome (OHSS). However, the retrieved oocytes were significantly fewer and a trend towards lower pregnancy rate (PR) could be noticed in most of the RCTs[8].

Five of these RCTs were examined in a meta-analysis published in a Cochrane review (Al-Inany H et al,. 2002) that confirmed the previous findings as regards the duration of the stimulation protocol, the amount of gonadotrophins used, the number of oocytes retrieved and the E2 levels on the day of hCG administration. Additionally, this meta-analysis showed a significant reduction in clinical pregnancy rate and failed to prove a significant preventive effect over severe OHSS[9].

However, a 5% higher clinical pregnancy rate did not match with an increase in live-birth rate according to a subsequent meta-analysis (Kolibianakis E.M et al., 2006) comparing agonist and antagonist[10].

In addition to that, the greater safety of GnRH antagonist over GnRH agonist has been definitively demonstrated in a recent version of the Cochrane review (Al-Inany H et al,. 2011)[11], in which a further clinical advantage has been detected, i.e. the reduction in the number of cycles cancelled due to OHSS risk. Furthermore, this review suggested that GnRH antagonist administration provides comparable results to traditional GnRH agonist stimulation, as opposed to previous works. In particular no significant differences concerning live-birth rates, ongoing pregnancy rates, miscarriage rates per clinical pregnancy rate, and rates of cancellation due to poor ovarian response were reported[11].

Despite the undeniable advantages provided by GnRH antago-

nist, their efficacy is still debated. Therefore, scientific research on the use of GnRH antagonist in COH for IVF/ICSI has been promoted even in recent years[8].

There were many studies in IVF cycles that compare the long agonist protocol with the antagonist protocol in PCOS patients. Most of these were used antagonist protocols either fixed or flexible and only two were used early initiation of the antagonist protocol[12].

Five of these RCTs were examined in a meta-analysis published by Mancini et al 2010[12]. The authors in this meta-analysis did not find a statistically significant difference between the use of a GnRH Antagonist protocol versus the standard long protocol in the incidence of pregnancy and of abortion in patients with PCOS undergoing IVF. The Antagonist protocol also seems to reduce significantly the incidence of OHSS. In this meta-analysis, they could not study the total doses of gonadotrophins used, nor the duration of stimulation or the number of cumulus-oocyte complexes. However, previous studies all agreed that the antagonist protocol is more patient friendly. Still, since the antagonist can be started alternatively on the first day of stimulation, on day 6, or according to the follicles diameter, to date no comparative studies have been made in PCOS to determine if there is a protocol that works best for this population[12].

The two Currently comparative studies have been published in PCOS patients between GnRH agonists and early initiation antagonists (Hwang et al., 2004[13] and Lainas et al., 2007[6]). In Hwang et al., 2004[13], the GnRH antagonist cetrorelix was started one day prior to initiation of stimulation with HMG and was compared with the long agonist protocol in 60 PCOS patients while in Lainas et al., 2007[6], antagonist ganirelix was started in the first day of stimulation and was compared with the long agonist protocol in 78 PCOS who received OC pill treatment for three weeks. In both studies full dose of antagonist (1 ampoule) was administered from the start to the end.

The current study is designed

to compare long agonist protocol with early initiation of antagonist (day-1 of stimulation) in PCOS patients undergoing ICSI who received OC pretreatement. It is differed in methodology from both studies of early intiation of antagonist protocol in PCOS patients as we start with the half dose (Cetrotide 0.125ml) from the first day of stimulation until the leading follicle reached 14 mm then full dose (Cetrotide 0.25ml) upto the day of hCG administration.

The goal of this study is maintaining low LH level throughout the follicular phase. This goal is clearly achieved when using the long GnRH agonist protocol, and it seems that because of these accomplishments the clinical results from the long agonist protocol were superior compared with results from the standard GnRH antagonist protocol.

This modification is similar in concept to the long GnRH agonist protocol and yet maintains the advantages of the GnRH antagonist protocol. The addition of the GnRH antagonist in early follicular phase of the cycle to the flexible antagonist treatment in this study achieves this goal. We believe that this novel modification in low resources countries optimize follicular recruitment and maximize the results without increase in the cost.

In the current study, the mean duration of stimulation was shorter in group B compared to group A. These results are comparable to results of Hwang et al., 2004, Lainas et al., 2007 and almost all comparative studies between long agonist ant antagonist protocols. The difference in our study was highly significant and this came in agreement with Lainas et al., 2007.

The significant reduction of number of ampoules of gonadotrophins in antagonist group when compared to the agonist group found in our study. Also, these results are comparable to results of Hwang et al., 2004 and most of comparative studies between long agonist and antagonist protocols. The difference in our study was highly significant and this came in agreement Hwang et al., 2004 but Lainas et al., 2007 did not comment on the amount of recomba-

nant FSH (rFSH) used.

Serum E2 at the day of hCG administration was found to be lower in group B compared to group A which support the results of Hwang et al., 2004 and most of comparative studies between long agonist and antagonist protocols. This difference of statistically significant value likes that of Hwang et al., 2004.

In one RCT conducted by Tehraninejad et al,. 2010[14], to evaluate the efficacy of antagonist in comparison with the GnRH agonist protocol in OCP pretreated PCOS patients undergoing their first ART cycle. They found that Serum E2 at the day of hCG administration was found to be lower in agonist group compared to antagonist group. They explained that, coasting was used in agonist group when serum estradiol was >3000 pg/mL. This was done in nine cases agonist group but not done in any case of the antagonist group.

Regarding retrieved oocytes number, no statistically significant difference was found between the studied groups. These findings are in accordance with the results of Hwang et al., 2004, Lainas et al., 2007 and all comparative studies between long agonist and antagonist protocols in PCOS patients included in Mancini et al,. 2010.

In the present study, comparable number of retrieved oocytes and MII oocytes in GnRh antagonist arm can be due to better synchronization of early antral follicles after partial blocking of GnRH receptor by the half dose of cetritide.

Fertilization rate was lower in group A compared to group B in the current study. This difference was statistically significant in contrast to the results of Hwang et al., 2004, Lainas et al., 2007. This came in agreement with Tehraninejad et al,. 2010, but with no significant difference.

Despite our study did not find statistically significant difference in the number of resulting embryos in the two groups, the number of grade A embryos was higher in group B than in group A and this difference was statistically

significant. This is came in agree with Tehraninejad et al., 2010. They found that the number of good quality embryos transferred was significantly higher in antagonist group. This is in contrast to the RCT conducted by Kurzawa et al., 2008[15], to evaluate embryological and clinical efficacy of GnRH antagonist and agonist stimulation protocols in non-obese women with PCOS. They found no difference in good quality embryos between agonist and antagonist group. This is due to their using flexible antagonist protocol for COH in antagonist arm while we used antagonist from the first day of stimulation which reduce the high LH in the early follicular phase. This may make follicular development more or less optimal.

In the current study, there were no statistically significant differences in the two groups as regard to chemical and clinical pregnancy rates. This is come in agree with the recent version of the Cochrane review (Al-Inany H et al., 2011), and most of recent comparable studies between agonist and antagonist. While pregnancy rates in our study were comparable to these of Tehraninejad et al., 2010 and Hwang et al., 2004, they were lower than that of Lainas et al., 2007 and Kurzawa et al., 2008. This is may be attributed to the high body mas index (BMI) of our patients since Pregnancy rate was found to be higher in patients with normal BMI than that of high BMI[16].

There was no statistically significant difference in the implantation rate in the two groups in our study. This is comparable to that of Hwang et al., 2004 while it is lower than that of Kurzawa et al., 2008. This is explained by, in Kurzawa et al., 2008, all PCOS women were non obese and absence of use OCP pretreatment.

In the current study, moderate OHSS according to Rizk and Aboulghar (1999)[17] was documented in 4 cycles of group A but was not documentd in any cycle of group B and this difference was statistically significant. This is come in agree with the last version of the Cochrane review (Al-Inany H et al., 2011) and Mancini et al., 2010.

In summary, this novel antago-

nist protocol offers a safe and efficient treatment for patients who present with PCOS who are at high risk of OHSS to standard IVF stimulation protocols. Larger RCTs with enough power are needed to further evaluate IVF outcome and the potential benefits of early initiation of GnRH-antagonist protocols in PCOs patients.

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LIPID PEROXIDATION AND LIPOPROTEIN CHANGES IN HEPATOCELLULAR CARCINOMA: CORRELATION WITH CHILD -PUGH AND MELD SCORE

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Abstract

Background: *Hepatocellular carcinoma constitutes (HCC) the 5th most frequent cancer worldwide. The liver plays a critical role in both the production and catabolism of lipids. This study was aimed to analyze the Serum lipid profile as well as C-reactive protein (CRP) and Malondialdehyde (MDA) among patients with chronic HCV infection suffering from HCC and its correlation with Child-Pugh and MELD score.*

Methods: *This study was carried out on 91 patients with HCC and 90 cirrhotic controls. The patients were classified according to Child-Pugh classification, MELD score and according to size of HCC.*

Results: *Serum levels of Cholesterol, triglycerides (TG), low-density lipoproteins (LDL), high-density lipoproteins (HDL) were significantly decreased in patients with HCC than control (p=0.005, 0.005, 0.008 and 0.009) respectively, however CRP and MDA are significantly increased compared to control (p=0.006). With progression from Child A to Child C there were significant decrease in lipids profiles and significant increase in CRP and MDA. In MELD score ≤9 lipids profiles were significant increases when compared with MELD score >9. Whereas in CRP and MDA showed significant decreases in MELD score ≤9 than MELD score >9 (p=0.045 and 0.001). Lipids profiles were significant increase in HCC ≤5cm than HCC> 5cm; however CRP and MDA showed significant decreases in HCC ≤5cm than HCC> 5cm.*

Conclusion: *There were significant alteration in the serum lipid pro-*

files, MDA and CRP parameters in patients with HCC and could be used as prognostic value.

Keywords: *Hepatocellular carcinoma, lipids, C-reactive protein and Malondialdehyde.*

Introduction

Hepatocellular carcinoma (HCC) constitutes the 5th most frequent cancer worldwide and its incidence is continuously rising in Europe and Northern America, which explained by spreading of hepatitis C virus infections.[1] In Egypt, HCC was reported for about 4.7% of chronic liver disease (CLD) patients.[2]

Most plasma apolipoproteins and endogenous lipids and lipoproteins, including apolipoprotein (a) (apo(a)) and lipoprotein(a) (Lp (a)), are synthesized in the liver. The normal metabolism and homeostasis of this lipids depend on integrated liver function.[3,4] Alteration in lipoprotein metabolism and changed plasma patterns of lipid and lipoprotein was demonstrated in advanced liver diseases.[5-7]

Many studies have indicated that cancer associated alternations in host metabolism are important factors for mortality determination because the disturbance in host metabolism could cause under nutrition and even cachexia.[8,9]

Serum C-reactive protein (CRP) could be expressed of the host defense reaction or as paraneoplastic syndrome and widely investigated as risk factor and prognostic variable in various human malignancies including hepatocellular carcinoma.[10,11]

Reactive oxygen species (ROS) mediated liver injury may trigger by three main mechanisms: lipid peroxidation, cytokine induction and Fas ligand induction.[12] It has been suggested that, oxidative stress is involved in the etiology and deterioration of liver cancer.[13,14] Those lipid oxidation products such as malondialdehyde (MDA) could act as tumor promoters and co-carcinogenic agents via their high cytotoxicity action.[14]

Therefore, we designed the current study to evaluate the serum lipid profiles (Cholesterol, triglyceride (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL)) as well as CRP and MDA among Egyptian cirrhotic patients with HCV infection suffering from HCC and its correlation with Child-Pugh, MELD score and tumor size.

Subjects and Methods

This study was carried out on 91 patients with HCC due to HCV infection (52 males, 39 females) and 90 cirrhotic related HCV infection without HCC as control group. All Patients matched for ethnicity, age and sex with age range (45-73 years). Patients were recruited from Tropical medicine and internal medicine department, Mansoura University during the period from March 2010 to June 2012. This study was approved by the Institutional Review Board of our university and obtaining informed consent form all study subjects.

The diagnosis of chronic HCV infection was based on the seropositivity of HCV specific antibody and positive HCV-RNA determined by the polymerase chain reaction (PCR). Patients were classified (table 1) according to Child-Pugh classification into Child A (n=26), B (n=31) and C (n=34), according to MELD score into 32 patients ≤9 score and 59 patients >9 score and according to size of HCC into 33 patients ≤5cm for single focal lesion of HCC and 58 patients >5cm for single focal lesion of HCC.

For the calculation of the CTP score, study used the Pugh score modification.[15] The Model for End-Stage Liver Disease (MELD) score was calculated using the original formula without including the cause of liver disease .16 (MELD) score = 0.957 x loge (creatinine mg/dL) + 0.378 x loge (bilirubin mg/dL) + 1.120 x loge (INR) + 6.43. MELD score calculator from the UNOS website (www.unos.org). HCC was diagnosed according to the diagnostic guidelines of the European Association for the Study of the Liver.[17]

2. Determinations of plasma lipid profiles, MDA and CRP:

Peripheral venous blood sam-

ples were obtained from patients and controls in the morning following an overnight fast. The samples were collected in sterile tubes and allowed to coagulate for 1 hour at room temperature. Then, serum samples were aliquoted in smaller containers that were marked with the patients' name, date and stored at -80°C until assaying. Serum levels of TG, cholesterol VLDL, HDL and LDL were determined by enzymatic method (Auto analyzer COBAS INTEGRA700, Roche,germany). Serum level of CRP was measured by turbidimetric immunoassay.[18] and MDA was determined by the thiobarbituric acid method.[20] Serum albumin, bilirubin, Alpha-Fetoprotein (AFP), creatinine, prothrombin time and aminotransferases (ALT and AST) were determined. HBsAg and anti-HCV antibodies were detected by enzyme-linked immunosorbent assay (ELISA). All patients included in the study were subjected to full medical history and thorough clinical examination included imaging using ultrasound scan, triphasic CT.

Statistics analysis:

The statistical analysis of data done by using Excel program and SPSS program (statistical package for social science) version 10. The description of the data done in form of mean±SD for quantitative data and frequency and proportion for qualitative data. The analysis of the data was done to test statistical significant difference between groups. For quantitative data student t-test was used to compared between 2 group paired sample t-test was used to compare one group at different measurements. P-value <0.05 is considered to be significant.

Results

Table (1) showed patients classification, Child A 26 patients, Child B 31 patients and Child C 34 patients. Patients with MELD score ≤9 were 32 and 59 patients with MELD score >9. patients with size of HCC ≤5cm were 33 and 58 patients with size of HCC >5cm.

Table 2 showed serum cholesterol 189.04 (23.02) mg/dL, TG 92.49 (20.94) mg/dL, LDL 119.89 (32.67) mg/dL and HDL 39.41 (7.11) mg/dL were significantly decreased (P<0.005, P<0.005, P<0.008 and P<0.009) in all studied patient groups as compared to

control group. There were highly significant increase in CRP 692.78 (64.71) mg/dL and MDA7.27 (1.49) nM/ml (P<0.006) in all studied patient groups as compared to control group.

Serum cholesterol 206.0 (17.47) mg/dL, TG108.18 (20.16) mg/dL, LDL 142.18 (34.30) mg/dL and HDL 44.09 (4.74) mg/dL were significantly increased in Child A group than Child B (P=0.018, P=0.0I5, P=0.043 and P=0.046, respectively) (table 3). Also these parameters were significantly increased in Child A group than Child C (P<0.001, P<0.002, P<0.001, and P<0.001, respectively) (table 4).

Serum cholesterol 185.89(16.97) mg/dL, TG 87.67 (11.86) mg/dL, LDL 117.44 (11.1) mg/dL and HDL 39.22 (5.40) mg/dL were significantly increased (P=0.036, P=0.041, P=0.007 and P=0.036, respectively) in Child B group as compared to Child C (table 5).

Serum levels of CRP 641.0 (52.64) mg/dL and MDA 6.32 (0.71) nM/ml were significant decreased (P<0.006 and P<0.039) in Child A patient group as compared to Child B (table 3) and were significantly decreased (P< 0.001 and P<0.006) in Child A patient group as compared to Child C (table 4). Moreover serum levels of CRP 706.67 (37.73) mg/dL and MDA 7.22 (1.1) nM/ml were significant decrease in (P<0.025 and P<0.037) in Child B patient group as compared to Child C (table 5).

As regard serum level of Lipoprotein patterns, MDA and CRP in patients according to MELD score this study showed that (table 6) serum cholesterol 196.1 (21.06) mg/dL, TG 98.1 (20.56) mg/dL, LDL 130.75 (28.83) mg/dL and HDL 41.65 (4.98) mg/dL were significantly increased (P=0.005, P= 0.0I5, P=0.002 and P=0.003) in patients with MELD ≤9 as compared to MELD >9. On the other hand, there was a serum levels of CRP 678.2 (56.05) mg/dL and MDA 6.75 (1.06) nM/ml were significant decrease in (P<0.045 and P<0.001)in patients with MELD ≤9 as compared to MELD >9.

Serum levels of Lipoprotein patterns, MDA and CRP were studied in patients according to size of HCC and our study revealed that

(table 7), serum cholesterol 186.1 (22.12) mg/dL,TG 95.12 (18.43) mg/dL, LDL135.62 (26.54) mg/dL and HDL 44.51(5.84) mg/dL were significantly increased (P=0.005, P =0.021, P=0.004 and P=0.002) in patients with HCC ≤5cm as compared to HCC >5cm. On the other hand, serum levels of CRP 689.31 (55.52) mg/dL and MDA 6.85 (1.26) nM/ml were significant decrease in (P<0.015 and P<0.001) in patients with HCC ≤5cm as compared to HCC >5cm.

Table (1): Classification of the study populations according to Child-Pugh, MELD scores and Size of HCC.

Child-Pugh classification			MELD sco		Size of HCC	
Child A	Child B	Child C	MELD ≤ 9	MELD > 9	HCC ≤5cm	HCC >5cm
n=26 (28.57%),	n=31 (34.06%)	n=34 (37.36%)	n=32 (35.16%)	n=59 (64.84%)	n=33 (36.26%)	n=58 (63.74%)

MELD, Model for End-Stage Liver Disease; HCC, Hepatocellular carcinoma.

Table (2): Comparison of Serum lipid profile, MDA and CRP in the study populations versus controls.

	Patents (n = 91)		Control (n = 90)		P value
	Mean	SD	Mean	SD	
Cholesterol (mg/dL)	189.04	23.02	213.64	22.56	0.005
TG(mg/dL)	92.48	20.94	115.91	23.86	0.005
LDL(mg/dL)	119.89	32.67	149.55	18.48	0.008
HDL(mg/dL)	39.41	7.11	45.73	3.95	0.009
CRP (mg/L)	692.78	64.71	621.55	75.26	0.006
MDA (nM/ml)	7.27	1.49	6.21	0.73	0.006

TG, Triglycerides; HDL, high-density lipoproteins; LDL, low-density lipoproteins; CRP, serum C-reactive protein; MDA, Malondialdehyde.

Table (3): Comparison of Serum lipid profile, MDA and CRP in patients Child A versus Child B patients.

	Child A (n = 26)		Child B (n = 31)		P value
	Mean	SD	Mean	SD	
Cholesterol (mg/dL)	206.00	17.47	185.89	16.97	0.018
TG(mg/dL)	108.18	20.16	87.67	11.86	0.015
LDL(mg/dL)	142.18	34.30	117.44	11.10	0.043
HDL(mg/dL)	44.09	4.74	39.22	5.40	0.046
CRP(mg/L)	641.00	52.64	706.67	37.73	0.006
MDA(nM/ml)	6.32	0.71	7.22	1.10	0.039

TG, Triglycerides; HDL, high-density lipoproteins; LDL, low-density lipoproteins; CRP, serum C-reactive protein; MDA, Malondialdehyde.

Table (4): Comparison of Serum lipid profile, MDA and CRP in Child A versus Child C patients.

	Child A (n = 26)		Child C (n = 34)		P value
	Mean	SD	Mean	SD	
Cholesterol (mg/dL)	206.00	17.47	166.43	16.27	0.001
TG(mg/dL)	108.18	20.16	74.00	12.26	0.001
LDL(mg/dL)	142.18	34.30	88.00	19.83	0.002
HDL(mg/dL)	44.09	4.74	32.29	6.55	0.001
CRP(mg/L)	641.00	52.64	756.29	41.44	0.001
MDA(nM/ml)	6.32	0.71	8.81	1.66	0.006

TG, Triglycerides; HDL, high-density lipoproteins; LDL, low-density lipoproteins; CRP, serum C-reactive protein; MDA, Malondialdehyde.

Table (5): Comparison of Serum lipid profile, MDA and CRP in Child B versus Child C patients.

	Child B (n = 31)		Child C (n = 34)		P value
	Mean	SD	Mean	SD	
Cholesterol (mg/dL)	185.89	16.97	166.43	16.27	0.036
TG(mg/dL)	87.67	11.86	74.00	12.26	0.041
LDL(mg/dL)	117.44	11.10	88.00	19.83	0.007
HDL(mg/dL)	39.22	5.40	32.29	6.55	0.036
CRP(mg/L)	706.67	37.73	756.29	41.44	0.025
MDA(nM/ml)	7.22	1.10	8.81	1.66	0.037

TG, Triglycerides; HDL, high-density lipoproteins; LDL, low-density lipoproteins; CRP, serum C-reactive protein; MDA, Malondialdehyde.

Table (6): lipid profile, MDA and CRP in the study populations according to MELD score.

	MELD ≤ 9 (n = 32)		MELD > 9 (n = 59)		P value
	Mean	SD	Mean	SD	
Cholesterol (mg/dL)	196.10	21.06	168.86	15.81	0.005
TG(mg/dL)	98.10	20.56	76.43	12.50	0.015
LDL(mg/dL)	130.75	28.83	88.86	21.80	0.002
HDL(mg/dL)	41.65	4.98	33.00	8.70	0.003
CRP(mg/dL)	678.20	56.05	734.43	73.91	0.045
MDA(nM/ml)	6.75	1.06	8.74	1.64	0.001

TG, Triglycerides; HDL, high-density lipoproteins; LDL, low-density lipoproteins; CRP, serum C-reactive protein; MDA, Malondialdehyde.

Table (7): lipid profile, MDA and CRP in the study populations according to size of single focal lesion of HCC.

	HCC ≤ 5cm(n = 33)		HCC > 5cm(n = 58)		P value
	Mean	SD	Mean	SD	
Cholesterol (mg/dL)	186.10	22.12	162.31	16.75	0.005
TG(mg/dL)	95.12	18.43	74.35	11.65	0.021
LDL(mg/dL)	135.62	26.54	82.65	23.21	0.004
HDL(mg/dL)	44.51	5.84	35.21	9.84	0.002
CRP(mg/dL)	689.31	55.52	785.23	68.89	0.015
MDA(nM/ml)	6.85	1.26	9.31	1.74	0.001

TG, Triglycerides; HDL, high-density lipoproteins; LDL, low-density lipoproteins; CRP, serum C-reactive protein; MDA, Malondialdehyde.

Discussion

Lipids are essential component of biological membranes, free molecules and metabolic regulators that control cellular function and homeostasis.[20] Liver plays a vital role in lipid metabolism. It contributes both in exogenous and endogenous cycles of lipid metabolism and transport of lipids through plasma. Synthesis of many apolipoproteins takes place in liver. Liver is the principal site of formation and clearance of lipoproteins. This shows liver is involved in many steps of lipid metabolism and lipid transport.[21] Hepatocellular injury or chronic liver diseases including hepatocellular carcinoma (HCC) may result in a distinctly abnormal pattern of plasma lipids, apolipoproteins and lipoproteins lipoproteins, which may be related to or regulated by various cytokines and/or metabolic cellular substances, or tumor factors, although the detailed mechanisms are not fully understood.[22] C-reactive protein (CRP) is one of the most important acute phase proteins produced predominantly by hepatocytes rising rapidly in response to inflammation, and serum CRP levels are routinely measured as markers for various acute and chronic inflammatory diseases.[10,23] Its production is regulated by proinflammatory cytokine.[24]

Results of the present study showed that, plasma levels of Cholesterol, triglycerides (TG), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were significantly decreased in patients with HCC than cirrhotic control patients.

It has been suggested that analysis of plasma levels of lipids, lipoproteins and apolipoproteins in the patients suffered from HCC reflects on the hepatic cellular impairment status. Studies revealed that alterations seen in the plasma levels of lipids, lipoproteins and apolipoproteins reflecting patients' pathologic conditions.[3] The results of this study were in agreed with the results of other. About 80% endogenous cholesterol are synthesized in the hepatocellular microsomes that contain cholesterol synthesis enzymes.[25,26]

In HCC and chronic liver diseases the synthesis and metabolism of cholesterol are impaired. It leads to a decrease in plasma cho-

lesterol levels.[5-7,27] In coordination with our result regarding serum triglyceride Motta et al has been demonstrated that plasma triglycerides (TG) decreased by 20-30% in the patients with HCC.[6] but In contrast, Alsabti et al, reported that serum TG in HCC patients were increased when compared to those with cirrhosis.[28]

In our study we found that HDL is reduced in patients with HCC. This result supported by Miller et al who found that, HDL and its major apolipoproteins, apoAI and apoAII, are frequently reduced in the patients suffered from cirrhosis or HCC.[21] Moreover Mehboob et al also found decreased levels of total cholesterol, triglycerides, LDL and HDL in chronic liver disease irrespective of its etiology.[29]

With progression from Child A to Child C and from MELD ≤9 to MELD >9 there were significant decrease in lipids profiles suggested that, changes in serum lipids my correlated with progression and severity of the disease. These results are rationally and are due to severe disturbance of lipid metabolism with progression from child A to child C and from MELD ≤9 to MELD >9. Jiang J, et al concluded that plasma levels of triglycerides, cholesterol, free fatty acids, HDL, low density lipoproteins Lpa, Apo A, and Apo B, were decreased in cases of hepato-cellular carcinoma they suggested that this may be due to hepato-cellular impairment and this also suggests poor prognosis.[3]

In this study the serum levels of lipoprotein were studied in patients according to size of HCC. Our result revealed that (table 7) serum cholesterol, TG, LDL and HDL were significantly higher in patients with HCC ≤5cm as compared to HCC >5cm indicated that more decrease in levels of lipoprotein with progression of HCC. This may be explained again by Jiang J, et al due to hepato-cellular impairment and this also suggests poor prognosis.[3] In contrast to our finding Lin C and Yin M found that HCC patients at III + IV stage showed significantly higher cholesterol, triglyceride, LDH and AFP than patients at I+II stage and healthy controls.[30]

In this study Serum CRP levels were increased in patients with

HCC than control cirrhotic group and showed significant increase with progression from child A to child C and from MELD ≤9 to MELD >9. Also CRP was increased in HCC >5cm more than HCC <5cm. These results suggested CRP as risk factor and indicator of poor prognosis among patients with HCC. Different studies showed increased Serum level of CRP in HCC.[31-33] Moreover Hashimoto et al demonstrated that the serum CRP level was correlated significantly with unfavorable tumor factors, such as tumor size and the incidence of portal vein invasion.[11] Also Nagaoka et al found that the prognosis of high sensitivity CRP-positive patients was poorer compared with H-CRP-negative patients.[34] On the other hand Lin et al concluded that serum CRP is not a good marker for HCC.[35] However, very high values of CRP in patients with cirrhosis may suggest the presence of a diffuse-type HCC. In fact several possible mechanisms have been proposed for the relationship between CRP and cancer. First, tumour growth can cause tissue inflammation and hence increase CRP levels.[36] Second, CRP could be an indicator of an immune response to tumour antigens.[37] Third, there is evidence that cancer cells can increase the production of inflammatory proteins, which could explain the high CRP concentrations in patients with cancer. Some cancerous cells have been shown to express CRP.[38] and cancer cell lines have been shown to secrete IL6 and IL8, which in turn induce the production of CRP.[39]

Many clinical studies, revealed the fact that MDA, which is a lipid peroxidation product, increases in various liver diseases. These findings point out the importance of MDA in the progression of Liver cirrhosis.[40] Reactive oxygen species are oxygen containing molecules that are produced during normal metabolism. The organism has enzymatic & non enzymatic antioxidant systems neutralizing the harmful effects of the endogenous ROS products. Under certain conditions, the oxidative (or) antioxidative balance shifts towards the oxidative status as a result of increase in ROS and / impairment in antioxidant mechanism.[41]

Reactive oxygen species degrade

polyunsaturated lipids, forming malondialdehyde. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form advanced glycation end products. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism.[42]

In this study Serum MDA levels were increased in patients with HCC than control cirrhotic group (p=0.006) and showed significant increase with progression from child A to child C and from MELD ≤9 to MELD >9. Also MDA was increased in HCC >5cm more than HCC <5cm.

Metabolism of various endo- and exogenous compounds and viruses generates reactive oxygen species, which could be involved in the pathogenesis of different liver diseases, including cirrhosis and hepatocellular cancer.[43,44]

Different studies in agree with our results found that MDA is increased in patients with HCC than cirrhotic patients. Czeczot et al found that a comparison of MDA level in supernatants prepared from cirrhotic, cancer and adjacent normal liver tissues indicated a higher MDA content in cancer tissue compared to control tissue or cirrhotic tissue.[45] Liu et al also found that MDA was increased in patients with HCC than Cirrhosis.[46]

With progression of disease from child A to child C, MELD ≤9 to MELD >9 and increased tumor size the level of MDA was progressively increased. This finding can be explained by the following, oxygen radical production, which increases with clinical progression of diseases, involves increased lipid peroxidation. The process of lipid peroxidation consists in oxidative conversion of polyunsaturated fatty acids to products known as malondialdehyde.[14,47]

In conclusion our study revealed a significant alteration in the serum lipid profile, MDA and CRP parameters in patients with HCC and serial evaluation of these parameters would be strongly advised in high-risk chronic liver disease patients. Analysis of serum levels of lipids, MDA and CRP

parameters in the patients suffered from cirrhosis and HCC may reflect the condition of hepatic cellular impairment, and may also be used as an indicator to evaluate patient's prognosis. It is suggested that variations in the levels of plasma lipids, MDA and CRP parameters may assist in describing the nature of HCC. Large-scale studies are to clarify the prognostic value of such changes in the light of probable predictability of malignant change in cirrhotic livers.

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LIPID PEROXIDATION AND LIPOPROTEIN CHANGES IN HEPATOCELLULAR CARCINOMA: CORRELATION WITH CHILD -PUGH AND MELD SCORE

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GINGER ADMINISTRATION HAS A PROTECTIVE EFFECT AGAINST HISTOCHEMICAL RENAL DAMAGE IN OBESE AND PASSIVE SMOKING ADULT RATS

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Abstract

Background: *Obesity is associated with a number of chronic diseases, such as cardiovascular disease, diabetes, and hypertension. As a direct link between obesity and kidney disease is increasingly appreciated, obesity is becoming an issue with which all nephrologists have to familiarize themselves Smoking, a well known risk factor for many diseases, was recently proven to play an important role in renal diseases. Ginger is one of the most commonly used spices around the world. Ginger has been used to help treating arthritis, colic, diarrhea, and heart problems. Experimental evidence exists for potential benefit of ginger on kidney function. The investigators demonstrated that dietary supplementation with ginger offered significant renal protection by activating antioxidant pathways.*

Objective: *To evaluate the effect of ginger on renal function of rats fed high sucrose diet and exposed to passive smoking.*

Materials and Methods: *Forty male Albino rats were divide into 5 groups 8 rat/ cage either not treated (control), or fed high sucrose diet, or fed high sucrose diet with dietary ginger supplementation (500 mg/ kg/d), fed high sucrose diet and exposed to passive smoking or fed high sucrose diet and exposed to passive smoking supplemented with ginger powder. After five weeks of treatment, final body weight was determined. Blood samples were collected, lipid parameters, blood glucose, serum urea and creatinine were determined. Kidney tissue isolated from rats for histopathological examination.*

Results: *Treatment with ginger had significant effect in reducing body weight. Ginger had the ability to reduce glucose level, lipid profile; (triglyceride-cholesterol), serum level of urea and creatinine in both*

obese rats and those exposed to passive smoking. In addition; the histological study of the renal tissue showing improvement after ginger administration in both obese and passive smoking rats.

Conclusions: *Ginger has a great ability to reduce body weight; blood glucose and lipid profile and has a protective effect against renal damage produced in obese rats and those exposed to passive smoking.*

Keyword: *ginger, obesity, renal damage, passive smoking.*

Introduction

Smoking and obesity are leading causes of morbidity and mortality worldwide[1]. The co-occurrence of overweight and smoking has substantial consequences for health. According to the Framingham study, the life expectancy of obese smokers was 13 y less than that of normal-weight nonsmokers[2].

Obesity is a risk factor for chronic kidney disease (CKD), Individuals older than 60 years of age have experienced the most rapid increase in prevalence because this age group experiences the greatest burden of chronic kidney disease, and this may be exacerbated by obesity[3]. Obesity almost certainly indirectly contributes to CKD because obesity associates with many dominant CKD risk factors such as diabetes, hypertension, and atherosclerosis. However, obesity may also directly lead to CKD[4].

Pathologic studies demonstrate that subjects with severe obesity develop proteinuria with pathologic findings of podocyte hypertrophy, mesangial expansion, glomerular enlargement, and focal segmental glomerular sclerosis in the absence of diabetes and hypertension[5].

The incidence of end-stage renal disease (ESRD) has risen considerably in the past two decades. This trend is partly due to the alarming rise in the incidence of type II diabetes over the same period, which in turn might be linked to the staggering increase in overweight and obesity[6]. Studies have indicated that a growing body of evidence suggests that in genetically obese animals, food restriction can prevent or greatly delay the onset of specific degenerative lesions, in particular

glomerulonephritis associated with obesity and diabetes[7].

Ginger is an aromatic root that has a strong flavor. It is used in recipes to add a spicy and sweet flavor and is very common in Asian cooking. Ginger can be found in the whole root form, as ginger powder, and is available in teas and ginger ale. Historically, ginger was used as a food preservative and as a remedy for indigestion. It is still used today to help treat nausea[8].

Ginger may be effective in stimulating weight loss in obese men and women. According to the National Kidney Foundation, more than 20 million Americans have chronic kidney disease; Current therapies may offer some benefit in slowing the progression of chronic kidney disease, but most patients ultimately end up with kidney failure. You may be tempted to try alternative therapies such as Ginger[9].

Smoking, a well known risk factor for many diseases, was recently proven to play an important role in renal diseases. Studies showed that cigarette smoking is a risk factor for the development and progression of chronic kidney disease (CKD) in community Smoking significantly increases the risk of CKD When compared to nonsmokers[10]. One study revealed that men who smoked were three times more likely to have reduced kidney function as compared to male non-smokers,

One of the ways smoking damages kidney function is by affecting the blood flow within the body. Smoking hardens the arteries and narrows the blood vessels, which can restrict blood flow to the kidneys and cause them to be less efficient[11].

A study in "Renal Failure" in April 2004 examined whether ginger could preserve kidney function following ischemia. The investigators demonstrated that dietary supplementation with ginger offered significant renal protection by activating antioxidant pathways[12].

Materials and Methods

Animals:

Six-wk-old male rats were housed in the Banha University

Animal in plastic cages 40 adult male albino rats; 8rats/cage, weighing 220-250 g, at room temperature of 25±2°C. Rats were allowed to acclimate to this facility for 1 wk prior to entry into an experimental study. Rats were given standard and modified diet and free access to water.

Composition of the diets used

Standard chow diet:

- The fat represented 4% of the total caloric requirement.
- The carbohydrates represented 44% carbohydrate (41 % starch and 3% sucrose) of the total caloric requirement.
- The protein represented 23% of the total caloric requirement.
- The fibers represent 14% of the total caloric requirement[13].

High sucrose diet:

- The fat represented 6% of the total caloric requirement.
- The carbohydrates 49.5% (4.5% starch and 47% sucrose) of the total caloric requirement.
- The protein represented 24% of the total caloric requirement.
- The fibers represent 9% of the total caloric requirement.
- The high-sucrose diet was obtained mixing 600 g sucrose and 60 g of soy oil to 1000 g of a previously triturated standard chow. Casein was added to achieve the same protein content as the standard chow[14].

The rat were divided into 5 groups:

Group I (control group): serves as control group, they received standard diet in which sucrose represents 3% of the total caloric requirement for 5 weeks and kept sedentary until the end of the experiment.

Group II (High Sucrose not exposed to passive smoking) HS group: rats that received 47 % sucrose in diet for 5 weeks and kept sedentary until the end of the experiment.

Group III (High Sucrose exposed to passive smoking) HSS group: that received 47 % sucrose in diet for 5 weeks and exposed to 6 cigarettes/day, 5 days/week for the last 4 weeks.

Group IV (High Sucrose not exposed to passive smoking with ginger administration HSG

group): that received 47% sucrose in diet and ginger powder was administrated in a dose 500 mg/kg by a gavage method for 5 weeks.

Group V: (High Sucrose exposed to passive smoking with ginger administration) HSSG group: that received 47 % sucrose in diet and ginger powder was administrated in a dose 500 mg/kg by a gavage method for 5 weeks and exposed to 6 cigarettes/day, 5 days/week for the last 4 weeks before taking samples.

Assessment of Obesity:

After 5 weeks of dietary treatments, the animals were anaesthetized (0.1ml intra peritoneal of 1% Thiopental Na) for the measurement of body length (nose-to-anus or nose-anal length). The body weight and body length were used to confirm the obesity through the obesity parameters body mass index (body weight g/ length cm2).

Exposure to passive smoking:

9 rats were divided into 3 groups the 1st group was exposed to 2cigarettes/day, the 2nd group was exposed to 4 cigarettes/day and the 3rd group was exposed to 6 cigarettes/day. All groups were exposed to passive smoking for 5 days/week for 4 weeks. At the end of the duration all rats were examined for serum urea and creatinine. The most effective dose was 6 cigarettes/day.

Chemical analysis:

At the end of the experimental period the rats were anaesthetized after 12 hour fasting by inhalation of diethyl ether. Blood samples were collected by intracardiac suction for serum separation, for the determination of urea (BUN) and creatinine level, fasting glucose, triglyceride, total cholesterol serum levels. These were investigated in Banha faculty of medicine at biochemistry analysis unit.

Pathological evaluation

A histological study was performed following a midline laparotomy to remove the kidneys. The kidneys were dissected and fixed in 10% formalin solution at room temperature. An experienced pathologist evaluated all samples. All fields in each section were examined and graded for necro-inflammation.

Statistical Analysis:

All data were expressed as mean ± S.D; data were evaluated by the one way analysis of variance. The calculations were performed by SPSS program version 17. Difference between groups were compared by Student's t-test with $P<0.05$ selected as the level of statistical significance.

Results

Blood biochemical parameters

Levels of Serum glucose, urea, creatinine and lipid profile (Triglycerides, Total cholesterol) and body weight index (BWI) measured in all groups are shown in Table 1. The serum level of glucose, urea, creatinine, triglycerides, total cholesterol and the body mass index were significantly increased in group II that received high sucrose diet in comparison to control group. The serum level of glucose, urea, creatinine, and triglycerides, total cholesterol were significantly decreased in ginger administrated group III in comparison to high sucrose group. High Sucrose group IV exposed to smoking showed significant increase in serum level of glucose, urea, creatinine, triglycerides, total cholesterol, and the body mass index in comparison to group of high sucrose not exposed to smoking. The serum level of glucose, urea, creatinine, triglycerides, and total cholesterol were significantly decreased in High Sucrose ginger administrated group V and exposed to smoking in comparison to High Sucrose group exposed to smoking.

Histological examination:

Group 1 (control group): The histological appearance of the kidney in the control group was normal. Light microscopic examination of the control group stained by H&E revealed that, the kidney showed normal characteristic renal tissue. Normal renal tubular structure and regular glomeruli (Fig. 1).

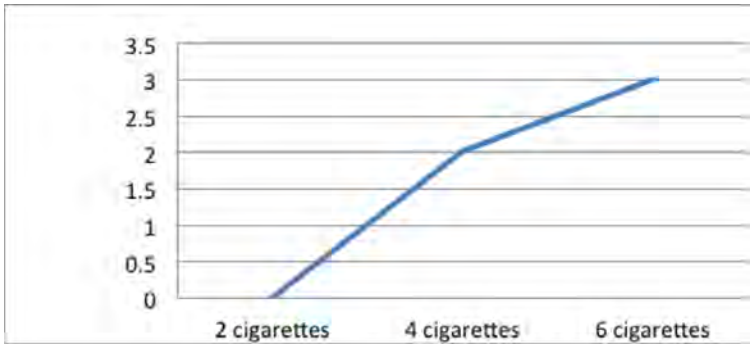
Histological examination of group II (HS) revealed appearance of hyaline degeneration of the renal tubular structure and congestion of arterioles (Fig.2). In group III (HSG) in which rats received ginger their renal tissue appeared nearly normal with mild hyaline changes in the tubular structure (Fig.3). The histological appearance in group IV (HSS) showed re-

nal damage in the form of tubular necrosis, interstitial hemorrhage and glomerular vascular damage (Fig.4). This damage was improved in group V (HSSG) in which the histological examination revealed moderate tubular damage and glomerular proliferation (Fig.5).

Table (1): Serum glucose, urea, creatinine and lipid profile (Triglycerides, Total cholesterol mg/dl) and body weight index. Results are expressed as the Mean ± SE. n = 8; High Sucrose group not exposed to smoking =HS, High Sucrose with ginger group=HSG, High Sucrose group exposed to smoking = HSS, High Sucrose with ginger group and exposed to smoking= HSSG.

	Control	HS	HSG	HSS	HSSG
Glucose (mg/dl)	98±1.56	154±1.64*	110±2.25#	171±0.821$	148.5±1.36@
Trigylc. (mg/dl)	99.5±3.8	139.8±3.1*	103.3±4.3#	158±4.4$	136.2±1.55@
T. choles.(mg/dl)	94.8± 4.9	110.6±4.3*	94.3±3.3#	135±2.66$	114.3±2.38@
urea(mg/dl)	14.8±2.9	39±3.11*	15±3.11#	44.7±3.61$	13.25±2.54@
creatinine(mg/dl)	0.36±.02	2.45±261*	1.32±.29#	3.61±.249$	0.413±.198@
BWI(g/cm2)	0.541±.024	0.87±.037*	0.577±.035#	0.8475±.029$	0.572+0.036@

*Significant difference ($p<0.001$) compared with normal control.
#Significam difference ($p<0.001$) compared with High Sucrose group not exposed to smoking.
$ Significant difference ($p<0.001$) compared with High Sucrose group not exposed to smoking.
@Significant difference ($p<0.001$) compared with High Sucrose group exposed to smoking.



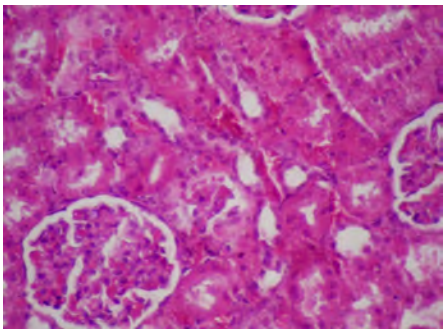


Fig. 1: normal renal tissue (control group) HE X200.

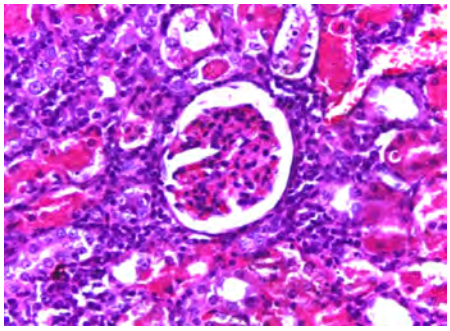


Fig. 2: degeneration of the renal tissue (HS group) With congestion of renal arterioles HE X200.

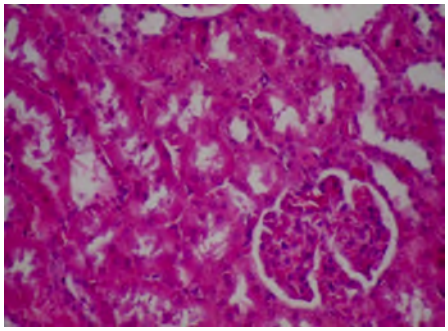


Fig. 3: normal renal tissue with mild hydropic changes (HSG) groupHEX200.

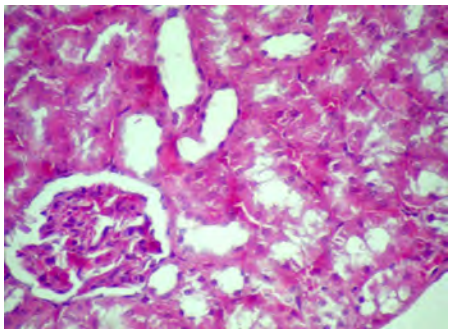


Fig. 4: Tubular necrosis, interstitial hemorrhage & glomerular vascular damage (HSS) group HEX200.

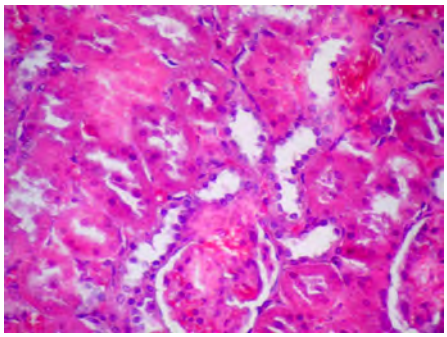


Fig. 5: moderate tubular damage & glomerular proliferation (HSSG) group HEX200.

Discussion

Ginger was used as a food preservative and as a remedy for indigestion. It is still used today to help treat nausea. Ginger has not been found to treat or cure kidney disease, but its benefits against indigestion and nausea can help dialysis patients who are feeling these symptoms. Because most dialysis patients are given a strict fluid restriction, natural ginger root and powder should be used rather than in a liquid form[15]. Our results revealed that ginger administration had significant effect on the kidney function in obese male rats and those exposed to passive smoking, However high sucrose diet resulted in obesity as evident by the body weight index. Obesity had significant effect on the renal function this appeared in significant increase in serum BNU and creatinine in obese rats and in obese rats exposed to passive smoking compared to the control group. In addition there were histological renal damage appeared in obese and passive smoking rats. Ginger administration caused significant decrease in serum urea and creatinine in obese and those exposed to passive smoking. These results are in agree with Experimental evidence exists for potential benefit of ginger on kidney function. A study in "Renal Failure" in April 2004 examined whether ginger could preserve kidney function following ischemia. Using an animal model, researchers clamped off the blood flow to the kidneys and then provided ginger as a dietary supplement to the rats. The investigators demonstrated that dietary supplementation with ginger offered significant renal protection by activating antioxidant pathways[16]. Investigators using animal models have also demonstrated ginger to provide protection from toxin-mediated kidney damage as can occur with chemotherapy or alcohol-induced damage; Ginger administration prevented the acute kidney injury caused by the chemotherapy agents. Another study published in the “Indian Journal of Experimental Biology” in 2010 demonstrated a similar benefit of ginger on kidney function following alcohol-induced injury[17]. In addition; Ginger may be effective in stimulating weight loss in obese men and women. Because of ginger's health benefits,

ginger has been useful in correcting certain digestive disorders that can greatly affect the BMI and cholesterol levels of an individual. Even though there are no current scientific or medical research on the effects of ginger for weight loss, ginger, which comes in many forms, has had a positive history of improving overall health and wellness[18]. In our results ginger significantly reduced body weight, the blood glucose and lipid profile compared to obese (high sucrose diet) rats and those exposed to passive smoking. In agreement; Not only does ginger play a large role in neutralizing intestinal imbalances but it also inhibits bad cholesterol buildup, or low-density lipoprotein (LDL), in the body's liver. Ginger helps lower cholesterol by significantly reducing serum and hepatic cholesterol levels. It slows cholesterol absorption by stimulating the conversion of cholesterol to bile acids. Consuming ginger can have a profound effect on high cholesterol levels that are often attributed to obesity among women and men[19]. In addition; Ginger reduces Triglyceride and Cholesterol levels and thus helps in improving cardiovascular health. It elevates the levels of high density Lipoproteins and lowers the risks of stroke and heart attack by improving the blood circulation[20]. Other study indicates that raw ginger possesses hypoglycemic, hypocholesterolaemic and hypolipidaemic potential. Additionally, raw ginger is effective in reversing the diabetic proteinuria observed in the diabetic rats. Thus, ginger may be of great value in managing the effects of diabetic complications in human subjects [21]. Ginger extract is among the top ten ingredients in weight-loss products. It is generally safe to take and it is believes to have anti-inflammatory and anti-nausea effect[22]. More study revealed that ginger and Arabic gum could be beneficial adjuvant therapy in patients with acute renal failure and CRF to prevent disease progression and delay the need for renal replacement therapy[23].

Ingesting pure ginger helps you quit smoking. Ginger has a warming and heating effect in the body, which promotes perspiration. When your body sweats it excretes toxins and relieves some chemical loads from the body. A great detox

for a smoking addiction. Also - Drink ginger tea. Instead of smoking try preparing a cup of ginger tea. This is effective because it helps form a new habit instead of smoking[24]. In spite of these benefits we should study accurately the dose and side effects of ginger as herbal supplements are not regulated by the government and may contain contaminants and high levels of other substances such as potassium that injured kidneys may not be able to process. The National Kidney Foundation, in addition to encouraging further research, recommends that patients with chronic kidney disease discuss taking any herbal supplement, such as ginger, with their health care provider prior to beginning therapy.

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REPRINT

BENHA MEDICAL JOURNAL

GINGER ADMINISTRATION HAS A PROTECTIVE EFFECT AGAINST HISTOCHEMICAL RENAL DAMAGE IN OBESE AND PASSIVE SMOKING ADULT RATS

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EFFECT OF VITAMIN E ON OXIDATIVE AND APOPTOTIC DAMAGE INDUCED BY STREPTOZOTOCIN IN RAT PANCREAS

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Abstract

Abstract: *The present study was designated to assess oxidative damage and its effect on apoptosis in pancreas of streptozotocin (STZ)-induced diabetic rats. The role of vitamin E for protection against such damage was evaluated. Thirty two adult male rats were randomly divided into four groups: group I, control, non-diabetic rats; group II, STZ-induced, untreated diabetic rats; group III, STZ-induced diabetic rats supplemented; with vitamin E for 4 weeks; group IV, STZ-induced diabetic rats supplemented; with vitamin E for 8 weeks . Glucose and insulin levels were estimated in blood samples. Malondialdehyde (MDA) and the activity of the glutathione peroxidase enzyme (GSH-Px) were determined in pancreatic tissues. Histopathological examination using H&E stain, as well as, immunohistochemical detection of apoptosis using BCL-X expression in cytoplasm of aciner cells of pancreas was also performed. Blood glucose was significantly increased while insulin was significantly decreased in STZ-induced diabetic rats as compared with controls. In rat pancreatic tissues, MDA was significantly elevated and GSH-Px enzymatic activity was significantly decreased in diabetic rats as compared with control group. Vitamin E supplementation to diabetic rats restored the pancreatic GSH-Px enzyme activity to almost control levels; in addition, MDA decreases as compared with untreated diabetic group. Prominent reduction of BCL-X expression in cytoplasm of aciner cells was found in diabetic rats supplemented with Vitamin E.*

Keywords: *Diabetes mellitus. Pancreas. Oxidative stress. Apoptosis. Vitamin E.*

Introduction

Diabetes is a disease in which the hallmark feature is elevated blood glucose concentrations due to a loss of insulin-producing pancreatic b-cells (type 1 diabetes) or through loss of insulin responsiveness in its target tissues like adipose and muscle (type 2 diabetes)[1].

β-cell mass is regulated by a balance of β-cell replication and apoptosis, as well as development of new islets from exocrine pancreatic ducts (neogenesis). Disruption of any of the pathways of β-cell formation or increased rates of β-cell death would result in decreased β-cell mass and thus reduced capacity to produce insulin[2].

Reactive oxygen species (ROS), which cause cellular damage via oxidation, have been implicated in the pathogenesis of diabetes mellitus. Persistent hyperglycemia in diabetes induces ROS production by glucose autoxidation, activation of protein kinase C (PKC), and increased flux through the hexosamine pathway. An important source of ROS production is nicotinamide adenine dinucleotid[3].

Apoptosis or programmed cell death is a cellular suicide program in which individual cells are destroyed while the integrity and architecture of surrounding tissue is preserved. It is involved in many physiological processes including tissue homeostasis, embryonic development, and the immune response. Under normal circumstances, apoptosis is highly regulated to maintain normal physiological function of the cells. In diabetes the pancreatic cells not only undergo apoptosis but also become necrotic and are unable to secrete insulin A study conducted by Butler et al61 indicated that increased apoptosis rather than decreased neogenesis proliferation might be the main mechanism leading to reduced β-cell mass in DM. Thus, decrease in the rate of apoptosis itself, may increase the β-cell mass via proliferation[4].

STZ-induced β-cell death in the pancreas is associated with oxidative stress caused by the production of excess intracellular ROS; furthermore, STZ may damage pancreatic tissue via imposition of oxidative stress, which in turn can induce apoptosis in pancreatic cells[5].

Hyperglycemia subsequent to diabetes causes oxidative stress, mainly leading to enhanced production of mitochondrial ROS. STZ has been proposed to act as a diabetogenic agent due to its ability to destroy pancreatic β-islet cells, possibly via the formation of excess free radicals. Furthermore, STZ-induced β-cell death is associated with oxidative stress caused by the production of excess intracellular ROS[6].

The activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase which is low in islet cells when compared to other tissues becomes further worsened under diabetic conditions. Earlier reports have shown apoptosis of β-cells and reduced insulin gene transcription by glycation mediated reactive oxygen species. The antioxidant treatment which suppresses apoptosis of β-cells was shown to preserve β-cell function in diabetic mice[7].

There is currently a strong interest in the effects of vitamin E and its relationship to diseases of aging. It is therefore logical to investigate associations between vitamin E and a disease like diabetes, particularly because of the multiple mechanisms by which ROS are generated by elevated blood glucose. Studies have shown that plasma a-tocopherol concentrations are lower in diabetics compared to controls[8].

Materials and Methods

Animals used:

This study was conducted on 32 adult male Wistar albino rats 6-8 weeks old, weighing between 170 and 200 g. Animals were fed a standard diet and housed in the animal laboratory at the medical research center at Benha faculty of medicine. They were placed at room temperature at 25 c with a 12:12-h light/dark cycle.

Groups of the experiment:

The animals were randomly divided into4 groups each consisted of 8 rats as follow:

Group (I): Control group injected with citrate buffer only.

Group (II): Untreated diabetic group.DM was induced by a single intraperitoneal injection of streptozotocin (STZ).

Group (III): Diabetic group re-

ceived vitamin E at a dose of 1 gm /kg/ day for 4 weeks[9].

Group (IV): Diabetic group received vitamin E at a dose of 1 gm /kg/ day for 8 weeks.

Vitamin E dissolved in olive oil and was given daily orally once the rats became diabetic.

Induction and diagnosis of diabetes mellitus:

Diabetes was induced by intraperitoneal (ip) injection of a single dose of STZ (40 mg/kg in freshly prepared citrate buffer pH 4.5). The animals were allowed to drink 5% glucose solution overnight to overcome drug induced hypoglycemia. Control rats were injected by the buffer alone[10].

Diabetes was verified 48-72 hours later by measuring blood glucose levels (after an overnight fasting) with the use of glucose oxidase reagent strips. Rats having blood glucose level of ≥ 300 mg/dl were considered to be diabetic.

Mechanism of streptzotocin (STZ) action:

STZ is diabetogenic because it selectively destroys the insulin producing beta cells. It is postulated that the selective beta-cell toxicity of STZ is related to the glucose similarity in its chemical structure. So it could be transported into the cell by the glucose transport protein 2(GLUT-2), but is not recognized by the other glucose transporters. This explains its relative toxicity to beta cells; since these cells have relatively high levels of GLUT-2[11]. STZ causes cellular toxicity and local immune responses. Cytotoxic effects of STZ are dependent upon deoxyribonucleic acid (DNA) alkylation by specific action with DNA bases and by free-radical generation during STZ metabolism[12].

Chemicals used:

*** Streptzotocin drug:**

It was purchased from Sigma-Aldrich Company (USA). It is presented in powder form, purity more than 99% to be dissolved in freshly prepared sodium citrate buffer pH 4.5.

*** Sodium citrate buffer pH 4.5:**

Preparation of 0.1MCitrate Buffer:

Weigh accurately citric acid 10.5 gm and sodium citrate 14.7 gm. Mix it with 500 ml water.

Make up volume to 1000 ml with distilled water.

Adjust pH 4.5 by sodium hydroxide[13].

*** Vitamin E:**

It was available in the form of dl-alpha-tocopheryl acetate soft gelatin capsule (1000 mg) from Pharco.

Procedure of the experiments:

After an overnight fasting, the animals were anesthetized with diethyl ether. The animals were fixed on operating table and the blood sample and the pancreas were taken as follow:

Blood sample collection:

A craniocaudal incision of about 2 cm is made, parallel and with slightly to the left of the sternum through the skin and pectoral muscles to expose the ribs. A blunt curved forceps is then binged between the 5th and 6th ribs, through the intercostals muscles. The gap is widened so that the rapidly beating heart becomes visible, then the blood sample were taken from the right ventricle.

Tissue preparation:

Each pancreas was quickly removed from the sacrificed rat, placed in ice cold saline solution and trimmed of adipose tissue. Each pancreas was finely cut, homogenized in 50 mM phosphate buffer, pH 7.4 and centrifuged at 10,000 x g for 15 min at 4°C using Beckman cooling Ultracentrifuge. The supernatant was used for determination of MDA and GPx.

Biochemical assessment:

Serum glucose was measured with a Beckman Analyser2 (glucose oxidase method; Beckman, Palo Alto, Ca- Alto, Ca - lif., USA)[14]. Plasma insulin was determined by a double-antibody radioimmunoassay (RIA) kit (Amersham Radiochemical Centre, Bucks, and UK)[15].

Glutathione Peroxidase Activity (GSH-Px):

Glutathione peroxidase (GSH-Px) activity was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide. 100 µL of enzyme sample was incubated for five minutes with 1.55 ml stock solution (prepared in 50 mM Tris

buffer, pH 7.6 with 0.1 mM EDTA) containing 0.25 mM GSH, 0.12 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50 µL of cumene hydroperoxide (1 mg/ml), and the rate of disappearance of NADPH with time was determined by monitoring absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that transforms 1 µmol of NADPH to NADP per minute. Results are expressed as units/mg protein[16].

Lipid Peroxidation contents (LPO):

The product of the reaction between malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) were measured by a modified method of[17]. For each sample to be assayed, four tubes were set up containing 100, 150, 200 and 250 µL of tissue homogenate, 100 µL of 8.1% SDS, 750 µL of 20% acetic acid, and 750 µL of 0.8% aqueous solution of TBA. The volume was made up to 4 ml with distilled water, mixed thoroughly and heated at 95oC for 60 minutes. After cooling, 4 ml of n-butanol was added to each tube, the contents mixed thoroughly, and then centrifuged at 3000 rpm for 10 minutes. The absorption of the clear upper (n-butanol) layer was read at 532 nm. 1, 1, 3, 3 tetraethoxy propane (97%) was used as the external standard. Results are expressed as nmoles TBARS/mg tissue.

Histopathological method:

After animal was scarified, pancreas was isolated and sectioned into small pieces. The sectioned pancreas tissues were fixed in 10% formalin solution embedded in paraffin and cut into a tissue section of 3-5 µm thickness. Tissue was fixed overnight on slides and subsequently stained with H&E, Slides were then observed under light microscope for histopathological analysis[18].

Immunohistochemical analysis:

Paraffin embedded tissue sections of 5 um were prepared on positively - charged slides to be stained with anti-BCl-x antibody using Biotin streptavidin immunoperoxidase technique[19].

Interpretation for immunostaining;

- Bcl-x was detected as cyto-

plasmic brown staining in examined tissues

- Stained sections were classified as[19]:

• Mild intensity → for mild brown cytoplasmic staining.

• Moderate intensity → for moderate brown cytoplasmic staining.

• Strong intensity → for strong brown cytoplasmic staining.

Statistical analysis:

All data were expressed as mean±S.D; data were evaluated by the one way analysis of variance. Difference between groups were compared by Student's t-test with $P<0.05$ selected as the level of statistical significance.

Results

Blood biochemical parameters:

Levels of serum glucose and insulin. Malondialdehyde (MDA) and the activity of the glutathione peroxidase enzyme (GSH-Px) in pancreatic tissues are shown in Table 1. The serum level of glucose was significantly increased in group that received streptozotocin, while serum insulin was significantly reduced in comparison to control group. Pancreatic level of MDA was significantly increased, while GSH-Px enzymatic activity was significantly reduced in group that received streptozotocin in comparison to control group. Administration of vitamin E causes significant decrease in serum glucose level and significant increase in insulin level in diabetic treated group in comparison to diabetic untreated group.

While pancreatic level of MDA was significantly decreased and GSH-Px enzymatic activity was significantly increased in diabetic treated group in comparison to diabetic untreated group.

Histomorphologic changes of pancreas:

Pancreatic sections stained with H&E showed that streptozotocin caused severe necrotic changes of pancreatic islets, especially in the center of islets. Nuclear changes, karyolysis, disappearing of nucleus and in some places residue of destructed cells were visible. Relative reduction of size and number of islets especially around the large vessel and severe reduction of β cells was clearly seen.

Study of pancreas of treated group showed increase size of islets and hyperchromic nucleus in section stained with H&E and also relative increase of granulated and normal β cells in group consumed.

Immunohistochemical changes of pancreas:

Figure 4 shows section in pancreas of control rat showing weak BCL-X expression in cytoplasm of aciner cells (strept - avidin - biotin) x200.

Figure5 shows section in pancreas of diabetic rat showing strong BCL-X expression in cytoplasm of aciner cells (strept - avidin - biotin) x200.

Figure6 shows section in pancreas of diabetic rat with vitamin - E administration for 4 weeks showing moderate BCL-X expression in cytoplasm of aciner cells (strept - avidin - biotin) x200.

Figure7 shows Section in pancreas of diabetic rat with vitamin - E administration for 8 weeks showing weak BCL-X expression in cytoplasm of aciner cells (strept - avidin - biotin) x200.

Table (1): Serum glucose, insulin, pancreatic MDA and pancreatic GSH-Px enzymatic activity. Results are expressed as the Mean ± SD. n = 8; DM= diabetes mellitus group. DME (4 week)= Diabetic group received vitamin E for 4 weeks. DME (8week)= Diabetic group received vitamin E for 8 weeks.

	Control group	DM group	DME (4 week) group	DME (8 week) group
Serum glucose (mg/dl)	116.13 ± 8.7	391.63 ± 26.5*	351.5 ± 13.6*@	207.8 ± 18.5*#
Serum insulin (μU/ml)	4.49 ± .56	1.13 ± .25*	1.48 + .2*@	3.38 ± .35*#
MDA (nmole/mg)	4.56 ± .3	10.75 ± .16*	10.3 + .27*@	5.6 ± .55*#
GSH-Px (units/mg)	10.86 ± .7	5.9 ± .9*	7.2 + .46*@	9.6 ± 1.6*#

**Significant difference (p<0.001) compared with normal control.*
#Significant difference (p<0.001) compared with diabetes mellitus group.
@ Significant difference (p<0.005) compared with diabetes mellitus group.

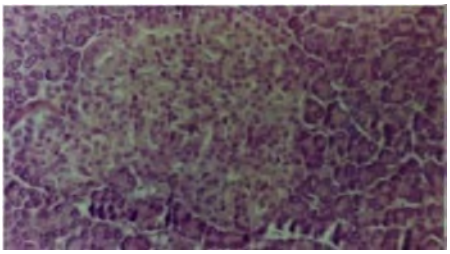

Fig. 1: Control group (H&E).

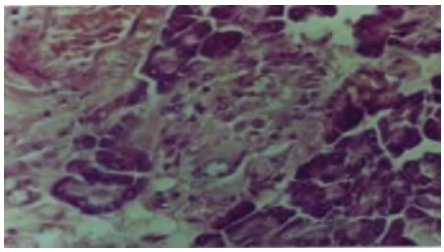

Fig. 2: Diabetic group (H&E).

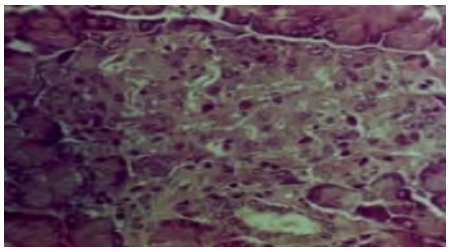

Fig. 3: Diabetic group received vitamin E (H&E).

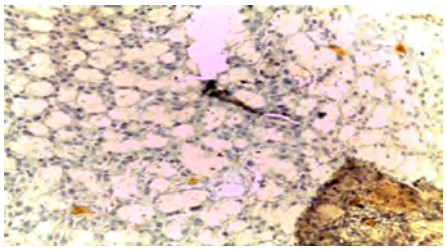

Fig. 4: Control group (strept - avidin - biotin) x200.

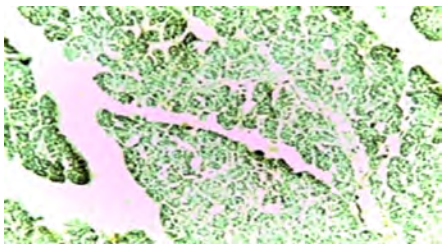

Fig. 5: Diabetic group (strept - avidin - biotin) x200.

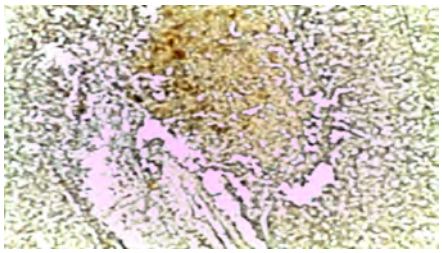

Fig. 6: Diabetic group received vitamin E for 4weeks.

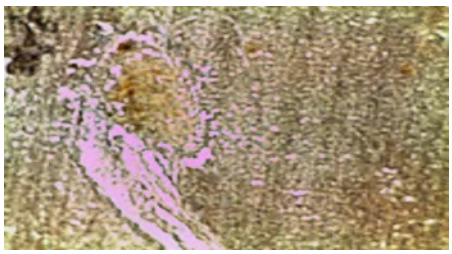

Fig. 7: Diabetic group received vitamin E for 8weeks.

Discussion

Damaged pancreatic islet cells are clinically associated with the development of diabetes as[19] reported that pancreas of diabetic rats displayed degeneration, necrosis and destruction of beta cells in the islets of Langerhans. STZ is a commonly used agent in experimental diabetes due to its selective destruction on beta cells of the islet of Langerhans. Depletion of intracellular nicotinamide dinucleotide (NAD), DNA strand breaks and methylation in islet cells has been proposed as the mechanism by which STZ destroys β-cells of the pancreas[20]. In addition, STZ administration also produced free radicals which directly causing oxidative damage to the beta cells of the pancreas. In this study, STZ-induced diabetes was successfully achieved as evidenced by significantly high level of blood glucose in diabetic rats. The histological finding of the pancreas also demonstrated that a remarkable tissue destruction with a very low intensity of beta cells in islets of Langerhans that could be observed.

Supplementation of vitamin E at 1gm/kg for 4 weeks was able to improve blood glucose levels by significance ($p<0.005$) and Supplementation of vitamin E at 1gm/kg for 8 weeks by significance ($p<0.001$). However, the improvement was not enough to restore blood glucose level to the normal state. Vitamin E is a potent antioxidant that is able to reduce oxidative stress in many pathological conditions[21]. We speculate that the reduction of blood glucose levels in this study may be due to its antioxidant activities. Furthermore, histologically, H&E staining showed that vitamin E supplementation was able to partially restore the histology of the pancreas. Therefore in this study, Vitamin E mainly acted as an antioxidant rather than antidiabetic because of its antioxidative actions on beta cells of the pancreas. Hence, the blood glucose levels did not reach its normal levels.

Our finding also in accordance with[20] and[22] whose had demonstrated that antioxidant supplementation can improve glycemic status[23] also proved that alpha lipoic acid able to reduce blood glucose and pancreatic damage in of type 1 induced diabetic rats.

Furthermore, tocotrienol supplementation significantly increased the insulin levels and reduced the blood glucose in diabetic induced rats in dose dependent manner [24]. In addition, vitamin E supplementation increased the production of insulin by protecting the destruction of beta cells[25]. According to[26] the destruction of pancreas in diabetic rats could be prevented with vitamin E because it's directly acts on the islets of Langerhans.

Previous study showed an increase of free radicals, lipid peroxidation and protein oxidation were occurred in diabetes[27]. Our study also demonstrate that MDA was significantly higher in the pancreas of diabetic group alone and these results are in line with study conducted by[28] which also found that the oxidative stress condition occurred in the pancreas of diabetic rats. The increase in oxidative stress is associated with the destruction of cellular component that continuously occurs during the pathogenesis of DM which may play an important role in tissue injury and the progression of diabetes complications.

Following 4weeks of vitamin E supplementation, the level of MDA in diabetic rats was significantly lower than diabetic rats alone but not reach the control level. While 4weeks of vitamin E supplementation, the level of MDA in diabetic rats was significantly lower than diabetic rats alone the level was almost similar to those recorded in control group. This result indicated the role of vitamin E in reducing lipid peroxidation which is beneficial in overcoming diabetes-related complications. According to[29] chronic administration of vitamin E for 28 days has increased MDA level in pancreas of rats. MDA is indicative of lipid peroxidation in the tissues studied. Accumulating MDA content in lipid bilayer membrane of pancreas can cause loss of its integrity and promote leakage of pancreatic enzymes into circulation. In addition [30] recorded that vitamin E depletes reactive oxygen species in cells and explains the maintenance of endogenous glutathione level and antioxidant enzyme activities. The antioxidant enzyme activities were not inhibited and normalized at similar level to control group. Increases antioxidant

enzyme activity and glutathione level indicates that TRF can restore the antioxidant status of pancreatic cells.

Lack of antioxidant defense mechanism also contributed towards increase in cellular susceptibility against oxidative injury. GSH is the antioxidant defense mechanism that is present in large amount in cells and it also possesses free radical scavenging property[31]. Our study showed that GSH-Px enzymatic activity was significantly decreased in diabetic rats as compared with control group and this in consistence with[32] who concluded that diabetic rats exhibited decreased level of GSH, which might be due to increased utilization of GSH for scavenging free radicals by GSH-Px.in addition Our study revealed that vitamin E supplementation to diabetic rats restored the pancreatic GSH-Px enzyme activity to almost control levels. Antioxidants such as vitamin C play an excellent role in preventing the cells from oxidative damage, which plays a major role in the protection of cells and tissue structures [33]. The decline of GSH in tissue is potentially due to either of lack of NADPH or excessive consumption of GSH for neutralization of peroxide radicals[34]. Supplementation of TRF to diabetic rats was able to restore the GSH level in diabetic conditions to nearly normal state and this is possibly due to the reduction of oxidative stress as shown in this study. The findings are in line with[35] who demonstrated that GSH is increased after the diabetic rats supplemented with antioxidants.

Our study revealed that there was prominent reduction of BCL-X expression in cytoplasm of aciner cells of pancreas was found in diabetic rats supplemented with Vitamin E. The findings are in line with[36] who demonstrated that BCl-xl, an anti-apoptotic protein coded by the "survival gene is involved in the inhibition of apoptosis. Marked overexpression of Bcl-xl, resulted in a severe defect in insulin secretion and hyperglycaemia in transgenic mice.

Conclusion

Vitamin E supplementation to diabetic rats restored the pancreatic GSH-Px enzyme activity to

almost control levels; in addition, MDA decreases as compared with untreated diabetic group. Prominent reduction of BCL-X expression in cytoplasm of aciner cells was found in diabetic rats supplemented with Vitamin E.

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EFFECT OF VITAMIN E ON OXIDATIVE AND APOPTOTIC DAMAGE INDUCED BY STREPTOZOTOCIN IN RAT PANCREAS

Noha Ibrahim Hussien MD
and Ola Ahmad EL-Gohary MD

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THE VALUE OF THE BILIARY AMYLASE IN THE DIFFERENTIATION BETWEEN MALIGNANT AND CALCULAR BILIARY OBSTRUCTION ERCP-BASED STUDY

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Abstract

Background: *Calcular obstruction is the most common cause for biliary obstruction followed by pancreatobiliary malignancies while other causes are far less common. The tissue sampling and diagnosis of pancreatobiliary malignancies is not usually easy and so, many tumour markers in the serum and bile have been reported for pancreatobiliary malignancy, such markers have limited specificity and sensitivity. The serum levels of such markers have been found elevated in some benign diseases. The level of the amylase enzyme in the bile has been evaluated by some authors as a candidate marker for differentiating pancreatobiliary malignancies from calcular obstruction.*

Aim of the work: *Our aim is to evaluate the biliary amylase as a candidate marker in the differentiation between calcular and malignant biliary obstruction.*

Patients and methods: *The present study was conducted on 64 patients with calcular or malignant obstructive jaundice. Between 2011 and 2013. they were divided into 2 goups, the first group with calcular obstructive jaundice (n=16) and the second group with malignant obstructive jaundice (n=48). ERCP was done in all cases and during the procedure bile samples were collected and the biliary amylase level was measured in all samples to evaluate the biliary amylase level as a marker which helps in the differentiation between calcular and malignant biliary obstruction.*

Results: *The present study has shown higher levels of the biliary amylase in calcular obstruction (median) 690.50 (range) 149-7510 and lower levels in malignant obstruction (median) 36 (range) 0.6-231, P<0.001.*

The area under the ROC curve was 0.977 for the biliary amylase in the malignant versus the calcular group (95% CI: 0.904 to 0.998). At a cutoff value of <=131 U/liter, biliary amylase differentiates malignant from calcular obstruction with sensitivity and specificity of 87.5% and 100%, respectively.

Conclusions: *The results of our study proves that the biliary amylase can be used as a reliable biomarker in the differentiation between malignant and calcular biliary obstruction with higher levels in calcular obstruction and lower levels in malignant obstruction.*

Key words: *Biliary obstruction – Biliary amylase – ERCP – Pancreatobiliary malignancy.*

Introduction

Choledocholithiasis is the most common cause for biliary obstruction followed by pancreatobiliary malignancies. The diagnosis and differentiation of such pancreatobiliary malignancies represent a chalange for the clinicians in spite of the advances in laboratory, radiological, endoscopic and histopathological evaluation[1,2,3].

Histopathological examination can simply diagnose pancreatobiliary malignancies but obtaining tissue samples from biliary strictures is not usually simple. Brush cytology is the most frequently used technique for tissue sampling at ERCP, and most of the biliary strictures detected during ERCP can be brushed. The procedure is relatively simple to perform, requires little time and is generally safe. Several studies have been performed to evaluate the cancer detection rate of the brush cytology since the technique was first described by in 1975, and although it has specificity close to 100% brush cytology has a sensitivity ranging from 30 to 57% in most published studies[4,5,6].

Many tumor markers in serum and bile have been reported for pancreatobiliary malignancy, but are with limited specificity and sensitivity. Also their serum levels have been found elevated in some

benign diseases, such as hepatitis, cirrhosis, pancreatitis and cholangitis, as well. Their use in the differential diagnosis is not well defined. These markers include Carcinoembryonic antigen (CEA), carbohydrate antigen (CA19-9), (CA 125), pancreatic elastase, the ratio of pancreatic elastase to amylase activity and, and the Biliary insulin-like growth factor[1,2,7,8,9,10].

The amylase enzyme in humans is secreted mainly from the pancreas and salivary glands while other tissues including the hepatocytes have minimal role in its secretion[11,12].

The most common clinical implication for Serum amylase level is its role as a biochemical marker for acute pancreatitis. However several non-pancreatic conditions can present with abnormal serum amylase levels. Some patients with pancreatitis have normal serum amylase level when a blood sample is examined[13,14,15,16].

The biliary amylase level has been evaluated for many clinical applications such as the diagnosis of acute pancreatitis[17], diagnosis and prediction of Pancreatobiliary Reflux and Occult Pancreatobiliary Reflux[17,18]. Pancreatobiliary reflux could be associated with some mucosal pre-cancerous changes such as hyperplasia and dysplasia of the gallbladder with increased cellular proliferation, and could be a possible risk factor for gallbladder carcinoma and so the biliary amylase level can be used for prediction of gall bladder cancer[19,20].

The biliary amylase represents a candidate marker in the differential diagnosis of the cause of biliary obstruction calcular or malignant irrespective of the pathology of biliary malignancies with its level lower in cancer patients than in gallstone patients and so[2,21].

Aim of The Work

The aim of this study is to evaluate the biliary amylase as a candidate marker in the differentiation between calcular and malignant biliary obstruction.

Patients and Methods

Patients:

The subjects of this study included 64 patients with calcular

or malignant obstructive jaundice. Those subjects were selected from patients attending the outpatient clinic and inpatient unit of Tropical Medicine and Endoscopy Unit of Dar Elshefa hospital in Cairo, the Mansoura University specialized medical hospital and the Mansoura university gastroenterology center between August 2011 and October 2013.

All the studied cases were classified into the following groups:

Group (1): Calcular biliary obstruction group:

- This group comprised 16 patients (25%), 10 males and 6 females with mean age 47.60 years.

- Their age ranged from (30-70 years) (mean±SD: 47.60±10.00 years).

Group (2): Malignant biliary obstruction group:

- This group comprised 48 patients (75%), 31males and 17 females with mean age 59.06 years.

- Their age ranged from (35-74 years) (mean±SD: 59.06±9.02 years).

In our study all the cases of calcular obstructive jaundice were diagnosed by a compination of clinical evaluation, laboratory evaluation, abdominal US, MRCP and ERCP for all patients with abdominal CT or MRI in some selected cases.

In our study all the cases of malignant obstructive jaundice were diagnosed by a compination of clinical evaluation, laboratory evaluation, abdominal US, MRCP, ERCP and histopathological examination for all patients with abdominal CT or MRI in some selected cases.

In our study we excluded all patients with other causes of Causes of hyperamylasemia and hyperamylasuria.

Methods:

The included patients were subject to the following investigations:

- History taking with stress on gastrointestinal symptoms.

- Clinical examination including general examination and local examination including the Abdo-

men, Chest and heart, Neurological examination, Dermatological examination for ulcers or any other skin lesion and Ophthalmological examination.

- laboratory Investigations including, Complete blood picture, Liver function tests. (Serum albumin, Serum bilirubin, Serum alanine transaminase (ALT), Serum aspartate transaminase (AST), Prothrombine time and its activity), Serum Creatinine, Viral markers (HbsAg, HBc IgG., Anti HCV Antibodies (3rd generation ELIZA) and biliary amylase level for all cases after bile samples collection during ERCP.

Tumor markers (CA19-9 and CEA) in the serum in some selected malignant cases.

- Radiological Investigations including, Abdominal ultrasound and MRCP for all cases, Abdominal CT in some selected cases and Abdominal MRI in some selected cases.

- Endoscopic Retrograde Cholangiopancreatography (ERCP). And bile sample collection from the CBD in all cases.

- Pathological examination of the lesions in all malignant cases, in some cases brush cytology was done and in others punch biopsy was taken by biopsy forceps.

ERCP: Preparation, Positioning, and Instrumentation

- Informed consents were taken from all patients, Patients must be an overnight fasting, IV line & nasal mask oxygen were assessed.

- Patients prepared for ERCP take prone position (chest and abdomen facing the table), with the left hand behind the patient's back. This position offers the best access for anterior-posterior fluoroscopy and X-ray imaging. A semi-prone or lateral position may have to be accepted in patients with difficult introduction to pylorus or obese patient to avoid hypoxemia[22].

- Propofol (Astra Zeneca, Wilmington, DE) (isopropylphenol) is a short-acting, intravenously-administered anesthetic agent in all patients in our study. Propofol can be administered by gastroenterologists and nurses with appropriate training are safe and effec-

tive, the dose is variable according to time-related maneuvers[23,24].

- Side view Olympus /or Pentax ERCP was introduced through the mouth to reach duodenal papillae and cannulation of CBD via papillotomes, cannulas or diagnostic catheters measuring a 6 or 7 Fr Teflon tube which tapers to a 3-5 Fr tip, used for injection of contrast into the ductal systems and aspiration of bile. Precut papillotomy is a technique that allows access to the bile duct and is mainly performed when previous attempts to cannulate with standard catheters of sphincterotomes have failed. It is a complex technique that requires expertise and careful patient selection[25,26,27].

- Bile samples (10 ml) were taken by a sterile syringe before injection of dye or contamination of blood from different maneuvers in test tubes not containing EDTA, heparin, or oxalate as these materials may interfere with the biliary amylase testing, any contaminated bile sample with blood or dye should be discarded.

- After bile samples were taken, cholangiography was done and endoscopic sphincterotomy was done in most cases and in calcular cases stone extraction was done.

- In malignant cases punch biopsy was taken if possible as in cases of ampullary carcinoma or cancer pancreas with duodenal infiltration and if not possible brush cytology was done specially in cases of cholangiocarcinoma

- Statistical analysis was carried out via Statistical package for social Science (SPSS) version 17 program on windows XP. Qualitative data were represented in the form of number and frequency, while quantitative data were represented in the form of mean ± standard deviation (mean±SD). Kolmogrov-smirnov test was used to test normality of quantitative data and all data were non-parametric. $\chi 2$, Mann-Whitney and Kruskal-Wallis tests were used to compare groups. Whereas, Spearman's rank test was used to determine correlation between variables. Receiver operating characteristic (ROC) curve was computed to detect possible cutoff val-

ues to differentiate malignant from calcular obstruction. Results were considered significant if p value is less than or equal to 0.05.

Results

• Demographic data in calcular and malignant groups.

There was no significant difference regarding the age and sex between calcular and malignant groups.

• CBC calcular and malignant groups.

There was no significant difference regarding the WBCs and platelets between the calcular and malignant groups but there is a significant difference regarding the Hb%(P=0.02).

• LFTs and serum creatinin in calcular and malignant groups.

There was no significant difference regarding the total bilirubon, direct bilirubin, serum albumin, SGPT and serum creatinin between calcular and malignant groups but there is a significant difference regarding the SGOT level (P=0.001).

• Biliary amylase in calcular and malignant groups

There is a significant difference regarding the biliary amylase with higher levels in calcular obstruction (median) 690.50 (range) 149-7510 and lower levels in malignant obstruction (median) 36 (range) 0.6-231, P<0.001.

The area under the ROC curve was 0.977 for the biliary amylase in the malignant versus the calcular group (95% CI: 0.904 to 0.998). For patients in malignant group, the biliary amylase proved to be useful for predicting malignancy. At a cutoff value of <=131 U/liter, sensitivity and specificity were 87.5% and 100%, respectively. with a positive likelihood ratio =1 and a negative likelihood ratio =0.13.

• Correlation between biliary amylase and other variables in the calcular group:

There was no correlation in the calcular group between Age, Hb%, WBCs, platelet count, total bilirubin, direct bilirubin serum levels of albumin SGPT, SGOT and serum creatinin with the Biliary amylase.

• Correlation between biliary amylase and other variables in the malignant group:

There was no correlation in the

malignant group between Age, Hb%, WBCs, platelet count, SGPT, SGOT and serum creatinin with the Biliary amylase.

There is significant correlation in the malignant group between the total bilirubin and the direct bilirubin with the biliary amylase (P=0.002 and P=0.001) respectively.

Also, there is inversely correlation between serum albumin and the Biliary amylase (P=0.003).

Table (1): Age and Sex in calcular and malignant groups:

	Calcular group (N=16)	Malignant group (N=48)	P value
Age (Mean±SD)	53.9 ±11.4	59.06±9.02	0.07
Sex			
Male	10(62.5%)	31(64.5%)	0.880
Female	6(37.5%)	17(35.5%)	

Table (2): CBC in calcular and malignant groups:

	Calcular group (N=16)	Malignant group (N=48)	P value
Hb% (Mean±SD)	12.45±1.38	11.70±0.97	0.02
WBCs (Mean±SD)	8.36±1.57	7.41±2.50	0.2
Platelets (Mean±SD)	2.46±1.17	2.22±1.05	0.263

Table (3): LFTs and serum creatinin in calcular and malignant groups:

	Calcular group (N=16)	Malignant group (N=48)	P value
Total bilirubin (Mean±SD)	8.16±5.62	11.25±7.41	0.209
Direct bilirubin (Mean±SD)	5.96±4.28	8.35±6.01	0.274
Albumin(Mean±SD)	4.18±0.34	4.18±0.44	0.263
SGPT (Mean±SD)	64.62±25.70	55.68±11.00	0.254
SGOT (Mean±SD)	55.62±21.97	38.68±6.57	0.001
Creatinin (Mean±SD)	0.93±0.18	98±0.19	0.461

Table (4): Biliary amylase in calcular versus malignant groups:

	Calcular group (N=16)	Malignant group (N=48)	*P value*
Biliary amylase Median (range)	690.50(149.7510)	36(0.6-231)	<0.001

Table (5): ROC curve of the malignant versus calcular groups:

Area under the curve	Standard Error	*P value*	*Cut off*	Sensitivity	Specificity
0.977	0.015	<0.0001	<=131	87.50	100.000

+ LR	-LR
1	0.13

Table (6): Correlation between biliary amylase and other variables in the calcular group:

	Rho	P
Age	0.331	0.211
HB	0.226	0.400
WBCs	0.041	0.879
Platelets	-0.329	0.213
Total Bilirubin	-0.089	0.743
Direct Bilirubin	-0.004	0.987
Albumin	-0.356	0.176
SGPT	0.227	0.398
SGOT	0.165	0.542
Creatinin	0.249	0.743

Table (7): Correlation between biliary amylase and other variables in the malignant group :

	Rho	P
Age	-0.185	0.284
HB	-0.189	0.198
WBCs	-0.880	0.550
Platelets	0.044	0.766
Total Bilirubin	0.429	0.002
Direct Bilirubin	0.469	0.001
Albumin	-0.420	0.003
SGPT	0.720	0.064
SGOT	0.119	0.422
Creatinin	0.068	0.646

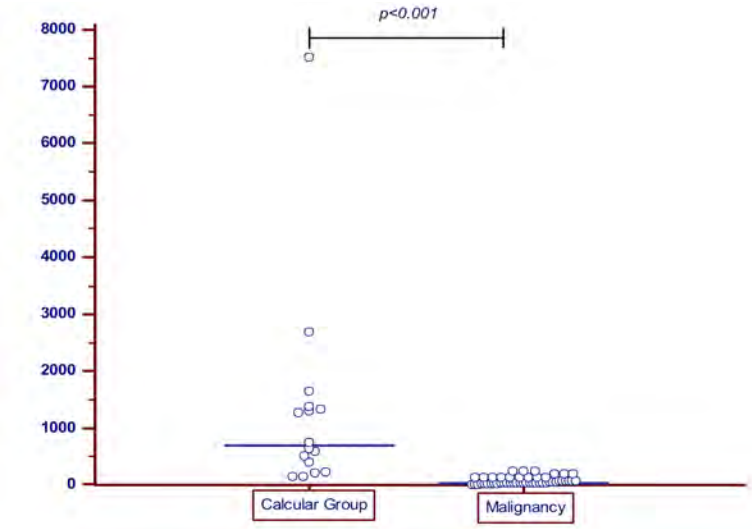

Fig.1: The scatter plot shows the distribution of the amylase levels in the bile from patients of malignant and calcular group.

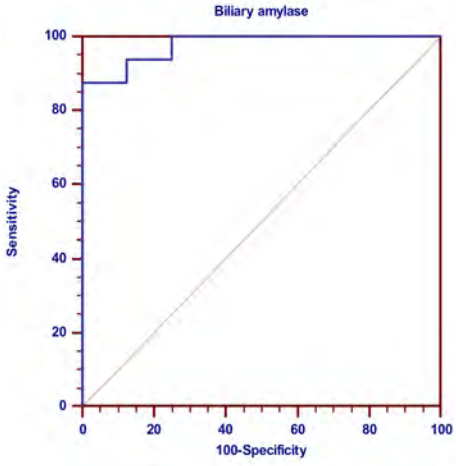

Fig.2: Receiver operating characteristic f ROC curve of the malignant versus calcular groups.

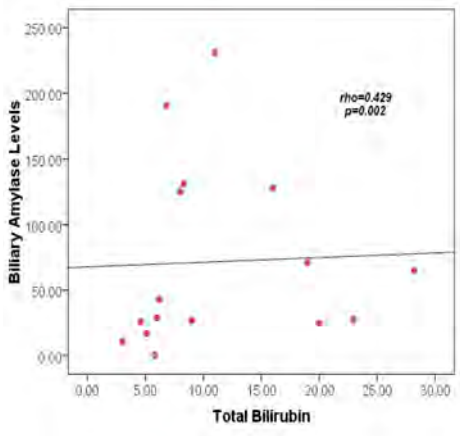

Fig.3: Correlation between the biliary amylase and the serum total bilirubin in the malignant group.

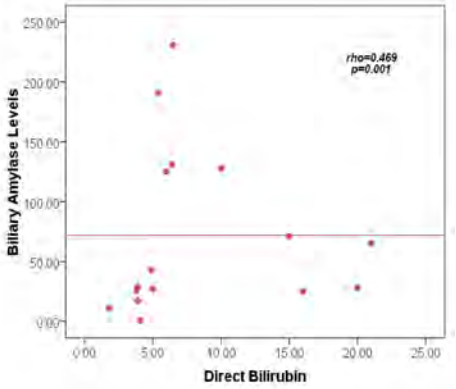

Fig.4: Correlation between the biliary amylase and the serum direct bilirubin in the malignant group.

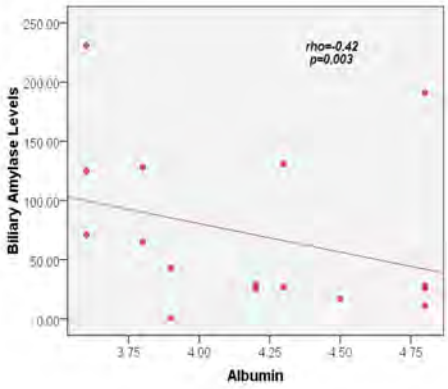

Fig.5: Receiver operating characteristic f ROC curve of the malignant and calcular groups.

Discussion

Our study proves that the biliary amylase level can be used as a reliable biomarker in the differentiation between malignant and calcular biliary obstruction with higher levels in calcular obstruction (median) 690.50 (range) 149-7510 and lower levels in malignant obstruction (median) 36 (range) 0.6-231, P<0.001. The area under the ROC curve was 0.977 for the biliary amylase in the malignant versus the calcular group (95% CI: 0.904 to 0.998). The biliary amylase proved to be useful for predicting malignancy. At a cutoff value of <=131 U/liter, sensitivity and specificity were 87.5% and 100%, respectively.

Chen et al., 2005 have shown similar results with markedly elevated amylase activity in the bile of patients with calcular obstruction (median) 228 U/L(range) 40-1965, compared to those with cancer (median) 32 U/L(range) 30-176, P<0.001. but in our study the amylase activity was much higher in the calcular group.

Chen et al., 2005 have shown some difference in the results with the area under the ROC curve of biliary amylase was 0.751 (95% CI: 0.69 to 0.81). At a cut-off value of 46 U/L, the biliary amylase distinguished patients with malignant obstruction from those with benign obstruction with a sensitiv-

ity of 66% and a specificity of 74%.

In our study there was no correlation in the calcular group between Age, Hb%, WBCs, platelet count, total bilirubin, direct bilirubin serum levels of albumin, SGPT, SGOT and serum creatinin with the Biliary amylase.

In our study there was no correlation in the malignant group between Age, Hb%, WBCs, platelet count, SGPT, SGOT and serum creatinin with the Biliary amylase.

In the present study there is a significant correlation in the malignant group between the total bilirubin and the direct bilirubin with the biliary amylase (P=0.002 and P=0.001) respectively. Also, there is inversely correlation between serum albumin and the Biliary amylase (P=0.003).

This correlation between the serum bilirubin and albumin with the biliary amylase needs further evaluation in both benign and malignant obstruction.

The amylase enzyme is responsible mainly for cleaving of the starch into smaller polysaccharides in the process of digestion. The main sources of amylase in humans are the pancreas and salivary glands, but it can be found in other tissues including the hepatocytes in very small quantities. Several isoforms of amylase can be identified by electrophoresis; the most abundant are the P form derived from the pancreas and the S form of salivary origin[11,12,21].

In cases of biliary obstruction the amylase enzyme detected in the bile is not derived from the hepatocytes or secreted by the hepatosytes from the serum with previous data in human and rabbits showing that the hepatocyte content of amylase is very low and the biliary output of amylase was so small that neither the hepatocyte itself secretes amylase into the bile nor does it act as an excretory pathway for serum amylase. Also, the hepatocytes would decrease or even stop the secretion of bile in response to an increased biliary pressure after biliary obstruction. Previous data has also proved the lack of correlation between the serum and bil-

iary levels of amylase in case of biliary obstruction, therefore the biliary amylase would not originate from the serum amylase secreted by hepatocytes[12,21,28,29,30].

Cases with pancreatobiliary reflux have elevated biliary amylase activity, including patients with anomalous pancreaticobiliary junction, juxtapapillary diverticulum, choledochocyst and even in normal populations where it is termed Occult Pancreatobiliary Reflux [31,32,33,34].

The biliary amylase level may therefore fall in patients with obstructive jaundice when the distal common bile duct is obstructed and pancreaticobiliary reflux is abolished. The biliary obstruction caused by malignancies is often complete and leads to a low possibility of pancreatobiliary reflux. Whereas gallstones induce an incomplete biliary obstruction usually enabling the pancreatic juice to reflux into the bile ducts. The half-life of amylase is relatively short, so increased amylase activity in bile may imply a recent pancreatobiliary reflux. These may responsible for the higher amylase activity in the bile of choledocholithiasis patients, whose biliary obstruction is usually partial and acute[21,35].

In conclusion, measurement of amylase in bile can be a useful biomarker which helps in the diagnosis of malignant biliary obstruction. At a cutoff value of <=131 U/liter, sensitivity and specificity were 87.5% and 100%, respectively.

Conclusions and Recomendations

The diagnosis and differentiation of pancreatobiliary malignancies represent a chalange for the clinicians and so there is continuous need for developing new biomarkers specific for them.

The biliary amylase can be used as a biomarker to differentiate malignant from calcular biliary obstruction with high specificity and sensetivity.

More studies are needed to evaluate the biliary amylase as a marker for malignant biliary obnstruction and to differentiate malignancy from other causes of bil-

iary obnstruction including calcular obstruction.

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THE VALUE OF THE BILIARY AMYLASE IN THE DIFFERENTIATION BETWEEN MALIGNANT AND CALCULAR BILIARY OBSTRUCTION ERCP-BASED STUDY

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SURGICAL MANAGEMENT OF MEDICALLY INTRACTABLE TEMPORAL LOBE EPILEPSY

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Abstract

Object: *The effect of temporal lobe epilepsy surgery on seizure frequency is well recognized but the neuropsychological changes that occur after surgery still not well known. The aim of this study was to compare seizure and Neuropsychological outcome in patients with medically refractory temporal lobe epilepsy using an anterior temporal lobectomy (ATL) or a selective amygdalohippocampectomy (SA).* ***Methods:*** *This study included patients with intractable TLE who received epilepsy surgery. Postoperative suzure outcome was evaluated using Engel classification. Memory functions were evaluated with Wechsler Memory Scale (WMS-R) pre- and one-year post-operatively. Post-operative gains and declines were evaluated in verbal and non-verbal memory scores. Changes or discrepancy of the score greater than 10 was defined as significant.* ***Results:*** *Total 24 patients were included in this study. Verbal-dominant memory impairment (VDMI) was most common (67%). VDMI was more frequent in Lt TLE (79% vs 50%) and non-VDMI was so in Rt TLE (10% vs 7%). Engel class. IA 79% in ATL group and 70%in SAH group. The distribution of significant improvement in full IQ after surgery in both groups (43% ATL/AH and 30% SAH) groups with minimal impairment percent (7% ATL/AH) but the majority of patients shows no significant difference in pre-post operative Full IQ scores (50% ATL/AH and 70% SAH). Significant gains in Full memory were observed in 70% of Trans sylvian amygdalohippocampectomy group and 36% of Anterior temporal lobectomy group.* ***Conclusions:*** *Comparing ATL and SAH, "neither SAH nor ATL can be recommended over the other option as a standard or guideline in the surgical management of TLE." Keeping the limited quality of evidence in mind it appears that the smaller resection*

type SAH is associated in the majority of reports with a similar seizure outcome to the larger ATL resection. Comparing neuropsychological sequelae or outcomes for SAH and ATL several authors have found a better neuropsychological outcome for SAH.

Keywords: *Hippocampal sclerosis, Epilepsy surgery, Temporal lobe epilepsy, Outcome, Engel classification.*

Introduction

The hallmark pathology of temporal lobe epilepsy and makes seizures resistant to medications is mesial temporal sclerosis. The surgically excised hippocampus in these patients almost invariably shows focal cell loss and gliosis. Patients with lesional epilepsy may have a primary brain tumor, vascular anomaly or a malformation of cortical development. (Cambier et al., 2001).

Traditionally, the standard surgical treatment has been an en bloc anterior temporal lobectomy (ATL). ATL involves resection of approximately 3-6 cm of anterior temporal neocortex (depending on language dominance), which permits the surgeon to access and resect mesial structures, including the amygdala and the hippocampus. (Spencer, 1991).

The available evidence suggests that for patients with well-defined mesial temporal onset seizures, particularly those with the syndrome of hippocampal sclerosis, seizure-free outcomes after SAH are equivalent to outcomes after procedures that involve more extensive temporal neocortical resections. (Paglioli et al., 2006).

Given the equivalence in seizure-free outcomes, SAH would be preferred over ATL if it could be clearly demonstrated that the more selective procedure would result in superior postoperative neuropsychological outcomes. Here, the results have been mixed. (Clusmann et al., 2002).

Methods

This study included patients (Total 24) with Drug-resistant TLE who received epilepsy surgery with Unilateral TLE. TLE patients were classified into two groups according to the surgical procedure (first group, 10 patients, trans sylvian Selective amygdalohippocampecto-

my, second group, 14 patients, Anterior temporal lobectomy with amygdalohippocampectomy). Post-operative seizure outcome was classified using Engel classification. Memory and IQ functions were evaluated with Wechsler Memory Scale (WMS-R) and Wechsler Adult Intelligence scale respectively pre- and one-year post-operatively to determine Post-operative gains or declines and Verbal or non-verbal dominant impairment. Changes or discrepancy >10 was defined as significant.

Preoperative evaluation:

The presurgical evaluation of potential candidates included a detailed history and clinical examination, Neuropsychological evaluation, EEG, long-term video/EEG recording, FDG-PET and high-quality MRI scan.

Neuropsychological Test Battery:

The IQ and Memory functions were evaluated with Wechsler Adult Intelligence scale(WAIS-R: VIQ, PIQ, FIQ) and Wechsler Memory Scale (WMS-R: verbal, non verbal, full) pre-operative and one year post operatively.

Two groups of patients enrolled into the study:

Group (A): Anterior temporal Iobectomy and amygdalo-hippocampectomy (14).

Group (B): Selective amygdalo-hippocampectomy (AH) (10).

Results

Seizure Outcome after one year Follow Up:

Seizure outcome in Engel classification according to type of surgery at the 1-year follow-up is provided in Fig 1. Majority of patients shows better seizure outcome after Temporal lope epilepsy surgery, Engel class IA (79% ATL/AH, 70% SAH), Engel II A (14% ATL/AH &10% SAH) Engle IIB (7% ATL/AH and 10% SAH) and (10% Engle IID SAH).

Neuropsychological outcome:

Full IQ:

Fig. 2 shows IQ scores difference before and 1 year after surgery for the ATL/AH and SAH groups. The distribution of significant improvement in full IQ after surgery in both groups (43% ATL/AH and 30% SAH).

Full memory outcome:

Significant improvement happened in 70% for full memory score in SAH

group and 36% in ATL/AH group Fig. 3.

Pathological findings:

The majority of patients show hippocampal sclerosis alone in 70%, HS associated with cortical dysplasia (CD) in 16.7, ganglioglioma in 8.3% and cavernous malformation in 4.2%.

Table (1): Summary of demographic and clinical data from 24 patients who underwent surgery for intractable TLE.

		Type of surgery	
		ATL/AH	**SAH**
No.		14	10
Age (Y)	Mean	32.8	37.7
	Std Dev	9.7	12.3
Sex			
F		9	8
M		5	2
Epilepsy onset (Y)	Mean	10.3	17.8
	Std Dev	7.1	9.7
Handedness			
L		1	0
R		13	10
Associated conditions			
Febrile Seizure		7	5
Meningitis, Febrile seizure		2	2
NO		5	3

ATL: anterior temporal lobectomy SAH: selective amygdalohippocampectomy.

Fig.1: Bar graph Postoperative Engel outcome class at the 1-2 years follow-up in patients with intractable TLE.

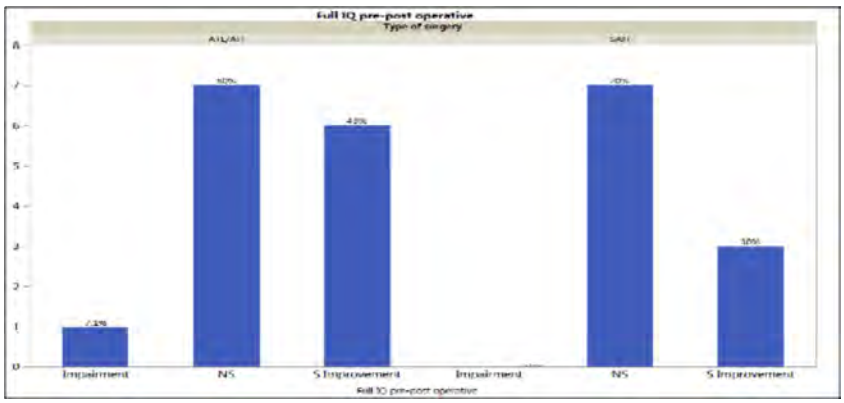

Fig.2: Bar graph showing Full IQ Pre-Post operative in surgery groups.

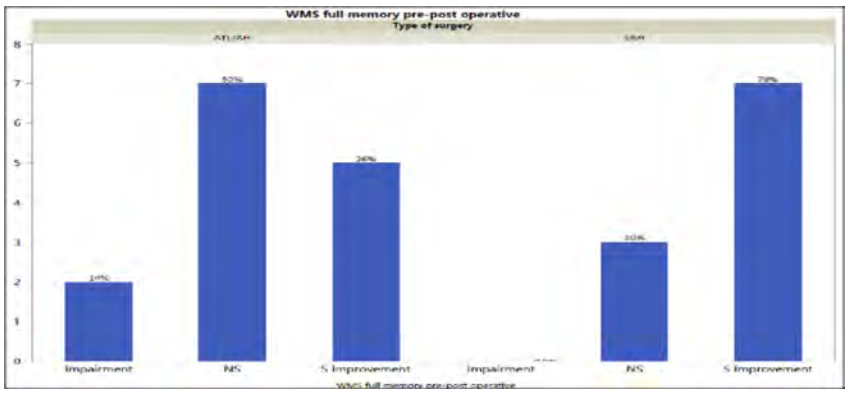

Fig.3: Bar graph showing the post operative full memory in both groups of patients.

Discussion

Surgical treatment of intractable temporal lobe epilepsy (TLE) is an efficient and well-established method (Helmstaedter et al., 2008). A meta-analysis reports a significantly improved seizure situation in about 60-70% of the surgically treated patients. Hippocampal sclerosis which is the most common pathological finding in adult temporal lobe epilepsy has been independently identified as a positive predictor of excellent surgical outcome with amygdalohippocampectomy. (Lee et al., 2002).

In the present study, 80% of

patients with hippocampal sclerosis shows post operative Engel Class I, all patients with ganglioglioma and cavernous malformation lesions shows Engel class I post operatively which is concordant with other studies.

There is also a major favorable rate of surgical success when there is detection of concordant of clinical, radiological and electrophysiological abnormalities that indicate unilateral temporal lobe. The present series also confirms that, all patients pre operatively show concordant radiological and electrophysiological information like other methods. (Smith et al., 2011).

In the present study, majority of patients shows better seizure outcome after Temporal lope epilepsy surgery, Engel class IA (79% ATL/AH, 70% SAH), Engel II A (14% ATL/AH &10% SAH) Engle IIB (7% ATL/AH and 10% SAH) and (10%Engle IID SAH) and these results are concordant with other researches.

Comparing ATL and SAH, McKhann et al. (2006) pointed out that "neither SAH nor ATL can be recommended over the other option as a standard or guideline in the surgical management of TLE." Keeping the limited quality of evidence in mind it appears that the smaller resection type SAH is associated in the majority of reports with a similar seizure outcome to the larger ATL resection (Schramm, 2008).

Comparing neuropsychological sequelae or outcomes for SAH and ATL several authors have found a better neuropsychological outcome for SAH. In a prospective study comparing SAH with anterior one-third lobectomy plus mesial resection (Helmstaedter et al., 2008); SAH appeared to be favorable for material-specific memory in right-sided resections.

Regarding IQ, the average of the IQ scores after surgery for the ATL/AH and SAH groups, shows significant improvement in full IQ after surgery in both groups (43% ATL/AH and 30% SAH) groups with minimal impairment percent (7% ATL/AH) but the majority of patients shows no significant difference in pre-post operative Full

IQ scores (50% ATL/AH and 70% SAH). This has been noted by other authors (Silvia et al.,2012), who point to an overall slight improvement, that might depend on absence of seizures post operatively.

Regarding postoperative memory changes, Significant gains in post-operative verbal memory scores (more than ten pre-post operative verbal memory score difference) were observed in 70% of SAH and 29% of ATL/AH. Significant improvement in non verbal memory 30 % in SAH group and 29% in ATL/AH group. 70% significant improvement in full memory score in SAH group and 36% in ATL/AH group, these results are concordant with other results that agree with the superiority of SAH when compared to ATL.

Summary and Conclusion

Comparing ATL and SAH, "neither SAH nor ATL can be recommended over the other option as a standard or guideline in the surgical management of TLE." Keeping the limited quality of evidence in mind it appears that the smaller resection type SAH is associated in the majority of reports with a similar seizure outcome to the larger ATL resection. Comparing neuropsychological sequelae or outcomes for SAH and ATL several authors have found a better neuropsychological outcome for SAH.

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SURGICAL MANAGEMENT OF MEDICALLY INTRACTABLE TEMPORAL LOBE EPILEPSY

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CESAREAN SKIN WOUND CLOSURE FOR WOMEN DELAYING THE 1st RETURN VISIT AFTER JANUARY 25th/2011

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Abstract

Objective: *Comparing 2 cesarean skin wound closure techniques in cases of delayed or even cancelled 1st return visit in our locality (Sharqya Governorate) after January- 25- 2011. The comparison was done between using vicryl suture with both ends of the suture left fixed inside the wound, to proline suture with both ends of the suture left outside the wound. Also the subcutaneous fat in both techniques was sutured only if it was >2 cm in thickness. The study also included a comparison of the effects of the alternative techniques for closure of the subcutaneous fat and skin on maternal health and satisfaction and also health care resources.*

Design: *clinical cohort observational study.*

Setting: *Zagazig University Hospital, Obstetrics and Gynecology Department. In period between july - 2011 to July 2013.*

Population: *A total of 200 women designed to have elective cesarean deliveries were allocated in 2 groups, each having 100 patients.*

Methods: *Group A; 100 patients with, their skin was closed by subcuticular technique using vicryl 0 suture with burying the suture ends inside the wound.*

Group B; 100 patients, their skin was closed by proline 0 suture with both ends outside the wound.

Removal of skin wound covering and wound cleaning by antiseptic spray containing ethyl alcohol and panthenol.

Main Outcome Measures: *Postoperative wound pain, infection, allergic reactions from sutures, skin separation (superficial separation of the skin layer), cosmosis, duration of hospital stay, and the delayed 1st return visit after cesarean section.*

Results: *No differences in both groups except: 1) skin separation not needing resuturing, in group B (proline group) was 10%, and it was 2% in group A.*

Conclusions: *the efficacy of skin closure by vicryl gave nearly the same results as proline, so it is better to close skin by vicryl with both ends buried in the wound in localities or countries with diffculties in transfer for the 1st return visit after cesarean.*

Keywords: *Cesarean section, proline, vicryl, subcuticular suture.*

Introduction

Cesarean section (CS) is a common operation but without agreed standards of operative techniques, and the materials to be used. Because a huge number of women undergo CS, even small differences in postoperative morbidity rates between techniques could be translated into improved health for substantial numbers of women, and significant cost savings. Improvements in health from optimizing CS techniques are likely to be more significant in developing counteries because the rates of postoperative morbidity in these counteries tend to be higher[1].

The subcutaneous fat (between the sheath and the skin) may be left to heal without suturing, or closed with a variety of techniques. Closing the subcutaneous fat may reduce the risk of some wound complications e.g. hematoma and seroma, but further research is needed to investigate how these outcomes affect the wellbeing and recovery of the patients[2,3].

Regarding the skin closure, the skin incision can be reapproximated by subcuticular suture immediately below the skin layer, by interrupted suture, or by staples[4]. A great variety of materials and techniques are used for skin closure after cesarean section and there is a need to identify which provide the best outcomes for women[4].

The 2 methods of skin closure for CS that have been most often compared are non-absorbable staples and absorbable subcutaneous sutures. Staples are associated with similar outcomes in terms of wound infection, pain, and cosmosis, when compared with sutures. If staples were removed before 4 days, there is an increased

incidence of skin separation and the need for reclosure compared with absorbable sutures. However, skin separation was variably defined across trials. It is unclear which technique of skin closure should be used at CS in order to get the best cosmetic result[5].

A variety of surgical techniques for all elements of the cesarean section operation are in use. Many have been rigorously evaluated in randomized controlled trials[1].

The large number of women undergoing CS throughout the world pass through a period of postoperative pain and a morbidity period. These women constitute a substantial portion of population and so, there is a load on the financial resources of health care system. Use of the appropriate technique to approximate the wound after CS would not only avoid financial load but also help in early recovery of the patient[6,3].

Methods

This is a randomized control trial done at Zagazig University Hospital, Obstetrics and Gynecology Department, between July 2011 and July 2013. Ethic approval obtained from Zagazig University Ethic Committee.

All patiens were designed to have elective CS. All patients were prepared for operative intervention. Information about the study were available to patients during consenting. The authors (W.S., M.S., and H.S.) discussed the procedures with patients and they accepted and consented.

Funding:
All patients were included in the study at fund of Zagazig University hospitals.

Study Population:
The 200 patients designed to have elective CS were in 2 groups:

Group A: 100 patients with elective CS. The skin closed by subcuticular vicryl 0 with burying the ends inside the wound.

Group B: 100 patients with elective CS, the skin closed by proline 0 with both ends outside the wound.

All patients, the subcutaneous fat was closed by vicryl 0 only if it is >2cm thickness.

All patients, were subjected to history taking, general examination, routine lab and obstetric US evaluation.

Inclusion criteria:

Previous 1 or 2 CS, Hb concentration 10-12 gm/dl. No placenta prevea. Single fetus. Average range amount of amniotic fluid.

Exclusion criteria:

>2 previous CS. Multiple pregnancy. Previa. Polyhydramnios.

All patients had CS steps similar to MRCOG guidelines except skin closure technique.

Pfannensteil incision.

Removal of previous CS scar.

Subcutaneous fat was closed only if fat thickness >2cm.

Skin was closed with 2 techniques: Group A by vicryl 0, sucuticular, with both ends hidden inside the wound at the angles of the incision. Group B by proline 0, subcuticular, with the ends remaining outside the wound at the angels of the incision.

Wound care after CS: removal of the skin wound covering after 3 days to remain without covering for cleaning by antiseptic spray containing ethyl alcohol and panthenol in both group.

Main outcome measures: Postoperative wound pain, infection, allergic reaction from sutures, wound gaping, cosmoses after 6 wks of CS, hospital stay, and return follow up visits after CS.

Results

All patients were in the childbearing period of life, 18-38 years. They were not anemic, all had hemoglobin concentration ≥11 gm/dl. (table 1)

No previous CS (1st time CS): 50 patients (50%) in group A and also in group B.

previous 1 CS: 40 (40%) in group A, and 38 (38%) in group B.

Previous 2 CS: 10 (10%) in group A, and 12 (12%) in group B.

Removal of previous 1 CS scar: 20/40 (50%) in groupA, and 23/38 (60.5%) in group B.

Removal of previous 2 CS

scar: 8/10 (80%) in group A, and 9/12 (75%) in group B.

Pain and need for more analgesia (another dose of diclofenac, pethidine, or both diclofenac and pethidine).

Hospital stay: up to 48 hours.

Infection 5%& 4% in A & B.

Allergic reaction at suture site 7%& 5% in A & B.

Superficial skin separation 2% in A (vicryl) & 10% in B (proline).

Wound Cosmosis 90%& 92% in A& B (table 4).

The 1st return visit after the CS (table 5): delayed for up to >40 day. The removal of proline in group B were at 1st return visit.

Table (1): Preoperative clinical criteria.

	Group A Range /number	Mean / percent	Group B Range /number	Mean / percent
1st time CS	50/100	50%	50/100	50%
Previous one CS	40/100	40%	38/100	38%
Previous 2 CS	10/100	10%	12/100	12%
Age (years)	18-38	28	20-37	28.5
Haemoglobin(gm/dl)	11-14	12.5	11-13	12

Table (2): Intraoperative, removal of the old CS scar.

	Group A number	Percent	Group B number	Percent
Previous 1 CS	20/40	50%	23/38	60.526%
Previous 2 CS	8/10	80%	9/12	75%

Table (3): Short term postoperative evaluation.

	Group A number	Percent	Group B number	Percent
Need for another NSAID injection	20/100	20	15/100	15%
Need for pethidine 50 mg injection	7/100	7%	5/100	5%
Need for both NSAID and pethidine	5/100	5%	6/100	6%
Hospital stay (hrs)	12-24	18	24-48	36

Table (4): Wound follow up.

	Group A number	Percent	Group B number	Percent
Wound infection	5/100	5%	4/100	4%
Wound allergic reaction from at suture site	7/100	7%	5/100	5%
Wound gaping	2/100	2%	10/100	10%
Wound cosmoses after 6 wks	92/100	92%	90/100	90%

Table (5): 1st Return visit after CS follow up of postoperative evaluation.

	Group A number	Percent	Group B number	Percent
From 7-14 days	20/100	20%	40/100	40%
From 15-28 days	8/100	8%	35/100	35%
From 28-40 days	15/100	15%	14	14%
More than 40 days	57/100	57%	11	11%

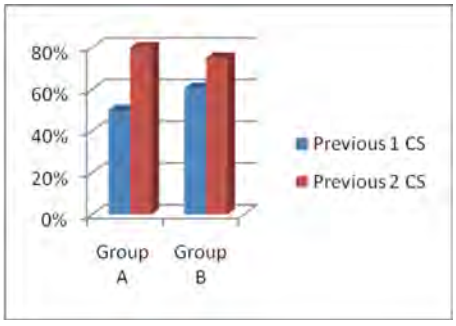

Fig. 1: Removal of old CS scar.

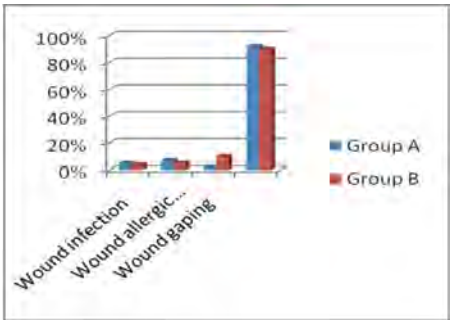

Fig. 2: Follow up of wound.

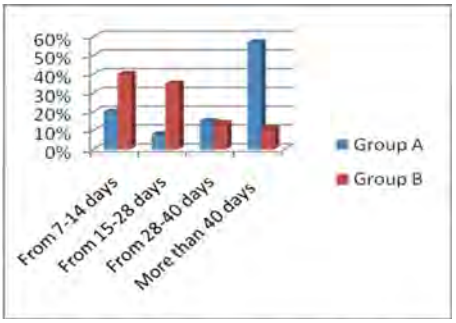

Fig. 3: 1st return vist after CS.

Discussion

Strengths:

Our study can be added to many recent studies that compared the effects alternative techniques for closure of subcutaneous fat and skin in CS on maternal health. This study was designed for women that delay the 1st return visit after CS. It is unique among others by being done in our locality with low socioeconomic standards (rural areas in Sharqyah Governorate) and difficulties in transfer for the 1st return visit after January - 25 - 2011. It is a comparative observational cohort study.

Cesarean is one of the most commonly performed surgical procedure in the world. Rates have increased in recent years – about 20-25% in many developed counteries and rates in other parts of the world vary widely[1]. yet the techniques used during this procedure often vary significantly among providers[13]. However, because huge numbers of women undergo CS, even small differences in postoperative morbidity rates between techniques could be translated into improved health for substantial numbers of women undergoing CS, and significant cost saving. Also, from women's point of view, occurance of a wound complications is the most important factor that influenced their satisfaction[1].

It is unclear which technique for skin closure could be used at cesarean section in order to get the best cosmetic result. Closing of the subcutaneous fat layer, however, negatively affects the cosmetic results and is associated with a longer admission time[14]. Suture closure of subcutaneous fat during CS delivery resulted in a 34% in risk of wound disruption in women with fat thickness >2. Subcutaneous fat appeared to be the only significant risk factor assossiated with abdominal wound infection after CS delivery[8]. In our study, we closed the subcutaneous fat layer only if its thickness was >2m.

Superficial wound disruption may be minimized by either closing large nondraining subcutaneous spaces or using continuous drainage. This needs randomized clinical trials when translating this information into routine sur-

gical practice[10]. In our study, the skin separation was superficial and more in cases with proline closure (10%), than in cases with vicryl closure (2%), that to say more time for skin approximation by vicryl allow better skin closure.

A type of suture material is the barbed suture (barbed suture is versus smooth surface suture e.g. vicryl). It is self anchoring and not requiring knots for wound closure but it has cosmosis and safety profile similar to that of the conventional suture technique[9], like that used in this study.

Out of 158 randomized patients, 83 patients underwent the innovative CS (Joel Cohen) incision which include a straight transverse skin incision 3cm above symphysis pubis. This method was not associated with decrease in puerperal mobidity [10]. It gave the same results regarding skin closure in this study.

Pfannenstiel skin incision closed with subcuticular suture following CS resulted in less postoperative discomfort and were more cosmetically appearing at the 6th week postoperative visit as compared to incisions closed with staples[11]. This gives the same results in our study in part of skin closure by subcuticular sutures.

Limitations:

Our study had some limitations as a result of: difficulty in follow up due to the delay in the 1st return visit after CS, and also due to no inclusion of the other complications of the CS that were not the aim of the study. A prospective multicenter study of 1,032 CS was performed to identify risk factors for post operative wound infection. The overall rate of wound infection was 6.6% (3.8% in elective cases and 7.5% following non-elective operations), with considerable interhospital variation[15]. This was different regarding the risk of wound infection as it was not in goals of our study.

Skin preparation with an antibacterial scrub in addition to standard povidone-iodine scrub do not appear to play as significant a role in the reduction of post-cesarean endometritis or wound infection as does intraoperative pelvic irrigation with antibiotic so-

lution[16]. A reduction in postoperative wound infection associated with redisinfection, while no benefit from adhesive plastic drapes could be demonstrated[17]. The study was different from our study design in that it compared different modalities for skin disinfection.

Interpretation:

Improvements in health from optimizing CS techniques are likely to be more significant in developing counteries because the rate of postoperative morbidity in these counteries tend to be higher. More women could therefore benefit from improvements in techniques[1].

The usage of skin closure with vicryl gives nearly the same results to that of proline. The technique of using vicryl with both ends hidden inside the wound was preferred to using proline with both ends outside the wound to avoid patient's need to return to hospital during periods of problems that prevent the 1st visit.

Much of the process regarding suture selection depends on the sugeon's training and preference. A wide variety of suture materials are available in each surgical locations and surgical requirement. Generally the surgeon selects the smallest suture that can adequately hold the healing edges and is able to achieve a tension-free closure. As the wound heals, the relative loss of suture strength over time should be slower than the gain of tissue tensile strength. Therefore nonabsorbable sutures are considered in skin. Sutures are no longer needed when the wound has reached maximum strength. Therefore, nonabsorbable sutures are considered in skin and also in fascia and tendons (slowly healing tissues). While mucosal wound (rapidly healing tissues)may be closed with absorbable sutures. Wound closure is affected by the initial tissue injury caused by needle penetration and subsequent tissue passage. Needle selection, surface characteristics of the suture (coefficient of friction) and suture coating material are important factors that justify surgeon's consideration[3].

Chemical treatments such as chromic salt lengthen the absorption time. Accelerated absorption may occur in patients with fe-

ver,infection or protein deficiency and mal lead to an excessively rapid decline in tensile strength. Accelerated absorption may also occur in a body cavity tha is moist, filled with fluid or if the sutures become wet or moist during handling prior to implantation[3].

Proline is a monofilament, non-absorbable synthetic suture that permits little or no saturation. It does not adhere to tissues and is useful as a pull-out suture.g. subcuticular closure. It also holds knots better than other monofilament synthetic materials. It is biologically inert and elicits minimal tissue reaction.

The goal of any skin closure technique is to produce appropriate skin approximation and adequate healing while minimizing pain, wound complications, scarring, and cost. The technique should be quick, cost effective and simple, while maximizing wound cosmosis and patient satisfaction [12]. So, both groups of patients in our study showed nearly the same result except: the higher incidence of skin separation with proline group, and the delay in the 1st return visit after CS in both groups.

Conclusion

This study was designed for cases that delay or cancell their 1st return visit. So, It is better for patients suspected to delay her 1st return visit to have her skin closed by subcuticular vicryl 0 with burying both ends inside the wound. This recommendation can be adopted in other counteries having problems like that in Egypt after January - 25 - 2011 that hinder the patient transfer for the 1st return visit.

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Contribution of Authorship:

W.S., M.S., and H.S. contributed to the protocol, coordinated the study, interviewed the parents, analysed the data, and drafted the article. W.S., M.S., and H.S. contributed to the revision and final approval of the article.

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ANTIOXIDANT EFFICACY OF ADRENOMEDULLIN VERSUS VITAMIN E AND C IN DIABETIC NEPHROPATHY IN RATS

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Abstract

Background: *Oxidative stress has crucial role in pathogenesis of diabetic nephropathy (DN). Despite satisfactory results from antioxidant therapy in rodent, antioxidant therapy showed conflicting results in combat with DN in diabetics.*

Aim of the work: *This study was designated to evaluate the effects of adrenomedullin (ADM) supplementation alone or combined with vitamins E and C on oxidative stress in streptozotocin (STZ) induced diabetic nephropathy in rats.*

Materials & Methods: *50 Sprague Dawley rats 6 weeks old weighing 300 to 350 gm were used. Diabetes was induced in 40 rats and the other 10 rats were received sodium citrate buffer(pH 4.5) intraperitoneal only and served as control group (Group I).*

The STZ induced diabetic rats were divided into four groups (n=10):

Group II: STZ – induced diabetic rats (DM group). Group III: STZ – induced diabetic rats treated with ADM s.c at a dose of 100 ng/kg for 14 days. Group IV: STZ – induced diabetic rats received α-tocopherol succinate (vitamin E) (500 mg/kg/day) in corn oil by gavage and ascorbic acid (vitamin C) (100 mg/kg/day) was provided as a supplement dissolved in the drinking water for 14 days. Group V: STZ - induced diabetic rats received ADM (100 ng/kg) s.c, α-tocopherol succinate (vitamin E) (500 mg/kg/day in corn oil; 3mg/kg/day) by gavage and ascorbic acid (vitamin C) (100 mg/kg/day) was provided as a supplement dissolved in the drinking water for 14 days. The study evaluated the effect of adrenomedullin and antioxidants (vitamins E & C) on body weight (BW), kidney weight (KW), KW/BW (%), 24 hours urinary albu-

min excretion (UAE), blood glucose (mg/dl) and serum creatinine (S.Cr), plasma malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), and glutathione in different rats groups.

Results: *Body weight and kidney weight of diabetic group (group-II) were decreased (P<0.001), while KW/BW ratio was significantly increased (P<0.05) compared with the control group (group-I). Urinary albumin excretion (UAE) was significantly increased in diabetic group (group II) in comparison to the control group (group I) (P<0.001). In comparison to diabetic group II, all other diabetic groups III, IV and V showed significant decrease in UAE (P<0.001). The glucose level of all diabetic groups was significantly increased (P<0.001) in comparison to the control group. Serum creatinine showed no significant change in between all diabetic groups. Plasma MDA in diabetic groups was significantly increased compared with the control group, while plasma SOD, CAT and glutathione were significantly decreased (P<0.001).*

Conclusions: *Adrenomedullin and vitamins E and C may decrease lipid peroxidation and increase the antioxidant enzyme level as well as decrease blood glucose to protect kidney from oxidative injury in STZ-induced diabetic nephropathy in rats.*

Introduction

Diabetes mellitus is a common but serious metabolic disorder associated with many functional and structural complications[1].

Diabetic nephropathy (DN) is the main cause of end stage renal disease requiring dialysis and glomerular sclerotic injury is an initial event that provokes renal dysfunction during processes of diabetic-linked kidney disease. Over the last decade, there has been an intense interest in oxidative stress and its role in the development of nephropathy in diabetic rats[2].

Clinical studies have demonstrated that hyperglycemia-induced oxidative stress play a central role in both pathogenesis and numerous pathophysiological mechanisms that trigger diabetic complications, primarily categorized into macroangipathy and microangiopathy, the latter of which includes nephropathy, retinopathy, neuropathy, and microvasccular damage to the cerebral artery[3]. A very common

finding in all tissues affected by diabetes, including the kidney, is the presence of increased oxidative stress that generated by the imbalance between reactive oxygen species (ROS) and the endogenous antioxidant forces[4]. The major source of oxidative stress is an enzyme reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived superoxide anion ($O2^{\cdot -}$) that directly damage cell and also activate signaling of cell proliferation, adhesion molecules, and fibrosis. $O2^{\cdot -}$ also inactivates endothelium derived nitric oxide[5]. NADPH oxidase is a critical component of both phagocytic and non-phagocytic cells, including fibroblasts, vascular smooth muscle cells, endothelial cells, renal mesengial cells, and tubular cells[6]. However, NADPH is considered the principle reducing power in the cell. An antioxidant enzymes glutathione reductase and catalase are critically dependent on NADPH. Reduced glutathione (GSH) is used to reduce an oxidant and in turn is converted into oxidized glutathione (GSSG). An enzyme glutathione reductase then converts it back to reduced glutathione using NADPH[7]. Also Xu et al.[8] reported that the main source of NADPH in cells is the enzyme glucose 6-posphate dehydrogenase (G6PD). Under high glucose conditions, G6PD activity is significantly impaired.

High glucose-induced ROS generation in mesengial cells is protein kinase C (PKC) dependent as in aortic smooth muscle and endothelial cells[9]. However, Nishikawa et al.[10] demonstrated that normalizing mitochondrial superoxde overproduction were able to inhibit the activation of PKC, advanced glycation end products (AGEs) formation, and increased aldose reductase pathway, suggesting that ROS produced by mitochondria play a critical role in high glucose-induced diabetic vascular complications.

Moreover, high glucose also induces intracellular ROS indirectly through AGEs and cytokines, including transforming growth factor beta-1 (TGF-β1)[11]. Advanced glycation end products (AGEs), a non - enzymatically glycated protein derivatives produced from the prolonged exposure of amino acids to high glucose level. The main pathological consequences of AGEs in-

teraction with their receptor in endothelial cells (RAGE) is the induction of intracellular ROS through the mitochondrial respiratory chain and the NADPH oxidase systems. There is compelling evidence that the formation and accumulation of AGE mediates the progressive alteration in renal architecture and loss of renal function. ROS generation induced by AGE might be involved in mesengial cell apoptosis[12].

On the other hand, the transforming growth factor- β1 (TGF - β1) is the key regulator of extracellular matrix (ECM) remodeling in the mesangium leading to mesengial expansion[13]. and of tubular epithelial-mesenchymal transition[14] leading to tubulointerstitial fibrosis. It increases intracellular ROS in mesangial and tubular epithelial cells through NADPH oxidase dependent pathway[15]. ROS induce AGEs and upregulate TGF-β1, both of which, in turn, induce ROS generation in mesengial cells, thus providing an amplification loop in high glucose signaling in diabetic kidney[16].

In the kidneys of diabetic animals, the expression of NADPH oxidase and its oxidative products is observed to increase, and the suppression of NADPH oxidase ameliorates renal damage and endothelial dysfunction in diabetics[17,18]. Adrenomedullin has been demonstrated to possess an antioxidant action; however, the relationship between NADPH oxidase and adrenomedullin in the kidneys of diabetic nephropathy remains to be elucidated.

On the other hand, adrenomedullin (ADM) is synthesized as part of a larger precursor molecule, termed preproadrenomedullin. In both rat and human this precursor consists of 185 amino acids[19]. Preproadrenomedullin contains a 21-amino acid N-terminal signal peptide that immediately precedes a 20-amino acid amidated peptide, designated proadrenomedullin N-terminal 20 peptide or PAMP[20].

It has also been suggested that a further biologically active peptide, termed adrenotensin, may be a product of the adrenomedullin gene[21].

The adrenomedullin gene is expressed in a wide range of tissues. The initial report on the distribu-

tion of adrenomedullin mRNA suggested that the highest levels of expression were seen in the adrenal medulla, ventricle, kidney, and lung[20]. Since the discovery that the adrenomedullin gene is more highly expressed in endothelial cells than even in the adrenal medulla[22], this peptide has come to be regarded as a secretory product of the vascular endothelium, together with nitric oxide (NO) and endothelin. Clearly, therefore, adrenomedullin expression is seen in all tissues of the body, and comparisons between tissues may simply reflect varying degrees of tissue vascularity. However, evidence from both immunocytochemistry and studies with cultured cell lines reveals that the adrenomedullin gene is expressed by many different cell types, in addition to vascular endothelial cells. In addition, many tumor cell lines express the adrenomedullin gene or have been shown to synthesize the immunoreactive peptide[23]. It is widely recognized that adrenomedullin is a product of vascular endothelial cells, but it appears that not all vascular endothelial cells synthesize the peptide. In some tissues, although adrenomedullin immunostaining has been demonstrated in certain cells, the vascular endothelial cells and smooth muscle cells remain unstained. This is particularly noticeable in the rat adrenal gland[24]. The question as to whether adrenomedullin, in common with many other regulatory peptides, is stored by some cell types has been raised. There is evidence that adrenomedullin is stored in secretory granules in the pancreas[25]. A positive correlation has been demonstrated between plasma renin angiotensin, aldosterone concentration, and adrenomedullin[26], with a negative correlation between adrenomedullin and glomerular filtration rate[26], creainine clearance, and sodium excretion[27].

Moreover, the Circulating adrenomedullin can affect renal function, and evidence exists for a role for locally produced adrenomedullin in tubular function. The first reported studies on renal function involved intrarenal arterial perfusion in anesthetized dogs was by Ebara et al.[28]. Adrenomedullin administration had no effect on heart rate or mean arterial blood pressure, but increased RBF, urine

output, and urinary Na^+ excretion in a dose-dependent manner, indicative of direct preglomerular and postglomerular arteriolar effects[28]. Subsequent studies found this effect to be mediated via an endothelial, NO-dependent mechanism[29]. In the anesthetized rat, intrarenal adrenomedullin infusion leads to increases in RBF, arterial conductance, glomerular filtration rate (GFR), Na^+ excretion, and urine flow[30]. Bolus administration of adrenomedullin peripherally significantly lowers mean arterial pressure and raises RBF, GFR, and urine flow; the latter three responses were significantly attenuated in the presence of L-NAME[31]. On the other hand, studies have been carried out in a rat model of heart failure. In this series of experiments, Nagaya and co-workers[32] demonstrated that intravenous infusion of a low dose of adrenomedullin to normal rats or those with heart failure led to significantly increased urine volume and Na^+ excretion without changing GFR, RBF or any other hemodynamic parameter[32]. High-dose adrenomedullin infusion did not increase urinary cGMP levels, suggesting that the renal actions of adrenomedullin may not be mediated totally by the NO pathway in these rats[33]. Contrary to this, Rademaker et al. showed that intravenous administration of adrenomedullin increased Na+ excretion without an increase in urine flow or creatine clearance in an ovine model of heart failure[34]. The discrepancy between these studies may be explained by the differences in renal perfusion pressure; however, it is unlikely that circulating levels of adrenomedullin regulate renal function physiologically[35]. However, it has been suggested that neutral endopeptidase (NEP) can potentiate the renal natriuretic and diuretic actions of intrarenal adrenomedullin infusion[36]. NEP is a membrane-bound metalloproteinase that cleaves endogenous peptides at the amino side of the hydrophobic residues. This ectoenzyme is localized in a number of tissues but is found predominantly in the kidney[37]. Substrates for NEP include bradykinin, AVP, and substance P, and the study described by Lisy et al.[36] concludes that adrenomedullin is also a substrate for this ectoenzyme. Inhibiting systemic NEP raises plasma adrenomedullin lev-

els significantly, supporting the conclusion that adrenomedullin is a substrate for NEP. NEP inhibition also potentiates an increase in Na^+ excretion in the absence of an increase in GFR or further increases in RBF in response to exogenous adrenomedullin. This indicates that a decrease in tubular Na^+ reabsorption is the mechanism for natriuresis. The identification of adrenomedullin in the inner medullary ducts correlates with the ability of this tissue to increase its permeability to water in response to adrenomedullin[38].

Adrenomedullin is a potent vasodilating peptide that is upregulated in cardiovascular diseases to counteract the disease process with its diverse physiological actions including antioxidative stress actions[39]. The plasma concentration of adrenomedullin also increased in the diabetic patients, and hyperglycemia increases the production of adrenomedullin in the vasculature[40]. The receptors for adrenomedullin are expressed in the kidneys, especially in the glomerulus and distal nephron, and the local action of adrenomedullin is increased in diabetic rats[41], thus suggesting that adrenomedullin may contribute to the dilatation of the glomerular capillary in the early phase of diabetic nephropathy. Although the organoprotective effects of adrenomedullin have been demonstrated in various cardiovascular diseases, the mechanisms underlying its renoprotection in diabetic nephropathy are still unclear.

However, Ascorbic acid or "vitamin C" is a monosaccharide oxidation-reduction (redox) catalyst found in both animals and plants. As one of the enzymes needed to make ascorbic acid has been lost by mutation during primate evolution, humans must obtain it from the diet; it is therefore a vitamin[42]. Most other animals are able to produce this compound in their bodies and do not require it in their diets. Ascorbic acid is required for the conversion of the procollagen to collagen by oxidizing proline residues to hydroxyproline. In other cells, it is maintained in its reduced form by reaction with glutathione, which can be catalysed by protein disulfide isomerase and glutaredoxins[43]. Ascorbic acid has direct antioxidant effects & is a redox catalyst which can reduce, and thereby

neutralize, reactive oxygen species such as hydrogen peroxide[44].

Vitamin C (ascorbic acid) water solubility allows it to be widely available in both the extracellular and intracellular spaces in most biologic systems where it can participates in reduction-oxidation reactions. It contributes to the regeneration of membrane-bound oxidant vitamin E, allowing it to function again as a breaking antioxidant[45].

On the other hand, Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties[46]. Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolising this form[47].

It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction[48]. This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidised α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol [49]. This is in line with findings showing that α-tocopherol, but not water-soluble antioxidants, efficiently protects glutathione peroxidase 4 (GPX4)-deficient cells from cell death[50]. GPx4 is the only known enzyme that efficiently reduces lipid-hydroperoxides within biological membranes.

Vitamin E (α-tocopherol) is a lipophilic endogenous molecules with important antioxidant function and working in close connection with vitamin C. Vitamin E plays a leading role in controlling excess oxidative radical formation at cell membrane[51] including mitochondrial membrane[52].

However, the role of adrenomedullin in the diabetic nephropathy is not well understood. Hence, the present study was designed to investigate the antioxidant effect of adrenomedullin versus common antioxidants (vitamins E & C) in diabetic nephropathy in rats.

Materials and Methods

Drugs & Reagents:

1- Adrenomedullin (ADM): Synthetic peptide, produced by SIGMA CHEMICAL CO. St. Louis, USA. It was dissolved in distilled water.

2- Ascorbic acid, vitamin C (Cevarol, Memphis): It is supplied in the form of ampoules (500 mg/5ml).

3- α-tocopherol succinate (Vitamin E): (Vitamin E capsule 100 mg, Cairo).

4- STZ (streptozotocin) was obtained from Sigma-Aldrich., St. Louis, MO, U.S.A.).

Other reagents were purchased from Sigma Chemical Co., St. Louis, USA.

Experimental Animals:

50 Sprague Dawley rats 6 weeks old weighing 300 to 350 gm were used. Diabetes was induced in 40 rats and the other 10 rats were received sodium citrate buffer (pH 4.5) intraperitoneal only and served as control group (Group I).

Diabetes was induced by a single intraperitoneal injection of streptozotocin, STZ, (55 mg/kg body weight)[53] dissolved in 0.1 mmol/l sodium citrate buffer(pH 4.5). 72 hours after STZ injection diabetes mellitus was confirmed by measuring tail blood glucose levels, using a reflectometer device (Lifescan, Milpitas, CA). Every animal received a daily injection of ultralente insulin (Novo, Copenhagen) s.c individually adjusted to attain blood glucose levels of 300 to 500 mg/dl, which was monitored every week.

The STZ induced diabetic rats were divided into four groups (n=10):

Group II: STZ - induced diabetic rats (DM group).

Group III: STZ - induced diabetic rats treated with ADM subcutaneously (s.c) at a dose of 100 ng/kg for route 14 days[54].

Group IV: STZ - induced diabetic rats received α-tocopherol succinate (vitamin E) (500 mg/kg/day) in corn oil by gavage[55] and ascorbic acid (vitamin C) (100 mg/kg/day) was dissolved in the drinking water[56] for 14 days.

Group V: STZ - induced diabetic rats received ADM (100 ng/kg) s.c, α-tocopherol succinate (vitamin E) (500 mg/kg/day) in corn oil by gavage and ascorbic acid (vitamin C) (100 mg/g/day) was dissolved in the drinking water for 14 days.

All rats had free access to water and standard rat chow through the study. Glucose level and body weight, kidney weight /body weight ratio (KW/BW ratio) were monitored every week according to [57]. Twenty four hour urine samples were collected in individual metabolic cages for measurement of urinary albumin excretion (UAE) one day prior to sacrifice.

After the experimental period, the animals were sacrificed, under pentobarbital anaesthesia (5 mg/kg), an anticoagulant (EDTA) was added to tube containing 5 ml blood which centrifuged at 2,000 g for 1 min at 4ºC to separate plasma from erythrocytes.

The biochemical parameters determined included:

1- Seum glucose level: glucose was determined after enzymatic oxidation in the presence of glucose oxidase[58].

2- Serum insulin level: A solid phase enzymatic amplified sensitivity immunoassay for insulin was performed on micro titre plate according to[59].

3- Serum creatinine: was measured by modified Jaffe method using creatinine - E kit (Yeong Dong Pharmaceutical Co.).

4- Malondialdehyde (MDA): was measured by the modified method of Ohkawa et al[60].

5- Catalase activity (CAT): was measured by Beutler's method[61].

6- Superoxide dismutase (SOD) activity: was determined by the method of Winterboum et al[62].

7- Glutathione: was measured by glutathione colorimetric assay kit[63].

8- Urine microalbumin concentration: was measured by rapid colorimetric method[64] using commercial kit (ABC diagnostic).

Statistical Analysis:

Data are expressed as means standard error (SE). Mann-Whitney U test was used to study the statistical significance of kidney weight, blood glucose and 24 hour urinary albumin excretion. While unpaired t-test was used for analysis of the remaining parameters. Correlation was expressed by Kendall's rank correlation, while regression formulation was obtained by regression analysis. A P value <0.05 was considered significant.

Results

Table (1): Body weight (BW), kidney weight (KW), KW/BW (%) and 24 hours urinary albumin excretion (UAE) of different rats groups (Mean ± SE).

Groups	BW (gm)	KW (gm)	KW/BW (%)	UAE (mg/24h)
Control (gpI)	329 ± 8.34	2.58 ± 0.12	0.0076 ± 0.0003	17.16 ± 0.25
STZ – induced DM (gpII)	200 ± 6.56	1.8 ± 0.06	0.0086 ± 0.0005	60.43 ± 2.56
P1	<0.001	<0.001	<0.05	<0.001
STZ - induced diabetic rats treated with ADM (gp III)	203 ± 5.64	1.56 ± 0.04	0.0079 ± 0.0004	41.35 ± 3.72
P2	NS	NS	NS	<0.001
STZ - induced diabetic rats received α-tocopherol succinate (vitamin E) and ascorbic acid (vitamin C) (gpIV)	217 ± 6.54	1.76 ± 0.03	0.0080 ± 0.0003	35.67± 4.53
P3	NS	NS	NS	<0.001
STZ - induced diabetic rats received ADM, α-tocopherol succinate (vitamin E) and ascorbic acid (vitamin C) (gpV)	213 ± 7.14	1.82 ± 0.02	0.0085 ± 0.0003	27.11 ± 3.06
P4	NS	NS	NS	<0.001
P5	NS	NS	NS	<0.05
P6	NS	NS	NS	NS

P1: statistical significance between CN group versus DM group
P2: statistical significance between DM group versus DM + ADM group.
P3: statistical significance between DM group versus DM + Vit E & C group.
P4: statistical significance between DM group versus ADM + Vit E & C group.
P5: statistical significance between ADM group & Vit E & C versus DM+ADM group.
P6: statistical significance between ADM group + Vit E & C group versus DM + Vit E & C group.

Table (2): Blood glucose and serum creatinine (S.Cr) of different rats groups (Mean ±SE).

Groups	Blood glucose (mg/dl)	S.Cr. (mg/dl)
Control(CN)	122.65 ± 2.04	0.88 ± 0.02
STZ – induced DM	423.60 ± 18.29	0.74 ± 0.03
P1	<0.001	NS
STZ – induced diabetic rats treated with ADM	452.73 ± 10.45	0.79 ± 0.01
P2	NS	NS
STZ – induced diabetic rats received α-tocopherol succinate (vitamin E) and ascorbic acid (vitamin C)	306.62 ± 6.28	0.73 ± 0.02
P3	<0.05	NS
STZ – induced diabetic rats received ADM, α-tocopherol succinate (vitamin E) and ascorbic acid (vitamin C)	335.32 ± 11.26	0.82 ± 0.03
P4	<0.05	NS
P5	<0.05	NS
P6	NS	NS

P1: statistical significance between CN group versus DM group
P2: statistical significance between DM group versus DM + ADM group.
P3: statistical significance between DM group versus DM + Vit E & C group.
P4: statistical significance between DM group versus ADM + Vit E & C group.
P5: statistical significance between ADM group & Vit E & C versus DM+ADM group.
P6: statistical significance between ADM group + Vit E & C group versus DM + Vit E & C group.

Table (3): Plasma malondialdehyde (MDA), Catalase (CAT), Superoxide disutase (SOD), and Glutathione in different groups (Mean ±SE).

Groups	MDA(nmol/ml)	CAT(unit/ml)	SOD(unit/ml)	Glutathione(unit/ml)
Control(CN)	3.74 ± 0.32	8.93 ± 0.36	435.78 ± 15.47	26.66 ± 1.06
STZ – induced DM P1	4.75 ± 0.21 <0.001	3.22 ± 0.25 <0.001	279.13 ± 24.04 <0.001	14.03 ± 0.83 <0.001
STZ – induced diabetic rats treated with ADM P2	6.27 ± 0.25 <0.05	5.63 ± 0.23 <0.01	418.14 ± 20.31 <0.001	25.84 ± 2.15 <0.001
STZ – induced diabetic rats received α-tocopherol succinate (vitamin E) and ascorbic acid (vitamin C) P3	5.45 ± 0.34 <0.01	4.84 ± 0.39 <0.05	407.13 ± 9.32 <0.001	27.13 ± 2.64 <0.001
STZ – induced diabetic rats received ADM, α-tocopherol succinate (vitamin E) and ascorbic acid (vitamin C) P4 P5 P6	5.21 ± 0.35 <0.001 <0.01 NS	7.76 ± 0.29 <0.001 <0.001 <0.001	435.35 ± 27.44 <0.001 NS NS	28.66 ± 3.02 <0.001 NS NS

P1: statistical significance between CN group versus DM group
P2: statistical significance between DM group versus DM + ADM group.
P3: statistical significance between DM group versus DM + Vit E & C group.
P4: statistical significance between DM group versus ADM + Vit E & C group.
P5: statistical significance between ADM group & Vit E & C versus DM+ADM group.
P6: statistical significance between ADM group + Vit E & C group versus DM + Vit E & C group.

Table (4): Correlation and regression analysis among Urinary albumin excretion (UAE), Kidney weight (KW) and Catalase (CAT), Superoxide dismutase (SOD), Reduced glutathione (GST)and Malondialdehyde(MDA).

Y	X	r	p
UAE; mg/24 hours	CAT; u/ml	- 0.732	<0.001
UAE; mg/24 hours	SOD; u/ml	-0. 524	<0.001
UAE; mg/24 hours	GST; u/ml	- 0.557	<0.001
UAE; mg/24 hours	MDA; nmol/ml	0.684	<0.001
Kidney weight	CAT; u/ml	- 0.514	<0.001
Kidney weight	SOD; u/ml	- 0.427	<0.05
Kidney weight	GST; u/ml	- o.411	<0.05
Kidney weight	MDA; nmol/ml	0.439	<0.001

Discussion

Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, or polyphenols[65], glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells[66].

Moreover, oxidative stress is thought to contribute to the development of a wide range of diseases including Alzheimer's disease, Parkinson's disease, the complications caused by diabetes, rheumatoid arthritis, and neurodegeneration in motor neuron diseases[67].

However, diabetic nephropathy (DN) is the common cause of end-stage of renal disease (ESRD). Diabetic nephropathy is a progressive and irreversible renal disease characterized by the accumulation of extra cellular matrix in glomerular mesangium and kidney interstitial tissue that eventually leads to renal failure[68].

Based on molecular mechanisms of diabetic nephropathy pathogenesis that mentioned in introduction, and increase of oxidative stress markers in experimental and diabetics patient, there is no doubt that oxidative stress plays a pivotal or central role in the initiation and progression of diabetic complications[69]. Besides epidemiological studies have demonstrated association between inflammatory and oxidative stress markers with cardiovascular and renal outcomes in chronic kidney disease (CKD) and ESRD[70]. Thus combined therapy with antioxidants and anti-inflammatory agent may be leads to satisfactory results.

The most known free radicals involving in the diabetic nephropathy pathogenesis are reactive oxygen species (ROS) such as superoxide ($-O_2$), hydroxyl (-OH), and

peroxyl (-RO_2) and non radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl) and reactive nitrogen species produced from similar pathways, which include the radicals nitric oxide (-NO) and nitrogen dioxide (-NO_2), as well as the non-radical peroxynitrite (ONOO-), nitrous oxide (HNO_2), and alkyl peroxynitrates (RONOO). Of these, -O_2, -NO, H_2O_2, and ONOO- have been the most widely investigated in the diabetic kidney. Products of lipid peroxidation especially ketone,hydroxyl radical and MDA are considered a known cofounder of diabetic nephropathy[53]. There are a number of enzymatic and no enzymatic sources of ROS in the diabetic kidney, including auto oxidation of glucose, transition metal-catalyzed Fenton reactions, advanced glycation, polyol pathway flux, mitochondrial respiratory chain deficiencies, xanthine oxidize activity, peroxidase, nitric oxide synthase (NOS) and NADPH oxidase[71]. An excessive amount of ROS production occurs before structural changes such as mesangial matrix deposition[5].

On the other hand, human body combat against free radicals by natural defense with antioxidant enzymes and exogenous antioxidants. Reactive oxygen species can be eliminated by a number of enzymatic and non enzymatic antioxidant mechanisms. Super oxide dismutase (SOD) immediately converts •O_2 - to H_2O_2, which is then detoxified to water either by catalase in the lysosomes or by glutathione peroxidase (GPX) in the mitochondria, catalase that converts H_2O_2 to O_2 and H_2O. Another enzyme is glutathione reductase, which regenerates glutathione that is used as a hydrogen donor by GPX during the elimination of H_2O_2. Non enzymatic antioxidants include vitamins A, C and E; glutathione; α-lipoic acid; carotenoids; trace elements like copper, zinc and selenium; coenzyme Q10 (CoQ10); and cofactors like folic acid, uric acid, albumin, and vitamins B1, B2, B6 and B12[72].

Meanwhile, the experimental data demonstrated that the metabolic abnormalities of diabetes cause mitochondrial superoxide over production. This increased superoxide production is the cen-

tral and major mediator of diabetes tissue damage[69].

Moreover, hyperglycemia not only generated ROS but also attenuated antioxidative mechanisms through glycation of scavenging enzymes[73].

So this present research aimed to study the role of adrenomedullin and antioxidants (Vitamin E and C) in STZ - induced diabetic nephropathy in experimental rats. The study evaluated the effect of adrenomedullin and antioxidants on body weight (BW), kidney weight (KW), KW/BW (%), 24 hours urinary albumin excretion (UAE), blood glucose (mg/dl) and serum creatinine (S.Cr), plasma malondialdehyde (MDA), catalase (CAT), superoxide disutase (SOD), and glutathione in different rats groups.

The involvement of oxidative stress in diabetic nephropathy was demonstrated in this study (Table-3) by the increase of lipid peroxidation products, MDA in plasma. These data are consistent with those of previous studies by Usui et al.[74]. Also it was founded that a decrease of SOD, CAT and GSH in plasma of STZ - induced diabetic rats (Table 3). This in agreement with that reported by[53].

The results of the present work regarding body weight (BW), kidney weight (KW), BW/KW and urinary albumin excretion (UAE) of various groups as shown in table (1). Body weight and kidney weight of diabetic group (group-II) were decreased ($P<0.001$), while KW/BW was significantly increased ($P<0.05$) compared with the control group (group-I). No significant difference of these parameters was found between the four diabetic groups. Urinary albumin excretion (UAE) was significantly increased in diabetic group (group II) in comparison to the control group (group I) ($P<0.001$). In comparison to diabetic group II, all other diabetic groups III, IV and V showed significant decrease in UAE ($P<0.001$). Plasma glucose and serum creatinine are shown in table (2), the glucose level of all diabetic groups was significantly increased ($P<0.001$) in comparison to the control group. Serum creatinine showed no significant change in between all diabetic groups.

Adrenomedullin (ADM) is a peptide hormone, originally isolated from human pheochromocytoma. ADM acts as a local autocrine and/or paracrine vasoactive hormone and has vasodilator and blood pressure lowering properties. ADM as a vasodilative molecule protects the vascular wall but its exact role is still uncertain. ADM is considered to play an important endocrine role in various tissues in maintaining electrolyte and fluid homeostasis. Its plasma concentration in healthy conditions is low. In hypertension, chronic renal failure and congestive heart failure its plasma concentration increases in a parallel manner with the severity of the disease. It is assumed that this peptide plays an important role in physiological and pathological conditions compensating the effects of vasoconstrictive molecules[75]. Ruzicska et al.[75] have proven that in diabetic angiopathies, the levels and production of vasoconstrictive factors and ADM are increased, while other relaxing substances such as nitric oxide (NO) are decreased. It is still uncertain whether the increased release of ADM is a compensatory mechanism or a coincidental event. Although the precise role of ADM in the pathogenesis of diabetic complications is still to be elucidated, the altered concentration of ADM in diabetes could indicate a certain interaction between ADM induction and vascular function. Hence, the induction of vascular ADM can be a new target of therapeutic approach to diabetic complications

However, the present study denoted a decrease in MDA with increase SOD, CAT and glutathione concentration in diabetic groups treated by ADM and vitamins (E & C). This was probably occur in part by scavenging the very reactive superoxide ($-O_2$), hydroxyl (-OH) and ROO by ADM[76] and antioxidant vitamins E and C[77] indicating reduction in lipid peroxidation and cellular injury.

Beside the ADM inhibited DM - stimulated MDA generation, also this study showed that DM significantly upregulated a set of redox-sensitive substances (MDA) and significant decrease of CAT, SOD and glutathione. These effects were blocked by an antioxidant,

vitamins E and C. ADM similarly inhibited the DM-induced upregulation of MDA via a receptor-mediated and PKA-dependent pathway, and the degrees of inhibition were similar to those by vitamins E and C. Meanwhile, the present study demonstrated that ADM potently blocked the DM-stimulated intracellular ROS (MDA) generation from NAD (P) H oxidase and the subsequent redox-sensitive gene expression via a cAMP-dependent mechanism, suggesting that ADM has protective effects against pro-oxidant stimuli[78].

Much of great interest about microalbuminuria derives from the fact that albumin excretion is a risk factor for renal failure[79] particularly for persons with diabetes mellitus and/or hypertension[80]. It predicts the onset of overt renal disease in diabetic patients and reflects glomerular dysfunction[80].

The results of the present work regarding effects of adrenomedullin on diabetic nephropathy in rats as demonstrated in table (1). The combination of ADM and vitamin E & C showed significant decrease in UAE in comparison to the ADM treated group alone. Also, there was no significant changes of glucose level between untreated and ADM treated diabetic groups, in comparison to DM group, the DM + vitamins E & C and ADM + vitamins E & C groups showed significant decrease in glucose level but with no significant difference between the two groups. No significant difference in serum creatinine between all groups. A greater increase in the urinary albumin excretion (UAE) was observed in the diabetic group (group II) than in groups treated with ADM and vitamins E and C. This demonstrated the occurance of early stage diabetic nephropathy in diabetic groups and these results were inagreement with O'Brien et al[81] and Tsuruda & Burnett[82], who revealed that diabetic mice exhibit significant increases in the NADPH oxidase expression and lipid peroxidation product formation in the kidneys. The presence of increased lipid peroxidation products in the glomeruli exhibited a positive correlation with the presence of mesangial matrix expansion. The

endogenous adrenomedullin plays an important role in protecting against oxidative stress in the kidneys via the suppression of NADPH oxidase and can prevent glomerulosclerosis. Also the oxidative stress can induce adrenomedullin production[83], and it is possible that adrenomedullin increases in patients with diabetes to counterbalance increased oxidative stress. Indeed, increased oxidative stress is associated with elevated plasma levels of adrenomedullin in hypertensive diabetic patients. The induction of adrenomedullin by high levels of glucose is dependent on PKC activation, while PKC also activates NADPH oxidase via translocation of p47phox and p67phox to the membrane components and produces oxidative stress that further increases adrenomedullin production. Moreover, it is possible to assume that endogenous adrenomedullin exerts a negative feedback action on oxidative stress via the suppression of NADPH oxidase[84].

The study by Brownlee[85] demonstrated that albuminuria (UAE) are more severe in the diabetic rats than in the control one and UAE was decreased significantly in STZ – induced diabetic rats treated with ADM and that was in accordance to our results shown in group III. Moreover, there were significant decrease in UAE in STZ – induced diabetic rats received ADM, α-tocopherol succinate (vitamin E) and ascorbic acid (vitamin C) more than STZ – induced diabetic rats treated with ADM only. Therefore, this study hypothesizes that endogenous adrenomedullin in diabetes may act to protect against the development of diabetic nephropathy. This is supported by evidence showing that the genetic predisposition to develop diabetic nephropathy is associated with the microsatellite DNA polymorphism of the adrenomedullin gene[86].

On the other hand, adrenomedullin could also play a regulatory role in the endocrine function of the kidney. It has been reported by Jensen et al.[87] that the level of local angiotensin II is increased in the kidneys of diabetes, although the circulating levels of renin are normal or even low in diabetic patients. Some reports have demonstrated that adrenomedullin increases the plasma renin

concentration and also increases the release of renin from isolated perfused kidneys as well as from primary cultured granular cells. On the other hand, chronic adrenomedullin administration in Dahl salt-sensitive rats inhibits increases in the plasma renin concentration, the aldosterone level, and the renal tissue angiotensin II levels. Studies by Charles et al.[88] raise the hypothesis that adrenomedullin counteracts the pathological activation of JGA and the renal renin-angiotensin system in diabetes and hypertension.

Furthermore, the ADM + vitamins E & C treated group showed significant increase in catalase enzyme (CAT) in comparison to the DM+ADM and DM+vitamins E & C treated alone with no significant differences in SOD and glutathione in combination group in comparison to DM+ADM or DM + vitamins E & C respectively.

Correlation & regression analysis of the parameters in table (4) showed that with the increase of UAE, KW, MDA, the SOD, CAT and glutathione in plasma were decreased.

Regarding vitamin C and diabetic nephropathy, serum vitamin C concentrations have been reported to be low in diabetic patients[89]. Although reduced concentrations of vitamin C were reported in type 2 diabetic retinopathy[90], the concentrations of serum vitamin C in type 2 diabetic patients with diabetic nephropathy have not been previously reported. Kenzo et al.[91] reported that no correlation was found between serum vitamin C levels and urinary albumin excretion levels in patients with diabetes. In contrast to this opinion, Li et al.[92] suggested that vitamin C protect renal lesions in diabetic nephropathy by inhibiting expression of type IV collagen.

Regarding vitamin E and diabetic nephropathy, Bursell et al.[93] proved that the oral vitamin E treatment normalized elevated baseline creatinine clearance in diabetic patients with type 1 diabetic without inducing a significant change in glycemic control in an 8-month randomized double-masked. This not consistent with the finding in present study which demonstrated that no significant

difference in serum creatinine between all groups. Furthermore, Hirnerová et al.[94] confirmed that the treatment with vitamin E favorably decreases microalbuminemia in diabetic nephropathy which was in agreement to this present study .

Gaede et al.[95] reported that vitamins E and C combination significantly improved Type 2 diabetes .This is in agreement with this work, it was founded that group treated with vitamins E and C and group treated with combination with ADM reduce blood glucose level.

In contrast, Ceriello[96] reported that vitamin E have failed to show beneficial effect on diabetic complication and high dose of vitamin E fail to prevent albuminuria while lower doses exacerbate renal injury[97].

Also, in contrast to results of present study, are those of Blum et al.[98] who reported that antioxidant supplementation studies have shown conflicting results in endothelial function and renal function outcomes in diabetic patients (Antioxidants per se have demonstrated minimal renoprotection in humans despite positive preclinical research findings. However, the classical antioxidants, such as vitamins E and C, do not appear to be helpful[96]. Some clinical evidences for the effectiveness of antioxidants on the treatment of diabetic nephropathy have not been established and there are several reports that indicated the absence of improvement and even worsening of diabetic nephropathy with antioxidant treatment[99].

Since oxidative stress appears to play an important role as an early etiologic factor in diabetic nephropathy and later progression so it is suggested that combination of antioxidant therapy as one of the most important treatment strategies for diabetic nephropathy for slowing of diabetic nephropathy before reaching to end stage renal disease.

Conclusion

Increased lipid peroxidation and decreased antioxidant enzymes exist in diabetic rats which promote the progression of diabet-

ic nephropathy. Combination between Adrenomedullin and vitamins E and C may decrease lipid peroxidation and increase the antioxidant enzyme level as well as decrease blood glucose to protect glomeruli from oxidative injury but of course, needs to be proven in clinical trials studies for clarification of the pathogenesis of diabetic nephropathy and development of novel and effective therapeutic strategies which are therefore high priorities.

Ethical approval: The study was performed in accordance with the ethical standards of the "local medical committee" of faculty of medicine in Mansoura University, Egypt.

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ANTIOXIDANT EFFICACY OF ADRENOMEDULLIN VERSUS VITAMIN E AND C IN DIABETIC NEPHROPATHY IN RATS

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POTENTIAL PROTECTIVE EFFECT OF MELATONIN ON ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN ALBINO RATS

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Abstract

Aim of the work: *The present work was conducted to determine the protective effect of the melatonin hormone on liver cellular damage induced by acetaminophen.*

Material & Methods: *The material of the study consisted of 50 male albino rats classified into 5 equal groups. The first group was the normal control group, the second was the acetaminophen induced liver damage group, the third was the melatonin treated group, the fourth was the combined melatonin and acetaminophen treated group and lastly the fifth was the melatonin pretreated group.*

Results: *It was found that acetaminophen administration to the rats produced considerable effects on the studied liver parameters (SGPT, SGOT and serum bilirubin).Melatonin treatment alone resulted in no effect on the normal hepatic functions. Melatonin given before and/ during exposure to acetaminophen proved to have a cytoprotective effect on induced liver damage as evidenced by normalization of liver function tests.*

Conclusion: *There is a protective effect of melatonin on acetaminophen-induced hepatotoxicity in albino rats.*

Introduction

Melatonin (N-acetyl-5 methoxy tryptamine) is synthesized within the pineal gland from tryptophan by N acetyl transferase enzyme, yet, it induces sleep resembling the natural type[1]. However, the exact mechanism by which melatonin helps people to sleep is not yet settled, but there are two possible mechanisms; firstly, it may alter the circadian rhythm, shifting the time of normal sleep to more desirable time of day, and

secondly, it may have a direct sleep inducing effect[2].

On the other hand, melatonin is the most potent free radical scavenger. It acts as antioxidant by stimulating glutathione peroxidase activity in tissues, which metabolize reduced glutathione to its oxidized form, so it converts hydrogen peroxide (H2O2) into water thereby reducing generation of hydroxyl radicals by eliminating its precursors[3]. Also the role of free radicals in liver cell injury was described by[4] who stated that free radicals can disrupt lysosomal membrane by their action on polyunsaturated fatty acids present in lysosomal membrane leading to release of lysosomal enzymes which have important role in the pathogenesis of hepatotoxicity, thus the use of appropriate antioxidants for the treatment of liver cell injury may be rationalized[5]. Neri et al.,[6] showed that melatonin increased production of tumor necrosis factor alpha, interferon gamma and interleukin-2 which are part of immune defense mechanisms against injury and cancer. Results by[7] indicate that melatonin exerts a therapeutic effect on CCl_4-induced acute liver injury in rats, possibly through its antioxidant action.

The liver is one of the most important organs of the body. It is essential for the metabolism of carbohydrates, fats, proteins, minerals, vitamins, hormones and drugs[8,9].

The heptotoxicity of acetaminophen (paracetamol) overdose depends on the metabolite by the hepatic mixed function oxidases[10]. There is evidence that an increase in cytosolic Ca^{+2} is involved in acetaminophen hepatotoxicity[11].

The intracellular free calcium concentration in hepatocytes is controlled by active transport of these ions across the mitochondria, endoplasmic reticulum and the plasma membrane[12]. The hepatic mitochondria are protected against high cytosolic calcium levels by the presence of glutathione, but became permeable to calcium when oxidative events convert glutathione (GSH) to oxidized glutathione (GSSH)[13]. The active translocation of Ca^{+2} through the plasma membrane is mediated by

Ca^{+2} ATPase which appears to be critically dependent on the glutathione status of the cell for its activity[14]. Impairement of this activity causes imbalance between passive influx of Ca^{+2} and its active extrusion from the cell, resulting in intracellular accumulation[15]. Calcium channels are macromolecular proteins traversing the lipid bilayer of membrane[16]. This directed me to search about the potential use of melatonin as a protective agent against lipid peroxidation and hepatotoxicity in vivo by its potent hydroxyl radical and free radical scavenger effect.

The aim of the present work is to clarify the protective effect of melatonin in acetaminophen - induced liver cell damage.

Materials and Methods

Animals:

The present work was carried out on 50 healthy male Sprague-Dawley rats, weighing 200-250 grams. All animals were purchased from Vaccine and Immunization Authority (Helwan, Cairo, Egypt) and kept under similar housing conditions (Animal House, Medical Physiology Department, Faculty of Medicine, Mansoura University, Egypt) in standard cages in groups of four to six animals per cage under controlled conditions (temperature 25±1 ºC, and a 12:12 light/dark cycle), with free water and food access for 1 week for acclimatization. All experimental procedures were approved by Medical Research Ethical Committee of Mansoura University, Egypt. The animals were divided into 5 groups as follows:

Group I (10 rats): The normal control.

Group II (10 rats): (Acetaminophen treated): Acetaminophen (Paracetamol tablet= 500mg) treated group to induce experimental liver damage and acute hepatic necrosis. This is done by intragastric administration of acetaminophen powder dissolved in 0.5 ml of distilled water in a dose of 700 mg/kg[10].

Group III (10 rats): (Melatonin-treated): This group received intragastric melatonin(Sigma-USA) only in a dose of 4 mg/kg daily[17] for 3 consecutive days.

Group IV (10 rats): (Combined melatonin and acetaminophen): This group received combined acetaminophen and melatonin in the previous doses simultaneously for 3 days consecutively.

Group V (10 rats): (A melatonin pretreated): This group received intragastric melatonin in a dose of 1 mg/kg/d for 2 weeks before acetaminophen-induced hepatotoxicity[18], during and after acetaminophen exposure. Six hours after the last administration, the animals were sacrificed and blood was collected to estimate:

1- Serum Glutamic Oxaloacetic and Pyruvic Transaminase (SGOT & SGPT)[19].

2- Total serum bilirubin[20].

In addition, liver tissues were taken for measuring the following parameters:

1- Malondialdehyde (MDA) level[21].

2- Glutathione peroxidase activity level[22].

3-Plasma prostaglandin E2 (PGE2) was estimated by using titerzyme PGE2 enzyme immunoassay kit, manufactured by Perspective Biosystems.

Statistical analysis:

Kruskal-Wallis non - parametric analysis of variants (ANOVA) was used to test for between groups variability, while Mann-Whitney u test was used to test for difference between each two groups in all variables. P value was considered significant if less than 0.05[23].

Results

The effects of acetaminophen on the liver functions showed a significant increase in SGOT, SGPT and serum bilirubin as compared to the control group (Table 1 & Figure 1). Melatonin alone had no significant effect on the liver function tests. Melatonin administration with acetaminophen or prior to it significantly protected the liver against the disturbed functions in comparison to acetaminophen treated group as shown in table 1 & figure 1.

Regarding table 2 & figure 2, it was found that acetaminophen administration resulted in significant increase in liver MDA level whereas it produced significant decrease in liver glutathione peroxidase activity and significant increase in PGE2. Furthermore, melatonin

administration resulted in significant decrease in liver tissue MDA and produced significant increase in glutathione peroxidase activity and significant increase in PGE2.

By these results, pretreatment and combined treatment with melatonin provided a significant protection against the acetaminophen - induced hepatotoxicity.

Table (1): Effect of acetaminophen, melatonin, combined and pretreated with melatonin and acetaminophen on some liver function tests (mean±SE).

Groups (10 animals)	Group I (normal control)	Group II (acetaminophen control)	Group III (melatonin treated)	Group IV (combined acetaminophen and melatonin)	Group V (melatonin pretreated)
SGOT (units/ml)	29.6 ± 6.54	217.5 ± 9.31 P1 <0.001	30.4 ± 6.55 P2 < 0.05	59.6 ± 5.87 P3 <0.001	30.7 ± 9.75 P3 <0.001
SGPT (units/ml)	6.7 ± 1.78	130.2 ± 7.1 P1 <0.001	7.5 ± 2.8 P2 < 0.05	8.8 ± 2.65 P3 <0.001	5.8 ± 2.1 P3 <0.001
Total serum bilirubin(mg/dl)	0.176± 0.04	0.68 ± 0.06 P1 <0.001	0.173 ± 0.03 P2 < 0.05	0.171 ± 0.03 P3 <0.001	0.174 ± 0.04 P3 <0.001

SE= Standard error
P1= Significance of difference between the means of acetaminophen treated and non treated rats (norma control)
P2= Significance of difference between melatonin treated group and normal control
P3= Significance of difference between combined and pretreated melatonin- acetaminophen and acetaminophen control
Test of significance P<0.05.

Table (2): Effect of acetaminophen, melatonin, combined and pretreated with melatonin and acetaminophen on liver tissue malondialdehyde (MDA) and glutathione peroxidase activity levels and plasma PGE2 levels (mean±SE).

Groups (10 animals)	Group I (normal control)	Group II (acetaminophen control)	Group III (melatonin treated)	Group IV (combined acetaminophen and melatonin)	Group V (melatonin pretreated)
Malondialdehyde (n mol/gm tissue)	12.8 ± 4.35	26.7 ± 9.8 P1 <0.001	7.0 ± 2.5 P2 < 0.05	8.7 ± 2.9 P3 <0.001	7.5 ± 3.2 P3 <0.001
glutathione peroxidase activity (u mol)	0.786 ± 0.13	0.545 ± 0.25 P1 <0.001	1.296 ± 0.65 P2 < 0.05	2.278 ± 0.47 P3 <0.001	1.964 ± 0.38 P3 <0.001
Plasma PGE2 (pg/ml)	368±12	2185 ± 384.74 P1 <0.001	803 ± 107.2 P2 < 0.05	965 ± 116.2 P3 < 0.05	850 ± 121.4 P3 < 0.05

SE= Standard error
P1= Significance of difference between the means of acetaminophen treated and non treated rats (normal control)
P2= Significance of difference between melatonin treated group and normal control
P3= Significance of difference between combined and pretreated melatonin- acetaminophen and acetaminophen control
Test of significance P<0.05.

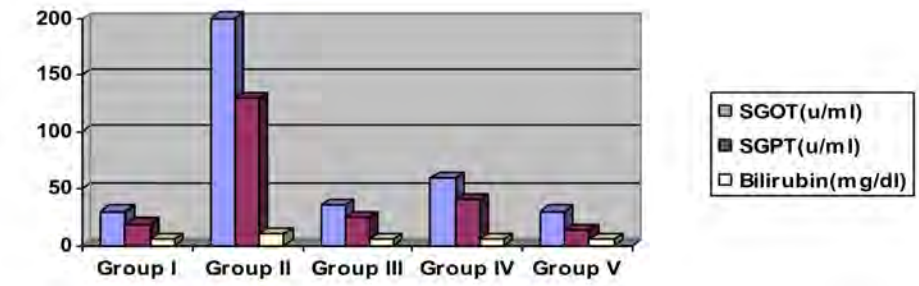

Fig.1: Effect of acetaminophen, melatonin, combined and pretreated with melatonin and acetaminophen on some liver function tests.

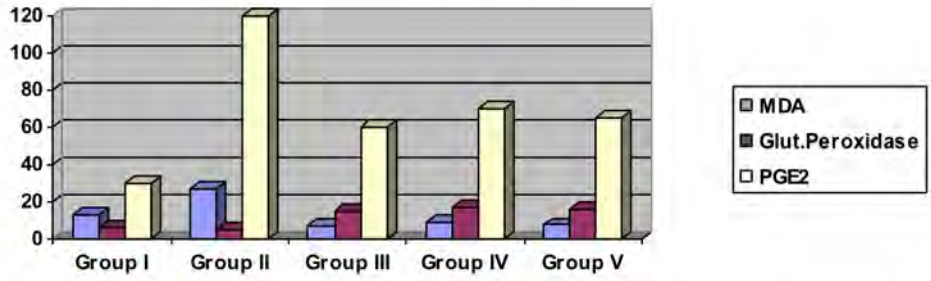

Fig.2: Effect of acetaminophen, melatonin, combined and pretreated with melatonin and acetaminophen on liver tissue malondialdehyde (MDA) and glutathione peroxidase activity levels and plasma PGE2 levels.

Discussion

The present work was conducted to clarify the effect of melatonin administration on experimentally induced liver injury before and/or during exposure to acetaminophen.

Charlos et al.,[24] showed that with acetaminophen overdose, more drug undergoes conversion to the electrophilic metabolites. N.acetylbenzoquinone imine (NAPQI), catalyzed by CYP 450[25], excessive generation of NAPQI results in depletion of hepatic reduced glutathione(GSH), covalent binding to cellular macromolecules, and ultimately liver injury[26].

In the present study, the acetaminophen treated group showed a significant increase in the hepatic serum glutamic oxaloacetic(SGOT) and pyruvic transaminases(SGPT) and total serum bilirubin. Mitochondrial oxidant stress and peroxynitrite formation have been implicated in the pathophysiology of

acetaminophen-induced (AAP-induced) liver injury and lipid peroxidation (LPO) might be involved in the injury mechanism[27]. An overdose of the analgesic drug acetaminophen (AAP) can lead to severe liver injury in humans and in experimental animals. Although the mechanism of this injury is still not entirely clear. It is undisputed that the metabolism of a fraction of the AAP dose by the P450 system is the initial step of the injury process[28]. The product of this reaction is a reactive metabolite presumably N-acetyl-p-benzoquinone imine (NAPQI)[29], which is detoxified by glutathione (Mitchell et al., 1973). However, if the formation of the reactive metabolite exceeds the capacity of liver glutathione, NAPQI will bind to cellular proteins[28]. Over the years, a number of proteins were identified that were modified by NAPQI binding[30]. There is increasing evidence to suggest that protein binding is an initiating event of cell injury which can be amplified through secondary processes[31]. One of these secondary effects of reactive metabolite formation and protein binding is mitochondrial dysfunction[32], which results in ATP depletion and oxidant stress[33]. Superoxide generated in mitochondria after AAP overdose can dismutate to form molecular oxygen and hydrogen peroxide, which is then reduced to water by glutathione peroxidase using electrons from GSH. The fact that mitochondrial glutathione disulfide (GSSG) levels increase substantially after AAP treatment is strong evidence for the increased hydrogen peroxide formation in mitochondria[34], although extramitochondrial sources of reactive oxygen cannot be excluded.

On the other hand, superoxide can react with nitric oxide (NO) to form the potent oxidant peroxynitrite[35]. The rate constant of the reaction between superoxide and NO is several times higher than the rate constant of superoxide dismutation with or without catalysis by superoxide dismutase[35]. Thus, an increased formation of superoxide in the presence of equimolar levels of NO can lead to increased formation of peroxynitrite in addition to hydrogen peroxide generation. Nitrotyrosine is a footprint for peroxynitrite forma-

tion[36]. Indeed, nitrotyrosine protein adducts can be detected in vascular endothelial cells and parenchymal cells after AAP overdose before cell injury, i.e., the loss of endothelial cell barrier function (hemorrhage) and ALT release, is observed[37]. Administration of pharmacological doses of glutathione accelerated the recovery of mitochondrial glutathione levels, which effectively scavenged most of the peroxynitrite and protected against cell injury[38]. These data suggested that peroxynitrite is a critical mediator of AAP hepatotoxicity[38]. Both hydrogen peroxide (through hydroxyl radical formation by iron-catalyzed Fenton reaction) and peroxynitrite (through hydroxyl radical–like decomposition products) can initiate lipid peroxidation[39], which can lead to oncotic necrosis of liver cells. Furthermore, inhibition of peroxynitrite formation by inhibitors of nitric oxide synthase was associated with an increase in lipid peroxidation after AAP treatment[40].

The effect of melatonin on normal liver functions was studied in the present work and no changes had been observed. This is in accord with the results obtained by[5].

In this study, a cytoprotective action of melatonin in acetaminophen induced liver injury has been demonstrated. Melatonin treatment before and/or during exposure to acetaminophen provided protection against the increase in hepatic enzymes and total serum bilirubin. These findings were in agreement with[41], where the antioxidant effect of melatonin were shown to prevent cell death caused by increase free radicals and by turn increase cytosolic and mitochondrial calcium content.

The present work has shown that acetaminophen - induced hepatotoxicity was accompanied by significant increase in serum MDA, decrease glutathione peroidase activity and significant increase in plasma PGE2 in comparison with control group. The significant increase in serum MDA is in agreement with finding of[42], who demonstrated augmented level of lipid peroxidation and defects in antioxidant systems in liver cell injury in rats. The increase in plasma PGE2 is in agreement with the results obtained by workers, who suggest-

ed that PGs may be important mediators of many inflammatory processes and cell injury[43].

Regarding the effects of melatonin on parameters relevant to the antioxidant activity, it was shown that liver malondialedhyde (MDA) level were significantly lower in groups III, IV and V compared to Groups I and II. This was attributed to its neutralizing effect on lipid free radicals thus breaking the chain of reactions where by lipid free radicals oxidize other fatty acids with reduction in the level malondialedhyde being the product of lipid peroxidation[44].

On the other hand, melatonin produced significant increase in liver glutathione peroxidase activity. The results of present study concerning antioxidant effect obtained by melatonin were consistent with those obtained by[45].

Furthermore, the significant increase in plasma PGE2 observed in this study, could be explained by the findings of[45] who reported that melatonin depresses 5-lipooxygenase pathway, thus, in turn it may increase the cyclooxygenase pathway and hence PG production.

However, melatonin caused significant increase in GSH activity, this increase could be attributed to the ability of melatonin to lower the glycosylation of proteins and thus increase the activity of glutathione peroxidase so increase the level of glutathione, the substrate upon which glutathione peroxidase enzyme acts thus increases its activity[46]. Another mechanism by which melatonin can increase GSH level is by increasing its production through increasing the level of mRNA so stimulating gene expression[47].

From the previous results it could be noticed that melatonin is a more potent antioxidant as indicated by the more reducing effect on MDA, increasing glutathione peroxidase activity, increasing PGE2 and improvement of liver function tests after induction of hepatotoxicity by acetaminophen.

Ethical approval: The study was performed in accordance with the ethical standards of the "local medical committee" of faculty of medicine

in Mansoura University, Egypt.

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PROGNOSTIC FACTORS AND ITS IMPACT ON SURVIVAL IN CANCER THYROID

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Abstract

Thyroid cancers represent approximately 1% of new cancer diagnoses each year. In Egypt, thyroid cancer constitutes 30% of endocrine malignancies. ***The aim*** *of the present study to detect differences in outcome between different types of thyroid cancer including the differentiated types (PTC and FTC) and the other types and to correlate the survival to epidemiological characters, histopathological features .*

This retrospective study reviewed 584 patients with histologically proven thyroid carcinoma during period from 2002 to 2011 with long term follow up.

Our ***results*** *showed that the papillary thyroid cancer accounting for 74.1% of the patients followed by follicular thyroid cancer which represented about 12.5% of the cases. Univariate analyses showed that old age, extrathyroid extension, large primary tumour size, distant metastases, involvement of lymph nodes and anaplastic carcinoma are significantly poor prognostic factors. Multivariate analysis showed that only the large primary tumour size, distant metastases, involvement of lymph nodes and tumour types retained the independent prognostic features.In PTC patients' series, old age, extrathyroid extension, large primary tumour size, distant metastases and involvement of lymph nodes were significantly poor prognostic factors.*

In Conclusion: *The prognosis of thyroid carcinoma in Egypt is similar to what happens worldwide. FTC patients showed significantly poorer survival than PTC. The significant prognostic factors are different between the two types. Although the distant metastasis is the most important factor in both types, PTC prognosis is widely and significantly affected by the age and extrathyroid extension in comparison to FTC.*

Keywords: *Thyroid cancer, Papillary, Follicular.*

Introduction

Thyroid cancers represent approximately 1% of new cancer diagnoses each year. The incidence of thyroid cancer has increased continuously over the last three decades on every continent except Africa, which is most likely due to insufficient screening[1]. Moreover, the increased incidence is mostly observed for small tumours, and the mortality rate for patients with thyroid cancer during the same period remained unchanged[2].

The average incidence of thyroid cancer in American women and men is 14.1 and 4.9 per 100,000 residents per year, respectively. The corresponding rates in European women and men are 12.9 and 5.0 per 100,000 residents per year, respectively[3]. In Egypt, thyroid cancer constitutes 30% of endocrine malignancies and 12-49% of head and neck tumours. It was also stated that thyroid cancer represents 1.5% of all cancers in Egypt. The rate among Egyptian females is 0.0027% with female to male ratio is less than 3[4].

Differentiated Thyroid carcinoma (DTC) is including two histological types, these are papillary carcinoma (PTC) and follicular carcinoma (FTC). Differentiated thyroid carcinoma was analyzed as a single group for clinical studies investigating prognostic factors and prognosis of patients. However, biological behaviors of these two carcinomas significantly differ. PTC frequently metastasizes to the regional lymph nodes and can show a high incidence of significant extrathyroid extension to adjacent organs. In contrast FTC more frequently metastasizes to distant organs such as the lung, bone, and brain than PTC[5].

Approximately 80-85% of all thyroid malignancies are PTCs. In addition to the classic form of PTCs, several morphologic variants, usually classified as 'biologically aggressive', have been identified; diffuse sclerosing, tall cell, columnar cell, solid/trabecular and insular variants[6]. However, recent works show that some of these variants (sclerosing and tall cell) are not related to poor outcome per se, but that tumour prognosis is rather dependent on the presence of aggressive features

including extracapsular and vascular invasion, larger tumour size and the presence of distant metastases[7,8]. FTC is the second most frequent subtype accounting for approximately 10-15% of all thyroid cancers[6].

There were another types of thyroid cancer including; anaplastic (ATCs) and medullary thyroid carcinomas (MTCs). ATCs are derived from thyroid epithelial cells, whereas MTCs are derived from calcitonin-secreting parafollicular cells. MTCs account for 3-4% of all cases of thyroid cancer[9]. They are often hereditary and frequently due to RET proto-oncogene mutations[10]. ATCs account for 1-2% of all thyroid cancers[11]. Primary lymphomas and sarcomas are rare[12].

The behavior of differentiated thyroid cancer can range from an indolent, clinically insignificant disease found incidentally with good prognosis in most cases are differentiated thyroid cancer, which is associated with a high 10-year survival rate (90 to 95%)[13].

Unlike most cancers, differentiated thyroid cancer recurrence does not necessarily correlate with increased risk of mortality. This is particularly demonstrated in young patients who have higher rates of local recurrence but low mortality risk. Several clinical features enable initial risk stratification, including patient age, size of the primary tumour, histology, gross extra-thyroidal extension, completeness of resection, involvement of the cervical lymph nodes, or distant metastasis[12].

Thyroid diseases are more prevalent in women particularly between puberty and menopause and carcinomas of the thyroid are more frequent in women than in men. The increased risk is also seen in women taking oral contraceptive pills and in pregnant women. These epidemiological data suggest a role of estrogen in the pathogenesis of thyroid diseases[14].

The aim of the present study was to detect differences in outcome between different types of thyroid cancer including the differentiated types (PTC and FTC) and the more aggressive type including the medullary and anaplastic variants and to correlate

the survival to epidemiological characters, histopathological features.

Patients and Methods

This retrospective study reviewed 584 patients with histologically proven thyroid carcinoma during period from January 2002 to December 2011. The study was carried out at the Clinical Oncology &Nuclear Medicine Departments, Mansoura University Hospital.

Data were collected from the files of patients in our department. The studied variables were data regarding general characteristics of patients including: age, sex, primary tumour size, number and multifocality of the primary lesion, type of thyroid carcinoma, subtypes of papillary thyroid carcinoma and follicular carcinoma, associated non- neoplastic lesions, extrathyroid extension, the number of lymph node metastasis, distant metastasis, TNM stage, type of surgical resection, redosurgery if was done, postoperative treatment, follow up of the patients, type of local recurrence (lymph node or at the thyroid site), presence or absence of metastasis, treatment modalities and survival including overall survival and disease free survival (OAS and DFS respectively).

Histological types of thyroid carcinoma were determined according to the system of World Health Organization[15]. TNM staging system was performed for these cases according to The American Joint Committee on Cancer[16].

Surgical treatment was done either hemi-thyroidectomy, near or subtotal thyroidectomy. Redo-surgery (completion thyroidectomy) was done in cases with post-operative big residual. Lymph node dissection was done in positive lymph node cases. Palliative resection was done in non-resectable cases.

Postoperative treatment consisted of radioiodine ablation (80-120 mCi) in patients undergoing at least a near-total thyroidectomy and thyroxine suppression therapy in differentiated thyroid carcinoma. Chemotherapy was used in medullary, anaplastic, some cases

in metastatic or non-resectable differentiated thyroid cancer. Radiotherapy was used in postoperative treatment in medullary thyroid cancer, whereas palliative radiotherapy was used in metastatic differentiated thyroid and anaplastic carcinoma.

Follow up of patients was done by means of three to six monthly outpatients' returns in first 5 years and then every 1-2 years. Follow up included physical examination, biochemistry tests for thyroid hormones assessments, thyroid stimulating hormone (TSH), and thyroglobulin levels, ultrasonography of the neck. whole body iodine scan (WBI 131Scan) every 6 months till it will become free twice then every year, radiological studies (including chest X-ray, abdominal ultrasound and bone scan, when indicated) were employed for detection of relapse. The survival data (overall survival, disease free survival) were retrieved from the archive of Clinical Oncology and Nuclear Medicine Department.

DFS was calculated from the date of surgery till the date of recurrence (either local or distant) and OAS was calculated from the date of diagnosis till the date of death of the patient. Mean follow-up time was 44 months.

Statistical analysis:

Data was analyzed by using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) program version 16. Survival curves were estimated by the Kaplan-Meier method with a log rank test toassess significance. Multivariate Cox proportional hazard regression models were used to evaluate any independent prognostic effect of the variables with 95% confidence interval. A p-value of <0.05 was considered to reflect a significance.

Results

The records of unselected 584 patients with histologically proven thyroid carcinomas were reviewed and retrospectively analysed. Of these patients, 31 % were males and 69% were females with an age range (3-84) years and mean of (43.1±15.339) years. The patients' characteristics are summarized in (Table 1). 54.3% of patients were more than 45 years of age, 37.3% showed lymph node involvement

while 8.7% and 7.9% developed distant metastasis and extrathyroid extension respectively. 6.7% of the tumours were multifocal in distribution.

The papillary thyroidcancer was the most prevalent type accounting for 74.1% of the patients followed by follicular thyroid cancer which represented about 12.5% of the cases. The anaplastic and medullary variants demonstrated smaller percentage of 7.9% and 5.5% respectively. Forty nine patients died during the follow up period (8.4%).

Prognostic features in the whole series;

Univariate analyses of various prognostic factors in the whole patients series using log rank test are summarised in table one.Our results showed that old age, extrathyroid extension, large primary tumour size, distant metastases, involvement of lymph nodes and anaplastic carcinoma are significantly poor prognostic factors in the whole patient series (Table 1). The Kaplan Meier plots for the tumour types in relation to the overall survival are shown in (Fig 1-3).

Multivariate COX hazard analysis showed that only the large primary tumour size, distant metastases, involvement of lymph nodes and tumour types retained the independent prognostic feature (Table 4).

Papillary thyroid cancer:

Univariate analyses of various prognostic factors in the PTC patients' series using log rank test are summarised in table two. Our results showed that old age, extrathyroid extension, large primary tumour size, distant metastases, involvement of lymph nodes are significantly poor prognostic factors (Table 2).

Multivariate COX hazard analysis showed that only patients age (HR=5.943, 95% CI=1.237-28.650, p=0.026), distant metastases (HR=12.968, 95% CI=3.924-42.854, p<0.001) and the extrathyroid extension (HR=3.527, 95% CI=1.070-11.626, p=0.038) are independent prognostic factors of survival.

Follicular thyroid cancer:

Univariate analyses of various prognostic factors in the FTC patients' series using log rank test are summarised in table 3. Our

results showed that only the distant metastases and degree of tumour invasion are significantly poor prognostic factors in the FTC patient series (Table 3). None of them retained independence in multivariate analysis in our patients' series.

Table (1): Patients' characteristics and prognosis in the whole series.

Variable	Number (%)	Overall Survival (%)	Log rank	*p* value
Gender			0.460	0.498
Female	403(69)	91.1		
Male	181(31)	92.8		
Patients' Age			**35.793**	**<0.001**
Less than 45 years	317(54.3)	97.2		
more than or equal to 45 years	267(45.7)	85		
Primary tumour size			**91.993**	**<0.001**
1 cm or less	52(9.5)	100		
more than 1 cm but not more than 2 cm	104(17.8)	99		
more than 2 cm but not more than 4 cm	241(41.3)	96.7		
more than 4 cm	153(26.2)	73.9		
Lymph node Stage			**42.725**	**<0.001**
N0	364(62.3)	97.3		
N1	218(37.3)	82.1		
Histologic type			**375.923**	**<0.001**
PTC	433(74.1)	97		
FTC*	73(12.5)	89		
Medullary^	32(5.5)	90.6		
Anaplastic	46(7.9)	45.7		
Development of distant metastasis			**77.399**	**<0.001**
No	533(91.3)	94.6		
Positive	51(8.7)	60.8		
Extrathyroid extension			**20.784**	**<0.001**
No	538(92.1)	92.9		
Probable	46(7.9)	76.1		
Multifocality			0.856	0.355
No	545(93.3)	91.9		
Yes	39(6.7)	87.2		

*including 19 cases (26%) of the Hurthle cell carcinoma variant.
^including one case of mixed medullary and papillary carcinoma.

Table (2): Incidence and Prognostic variables in PTC in relation to overall survival.

Variable	Number	Overall Survival (%)	Log rank	*p* value
Gender			0.184	0.668
Female	302	96.7		
Male	131	97.7		
Patients' Age			**12.465**	**<0.001**
Less than 45 years	260	98.8		
more than or equal to 45 years	173	94.2		
Primary tumour size			**14.876**	**0.002**
1 cm or less	47	100		
more than 1 cm but not more than 2 cm	93	100		
more than 2 cm but not more than 4 cm	189	96.8		
more than 4 cm	72	90.3		
Lymph node status			**22.564**	**<0.001**
N0	274	100		
N1	159	91.8		
Lymph node status (≥45)			**15.551**	**<0.001**
N0	100	100		
N1	73	86.3		
Development of distant metastasis			**102.496**	**<0.001**
No	414	98.6		
Positive	19	63.2		
Extrathyroid extension			**24.563**	**<0.001**
No	401	98		
Probable	32	84.4		
Multifocality			0.006	0.940
No	399	97		
Yes	34	94.1		
Type			3.788	0.150
Classic	298	98		
Follicular	108	94.4		
Others*	13	100		

* Including encapsulated, tall and columnar cell variants.

Table (3): Incidence and prognostic variables in FTC in relation to overall survival.

Variable	Number	Overall Survival (%)	Log rank	*p* value
Gender			0.051	0.822
Female	52	88.5		
Male	21	90.5		
Patients' Age			1.305	0.253
Less than 45 years	34	94.1		
more than or equal to 45 years	39	84.6		
Primary tumour size			2.748	0.432
1 cm or less	2	100		
more than 1 cm but not more than 2 cm	5	80		
more than 2 cm but not more than 4 cm	34	94.1		
more than 4 cm	32	84.4		
Lymph node status			0.446	0.504
N0	67	88.1		
N1	4	100		
Lymph node status (≥45)			0.407	0.524
N0	35	82.9		
N1	3	100		
Development of distant metastasis			**15.552**	**<0.001**
No	52	98.1		
Positive	21	66.7		
Extrathyroid extension			1.975	0.160
No	70	90		
Yes	3	66.7		
Multifocality			0.006	0.940
No	399	97		
Yes	34	94.1		
Type			2.753	0.097
Classic	54	92.6		
Hurthle cell carcinoma	19	78.9		
Degree of invasion			**8.522**	**0.004**
Minimally invasive	40	97.5		
widely invasive	28	78.6		

Table (4): COX proportional hazard analysis of the predictors of overall survival in the whole series of thyroid cancer patient.

Variable	*p*value	Hazard Ratio (HR)	95 % CI	
			Lower	Upper
Patients' age	0.067	2.104	0.950	4.660
Tumour size	**0.008**	2.496	1.264	4.927
LN stage	**0.003**	3.083	1.479	6.426
Tumour types	**<0.001**	2.339	1.787	3.063
DM	**0.010**	2.248	1.214	4.164
Extrathyroid extension	0.195	1.595	0.788	3.229

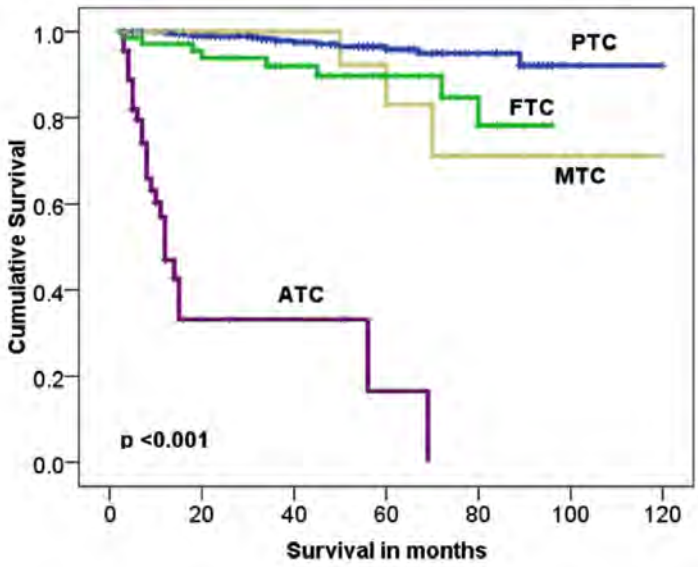

Fig.1: Kaplan Meier plot for the tumour types in relation to the overall survival.

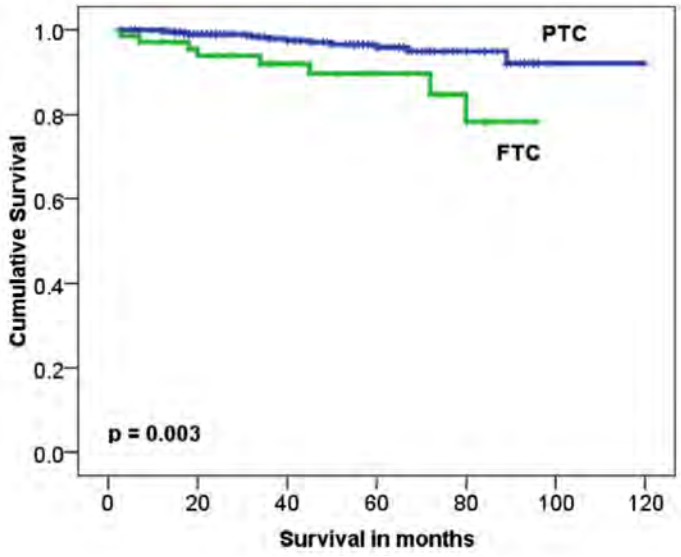

Fig.2: Kaplan Meier plot for the tumour types (PTC and FTC) in relation to the overall survival.

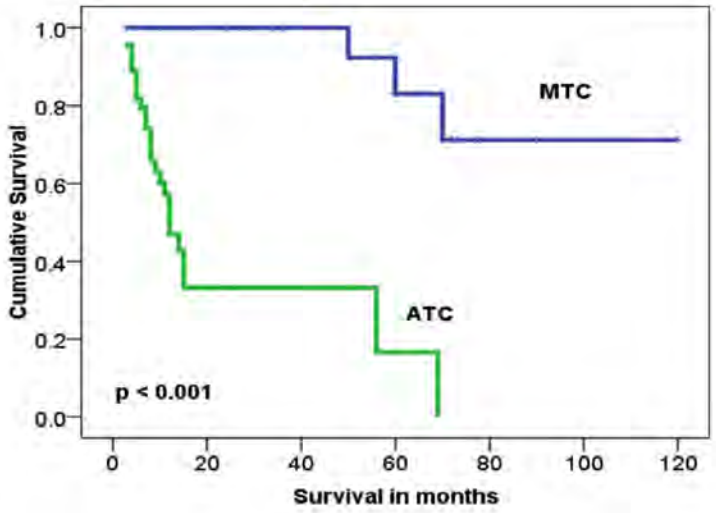

Fig.1: Kaplan Meier plot for the tumour types (MTC and ATC) in relation to the overall survival.

Discussion

Approximately 80-85% of all thyroid malignancies are papillary cancers[6]. Follicular carcinoma is the second most common type accounting for 10-15% of all thyroid cancers. Other uncommon types of thyroid cancer include anaplastic and medullary thyroid carcinomas. Anaplastic carcinoma derived from thyroid epithelial cells and account for only 1-2% of thyroid cancer, while medullarycancer is derived from the parafollicular cells and account for 3-4%[11]. In agreement with our results, these incidence show close similarity to the current study regarding the tumour type distributionindicating that thyroid cancer patients in Egypt show no noticeable differences in incidence and distribution.

Although the papillary and follicular carcinomas are derived from the follicular cells, however, the biological behaviors of these two carcinomas significantly differ. PTC frequently presented with regional lymph nodes metastases and can show a high incidence of significant extrathyroid extension. In contrast FTC more frequently metastasizes to distant organs such as the lung, bone, and brain[5].

Our results showed that, the significant prognostic factors are different between the two types. Although the distant metastasis is the most important factor in both types, PTC prognosis is widely and significantly affected by the age, lymph node metastases, and extrathyroid extension in comparison to FTC which is affected by extrathyroid extension.

In conclusion, our study, the prognosis and biological behaviour of thyroid carcinoma in Egypt is similar to what happens worldwide. FTC patients showed significantly poorer survival that PTC. The significant prognostic factors are different between the two types. Although the distant metastasis is the most important factor in both types, PTC prognosis is widely and significantly affected by the age and extrathyroid extension in comparison to FTC.

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PROGNOSTIC FACTORS AND ITS IMPACT ON SURVIVAL IN CANCER THYROID

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RESPONSE AND SURVIVAL BENEFIT OF CONCURRENT CHEMORADIOTHERAPY (CCRT) WITH CAPCITABIN AND CISPLATIN FOR LOCALLY ADVANCED INOPERABLE ESOPHAGEAL CANCER

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Abstract

Background: *Esophageal cancer is the ninth most common cancer worldwide and is highly malignant. The prognosis for patients with T3 or T4 lymph node positive disease is particularly poor. CRT with concurrent cisplatin and 5FU is associated with grade 3 or 4 toxicity in 93% of patients, excessive toxicity significantly compromises patient compliance*

Objectives: *This study was performed to investigate the safety and efficacy of concurrent chemoradiotherapy (CCRT) combined with an orally active capcitabin plus cisplatin for locally advanced esophageal cancer (LAEC).*

Methods: *Between July 2007 and May 2008, 30 patients with stage III locally advanced inoperable esophageal cancer were enrolled in this study. CCRT comprised 2 courses, a 30-Gy radiotherapy over 3 weeks plus daily oral Capcitabin in a dose of (45 mg/m2/day) for 2 weeks and 24 hour Cisplatin infusion (50 mg/m2) on day 8. An identical course administered after a 3-weeks break.*

Results: *The overall survival rate was 60% at 12 months while the overall progression free survival rate was 40% at 12 months. Endoscopic CR rate was 52.7%, PRR was 30.43% and progressive disease was detected in 13.05%. The most frequent hematologic adverse event was grade III and IV neutropenia (50%).*

Conclusion: *CCRT combined with capcitabin plus cisplatin showed promising safety and efficacy. Potentially, this combination therapy could become a baseline medication for patients with LAEC.*

Introduction

Esophageal cancer is the ninth most common cancer worldwide and is highly malignant (1). Although advanced diagnostic techniques have improved the detection of early-stage EC, locally advanced and metastatic cases are still encountered. Surgical resection is curative only during early stages; local recurrence and distant metastases remain problematic after surgical resection of advanced tumors[1]. The prognosis for patients with stage T3 or T4 lymph node-positive squamous cell carcinoma (SCC) is particularly poor[2]. Multimodality approaches based on preoperative chemotherapy or preoperative and definitive chemoradiotherapy (CRT) have been evaluated as these interventions allow locoregional control and long-term survival in locally advanced EC (LAEC)[1]. Randomized trials of CRT with cisplatin and continuous 5-fluorouracil (5-FU) infusion as a definitive, neoadjuvant treatment yielded a significant survival benefit[3,4].

However, the prognosis for patients with LAEC remains disappointing with a median survival time (MST) of just 16.0 months [1-4]. Additionally, CRT with concurrent cisplatin and 5-FU is associated with grade 3 or 4 toxicity in 93% of patients[5], while 20% experience life-threatening side effects[3], and treatment-related mortality amounts to 7%[6]. Excessive toxicity significantly compromises patient compliance. As progress in LAEC treatment is limited, new CRT regimens with good safety and high efficacy are in demand. Most patients prefer oral over intravenous chemotherapy if efficacy is maintained, so oral administration is desirable. It is a fourth-generation, novel, orally active fluoropyrimidine with enhanced anticancer activity and reduced gastrointestinal toxicity[7]. The response rate (RR) of gastric cancer to capcitabin is higher and the incidence of toxicity is lower compared with 5-FU[8]. Accordingly, 5-FU is superseded by capcitabin in its efficacy in solid tumors. Furthermore, as capcitabin enhances radiotherapy[9], it could prove to be an effective chemotherapeutic agent in CCRT.

Patients and Methods

Inclusion Criteria:

Between July 2007 and May

2008, we enrolled 30 patients from Clinical Oncology and Nuclear Medicine Department and Oncology Center Outpatient Clinics to this study. Eligible patients had pathologically confirmed LAEC, defined as clinical stage III of cancer according to the International Union against Cancer (UICC), tumor-node-metastasis system (TNM), were previously untreated, and >40 years of age[11]. Patients were required to have an Eastern Cooperative Oncology Group performance status of 0-2 and were able to take capcitabin. They had adequate bone marrow function (neutrophil count >1,500/µl, platelet count >100,000/µl, and hemoglobin >9 g/dl), adequate liver function (serum bilirubin <1.5 mg/dl and adequate renal function (serum creatinine <1.2 mg/dl and blood urea nitrogen <25 mg/dl), and an expected survival time >3-6 months.

Study Endpoints:

The primary endpoints were evaluation of toxicity, clinical response, and compliance with treatment completion. Patients were considered to have completed treatment if they received 2 courses each of CCRT. Secondary endpoints were overall survival (OS) and progression-free survival (PFS).

Evaluation before Treatment:

At study entry, disease extent was determined by physical examination, a barium swallow study, upper digestive endoscopy, and computed tomography (CT) scanning. Similarly, TNM stage before treatment was determined using CT.

Treatment Schedule:

The treatment schedule is outlined in figure 1. CCRT included 2 identical courses separated by a 3 weeks resting period. Treatment courses comprised a 30-Gy radiotherapy over 3 weeks (2 Gy per fraction, 5 times a week), with daily oral, (45 mg/m2/day of capcitabin; for 2 weeks from day 1, and a 24-hour cisplatin infusion (50 mg/m2) on day 8. For patients who showed an objective response to CCRT, chemotherapy consisting of (45 mg/m2/day; capcitabin for 2 weeks (starting on day 1) and a 24-hour cisplatin infusion (50 mg/m2) on day 8 was administered followed by 21 days without (4 cycles after end of the 21 days cisplatin).

Dose Modifications:

Dose adjustments were made for capcitabin and cisplatin during treatment if severe toxicity occurred. For grade 3 hematologic toxicity, the dose was reduced by 25% in the subsequent course. For grade 4 hematologic toxicity or grade 3 nonhematologic toxicity, both chemotherapy and radiotherapy were stopped. After toxicity had resolved, reduced doses of capcitabin (50%) and cisplatin (75%) were administered in the subsequent courses.

Radiotherapeutic technique:

Split-course radiotherapy was delivered at 2 Gy per day up to a total dose of 60 Gy using a 6- or 10-MV linear accelerator. The primary tumor with proximal and distal margins <5 cm and the surrounding regional nodes were treated with 30 Gy over 3 weeks period and another identical course of radiotherapy after 3 weeks resting period, with opposed anteroposterior fields. Supraclavicular lymph nodes were included in the field when the primary tumor was located above the carina. Celiac nodes were included for distal esophageal primary tumors. The dose to the spinal cord was limited to <45 Gy.

Adverse events and response evaluations:

Patients were hospitalized during CCRT, hemogram and blood biochemistry were examined twice weekly. Ability to swallow, body weight, performance status, and related symptoms were checked. Toxicity of treatment was evaluated according to the Common Toxicity Criteria in the National Cancer Institute[12]. Clinical response was assessed according to RECIST (Response Evaluation Criteria in Solid Tumor)[13]. Primary lesion were estimated by endoscopy according to the response evaluation criteria of the Japanese Society for Esophageal Diseases[14]. Endoscopy and CT were done at 3-month intervals for 2 years after completion of the treatment course.

Statistics:

The required sample size was calculated based on a target complete response (CR) of 50% and a minimum CR of 35% with an a P-value of 0.05 considered statistically significant. Time intervals were calculated from treatment in-

itiation to death or last follow-up day. OS and PFS curves were plotted using the Kaplan-Meier estimator.

Results

Demographic variables of the patients:

Between July 2007 and May 2008 we enrolled 30 patients into this study. Their demographic variables at study entry are presented in table (1). All patients had stage III LAEC adenocarcinoma of esophagus.

Overall treatment outcomes:

Five patients (16.7%) did not complete the CCRT due to disease progression, two patients (6.7%) did not complete CCRT due to treatment toxicity, while 23 patients (76.6%) completed treatment course.

Adverse Events:

Toxicities are summarized in table 2. The most frequent hematologic adverse events were grade 3 and 4 neutropenia (50%), G2 thrombocytopenia (66.7%), and anemia (50%). Adverse events began on days 5-10 and nadir was on day 13 (range 10-17). One patient died on day 20 from febrile bone marrow aplasia. The most common non-hematological adverse effects were grade 3 nausea (83.3%), G3-4 esophageal pain in (76.71%) and G3-G4 oral mucositis (40%) and renal dysfunction with elevated serum creatinine (G2) detected in (10%) of cases. Adverse event from the first course of CCRT decreased during the 2-week interval.

Survival Rates and PFS Values:

The survival and PFS curves for all patients are shown in figure 2. The overall survival rate was 60% at one year and the overall PFS rate was 40% at 1 year.

Response rates:

Response rates are summarized in table 3a/3b endoscopic CR rates were 52.7% with as 95%CI 59.9-81.9, partial endoscopic RR were 30.43 with 95%CI (14.3-32.4), progressive disease in 13.05% with 95%CI (11.2-28.1) during CCRT, while SD was detected in 4.35% of treated cases. On other hand overall response rates assessed by RECIST criteria revealed CR in about 43.5% with 95% CI (55.5-78.2) and PR in 21.7% with 95% CI (13.5-78.2), SD in 17.4% with 95% CI (1.6-13.8) and PD in 17.4% with 95% CI (0.9-11.9).

Table (1): Patients demography at study entry.

Patients characters	No.	%
Age (years):		
>60	7	23
60-70	13	43
<70	10	33
Sex:		
Male	16	53.4
Female	14	46.6
Performance status:		
0	6	20
1	21	70
2	3	10
Location:		
Upper	7	23.3
Middle	18	60
lower	5	16.7
↓ 5 cm	12	40
↑ 5cm	18	60
UICC T.N.M system:		
T3. N1-Mo	10	33.3
T1-2. N2-Mo	7	23.3
T4a. No-Mo	13	43.4

Table (2): Toxicity by grade.

Toxicity	G2 (n)	G3 (n)	G4 (n)	G2 (%)	G3/G4 (%)
Hematologic toxicity:					
Neutrophils	15	10	5	50	50
Platelets	20	5	5	66.6	33.3
Hemoglobin	15	7	3	50	33.3
Febrile bone marrow aplasia	0	0	1	0.0	0.03
Nonhematologic toxicities:					
Nausea	5	10	15	16.7	83.3
Vomiting	3	7	20	10	90
Anorexia	4	5	21	13.3	86.7
Esophageal pain	7	3	20	23.3	76.7
Oral mucositis	3	10	17	10	90
Fatigue	7	8	14	23.3	73.3
Creatinine	3	7	20	10	90
Diarrhea	5	20	5	16.7	83.3
Constipation	20	7	3	66.7	30
Hyperpigmentation	23	5	2	76.7	23.3
Sensory neuropathy	10	15	5	33.3	66.7

Table (3a): Response rates for the 23 patients with LAEC who completed the course of therapy.

Response rates	No.	%	95% CI
RR for the primary lesion assessed by endoscopy:			
CR	12	52.17	(59.9-81.9)
PR	7	30.43	(14.3-32.4)
SD	1	4.35	(0.9-11.9)
PD	3	13.05	(11.2-28.1)
CR complete response PR partial response	SD stable disease PD progressive disease		

Table (3b): Response evaluation criteria in solid tumors.

Overall PR assessed by RECIST criteria			
	No.	%	(95% CI)
CR	10	43.5	(55.5-7.2)
PR	5	21.7	(13.5-34.0)
SD	4	17.4	(1.6-13.8)
PD	4	17.4	(0.9-11.9)

CR= complete response, PR= partial response, SD= stable disease, PD= progressive disease

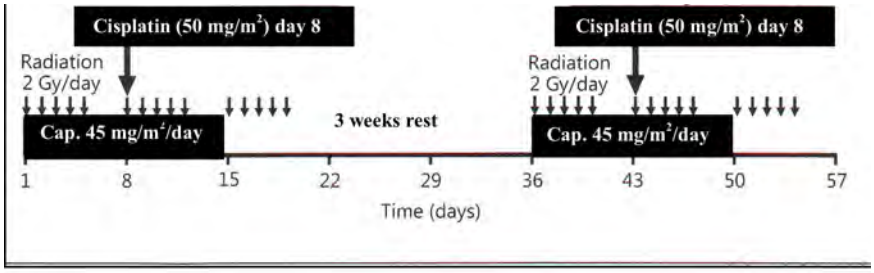

Fig. 1

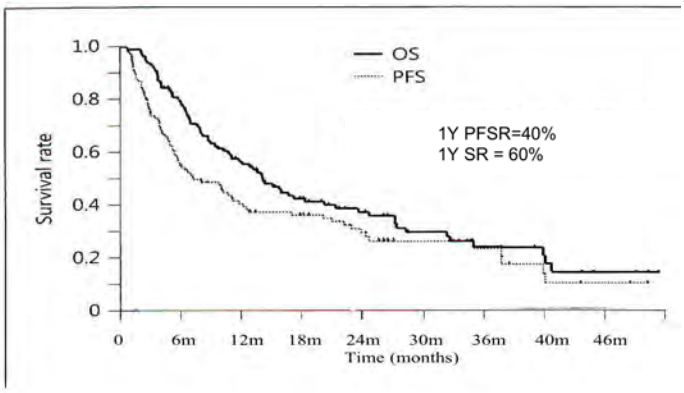

Fig. 2

Discussion

Recently, attempts have been made to increase the efficacy of conventional CCRT with a combination of 5-FU and cisplatin for LAEC. The Radiation Therapy Oncology Group recommended a study of high dose RT of (64.8 Gy) versus standard dose of RT (50 Gy) of radiation during CRT. However, the increase in the radiation dose did not reduce Locore-gional failure rates (56 vs. 52%) or increase survival[15]. Similarly, CRT with extensive radiation fields did not also improve survival rates [16]. Hence, new regimens with alternative radiosensitizing and biochemical modulation of chemotherapeutic agents are under investigation to improve definitive CRT outcome. Radiotherapy, concurrent with cisplatin, carboplatin, paclitaxel, or irinotecan, is well tolerated and may be of benefit in LAEC patients[17,18]. Several studies reported strong antitumor effects for docetaxel-based CRT/ with CR rates of 11-71.2%[19]. In a phase II, randomized trial of CRT with FOLFOX4 or cisplatin and 5-FU, CRT with FOLFOX4 achieved a better endoscopic CR (44.7% vs. 29.3%) with modest toxicity[20].

50% of the patients developed grade 3 or 4 leukocytponeia. In this trial, CCRT with capcitabine and cisplatin was divided into 2 courses with 3 weeks resting period to avoid elevated myelosuppression. The most common adverse event was neutropenia which was not fatal (except for 1 case) and improved during the 3 weeks resting period. Nonhematological toxicities were mild to moderate. Adherence to CCRT results in good outcome, is a gentle regimen for patients with LAEC, especially those who are weak or elderly, and increases compliance. Nonhematologic toxicities are important factors in maintaining CCRT. Conventional 5-FU CRT causes nonhematologic toxicity resulting from protracted venous infusion of 5-FU, including oral mucositis, esophagitis, diarrhea, thrombosis, and life-threatening toxicities, which require hospitalization in some patients [5,6]. A slow infusion of cisplatin over 24 hours minimizes gastrointestinal toxicities and renal dysfunction[22].

In patients undergoing CCRT, achieving an endoscopic CR is es-

sential. The RECIST guidelines[13] do not discuss endoscopic CR criteria in detail, but recently, endoscopic CR was used for quality of life evaluation and as a surrogate endpoint for OS[23]. After CRT, local relapses were often detected by endoscopy as esophageal ulceration (sloughing)[23]. Therefore, endoscopic evaluation is important to confirm not only CR but also primary lesion recurrence, and it could be of a great significance in the assessment of new treatment strategies for patients with LAEC [16,19,20,23,24]. Tumor persistence and progression within the radiation field are the main causes of treatment failure, and reducing the risk of locoregional treatment failure is under investigation[14,15,16]. Improved treatment success was evident for locoregional lesions in this trial, supporting our prediction that capcitabin better antitumor and biochemical effects with cisplatin than UFT or 5-FU in the treatment of LAEC.

In conclusion capcitabin can be conveniently administered with no negative impact on quality of life. Unlike 5-FU and cisplatin, capcitabin plus cisplatin appears to provide a safe combination with radiotherapy. Accordingly, CCRT combined with capcitabin plus cisplatin showed good efficacy with acceptable toxicity and could become an alternative of stage III adenocarcinoma of LAEC.

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RESPONSE AND SURVIVAL BENEFIT OF CONCURRENT CHEMORADIOTHERAPY (CCRT) WITH CAPCITABIN AND CISPLATIN FOR LOCALLY ADVANCED INOPERABLE ESOPHAGEAL CANCER

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SPECIES DISTRIBUTION AND ANTIFUNGAL SUSCEPTIBILITY TESTING OF CANDIDA ISOLATES FROM PATIENTS IN PEDIATRIC AND NEONATAL INTENSIVE CARE UNITS

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Abstract

This study targets high risk population for fungal infection. Candida species distribution varies according to geographic distribution and patient population. Type of species guide type of antifungal treatment.

Objective: *determining species distribution in Egyptian neonatal and pediatric intensive care units and their susceptibility to the available antifungal drugs.*

Study design: *a prospective study from September 2011 to November 2012. 320 samples were collected from patients in pediatric and neonatal intensive care units and 95 from hands of health care personnel to isolate different Candida species to be identified to the species level and tested for susceptibility to different antifungals.*

Results: *The overall Candida isolation rate among patient population was 19.4%. Candida albicans was the most common species isolated followed by C. tropicalis and C. parapsilosis, C. dubliniensis, C. glabrata, C. krusei, and C. famata. Candida parapsilosis was the most common species isolated from neonatal intensive care unit patients. Candida was isolated from 17.9% of personnel samples with C. parapsilosis as the most common species. Amphotericin B was the drug of choice. Voriconazole and fluconazole were effective except with C. glabrata.*

Conclusion: *Candida albicans is still the commonest Candida species isolated from patient population. Non-albicans Candida is increasing especially C. tropicalis and C. parapsilosis in pediatric and neonatal population that's why amphotericin B was the most active antifungal.*

Candida was isolated from health care personnel indicating the need for strict infection control measures.

Keywords: *Candida species, Pediatric Intensive Care Unit, Neonatal Intensive Care Unit, antifungal susceptibility testing.*

Introduction

Over the past 20 years, there has been a significant increase in Candida spp. infections worldwide, particularly in patients admitted to intensive care units. Candida bloodstream infections are life-threatening; they prolong hospital stays and are associated with high morbidity and mortality[1].

Candida albicans is still the Candida species most commonly isolated from neonatal patients with invasive candidiasis, but there has been a significant rise in isolation rates for other species in recent years. Prophylaxis with azole agents may increase the risk of infection with resistant Candida spp.[2].

Determining a pathogen's antifungal susceptibility is an important step in effective treatment[3]. However, it takes considerable time to isolate and identify organisms from patients with invasive fungal infections, and to determine resistance profiles. Delayed initiation of appropriate antifungal therapy can raise the risk of mortality and morbidity in infants, and also increases the likelihood of cross-contamination[4].

The opportunistic Candida species existing as parts of commensal microbiota in humans are usually the etiological agents causing infections. Thus, it is important to investigate whether isolates from various sources and various patients with different ages and in different hospital units have distinct characteristics[5].

Aim of The Work

The aim of this work is to investigate the distribution of various Candida species among clinical and health care personnel samples in the neonatal and pediatric intensive care units of Mansoura University Children's Hospital to detect whether Candida albicans or non-albicans Candida species predominate and detect

the difference in their antifungal susceptibility.

Patients and Methods

This is a prospective study carried out from September 2011 to November 2012 in intensive care units in Mansoura university children hospital. Three hundred and twenty patient samples were obtained (188 from Pediatric ICU, 120 from Neonatal ICU and 12 from Surgical ICU) and 95 samples from hands of health care personnel. Demographic and clinical data were collected. Samples were collected from each patient according to the site of infection. Samples from non-sterile sites were cultured more than once to ensure the pathogenic role of isolated organism. Candida species were identified using Sabouraud's Dextrose Agar: (Oxoid, England), Germ tube test, Corn meal agar, CHROMagar Candida, API 20C-AUX (Bio-Merieux, France). Antifungal Susceptibility Testing was performed for Amphotericin B (Bristol Myers Squibb Company), Fluconazole (Seidico Company) and Voriconazole (Pfizer Company) according to Clinical and Laboratory Standards Institute (CLSI) M27-A2 criteria[6].

Results

The 320 patients' samples included 103 (32.2%) urine, 130 (40.6%) blood, 20 (6.2%) CVC, 47 (14.7%) ETA, 2 (0.6%) CSF and 18 (5.6%) wound samples. Candida was isolated in 62 of the 320 samples, 43 (69.4%) were from males and 19 (30.6%) were from females; however this result was not statistically significant (P value= 0.909). Of the 62 patients' isolates, 24 (38.7%) were from patients aged 3-11 m, 15 (24.2%) from patients aged 1-5 y, 14 (22.6%) were from patients aged 0-2 m, then 8 (12.9%) were from patients aged ≤28d and lastly one isolate (1.6%) was from patient aged 6-12 y. All the neonates infected with Candida were preterm, 60% of them were VLBW, while LBW and ELBW were 20% each.

The length of ICU stay >1 week was associated with statistically significant high rates (90.3%) of Candida infection (P value <0.0001). Mortality was significantly higher (P value <0.0001) among patients infected with Candida (56.5%) than other patients

(43.5%). Urinary catheterization was the most commonly associated predisposing factor with Candida infection in this study 54 (87.1%), followed by MV 43 (69.4%), CVC 25 (40.3%), TPN 21 (33.9%) and lastly VPS was found in only one patients (3.2%). Surgery was a predisposing factor in 12.9% of the Candida infected patients. Of 62 patients positive for Candida, 46 (74.2%) were on broad spectrum Caphalosporins and 29 (46.8%) were on Vancomycin. Corticosteroids was administered in 30 (48.4%) of the patients positive for Candida, while Empirical Fluconazole was administered in 21 (33.9%).

Candida isolation rate in urine samples (67.7%) was significantly higher than other clinical specimens, followed by blood (27.4%), CVC (3.2%) and ETA (1.6%) (P value <0.0001). No Candida species were found in the 2 CSF or 18 wound samples.

Candida isolation rate from patients' samples collected from PICU was 24.7 %. Urine samples showed the significantly highest Candida isolation rate (48.8%) (P value= 0.001). In NICU Candida isolation rate was 9.3%. Blood samples showed the highest Candida isolation rate (12.8%) followed by urine (11.8%), CVC (7.1%) then ETA (5%). However the result was not statistically significant (P value = 0.818). Candida isolation rate from SICU was 20%. Blood samples showed high Candida isolation rate (37.5%) among clinical specimens. Other specimens did not yield any Candida species, however the result was not statistically significant (P value = 0.350).

Candida albicans was the most commonly isolated species (43.5%) followed by C. tropicalis (22.6%), C. parapsilosis (17.7%), C. glabrata (6.5%), C. krusei (4.8%), C. dubliniensis (3.2%) and lastly C. famata (1.6%) Fig. (1).

Candida albicans was the most commonly isolated species from urine samples (45.2%) followed by C. tropicalis (31%), C. glabrata (9.5%), C. parapsilosis and C. krusei (4.8%) each and C. famata and C. dubliniensis (2.4%) each. From blood specimens C. parapsilosis was the most commonly isolated species (47.1%), followed by C. al-

bicans (35.3%) and C. tropicalis, C. dubliniensis and C. krusei (5.9%) each. However the difference in species distribution in relation to the type of specimen was not statistically significant (P value=0.240).

In the PICU, C. albicans was the most commonly isolated species (44.9%) followed by C. tropicalis (26.5%), C. parapsilosis (14.3), C. glabrata (6.1%), C. krusei (4.1%), then 2% of each of C. dubliniensis and C. famata. In the NICU, C. parapsilosis was the most commonly isolated species (40%), followed by C. albicans (30%), then C. tropicalis, C. glabrata and C. krusei 10% each. While in the SICU, 2 isolates (66.7%) were C. albicans and one (33.3%) was C. dubliniensis. However the difference in species distribution in the three ICUs was not statistically significant (P value = 0.167).

Of the 95 personnel samples, 17 (17.9%) yielded Candida species. Candida parapsilosis was the most commonly isolated species in personnel sample. However, C. albicans and C. tropicalis were equally the most commonly isolated species in the PICU (37.5%) each. In the NICU C. parapsilosis was the commonest (80%) and in the SICU, C. albicans was the commonest, however the results were not statistically significant (P value=0.285).

Table (1) and Fig. (2) show the in vitro susceptibility pattern of the Candida isolates to Amphotericin B, Fluconazole and Voriconazole according to M 27A2.

Table (1): In vitro activity of Amphotericin B, Fluconazole and Voriconazole against isolates of Candida species according to M 27A2.

Antifungal susceptibility		Candida species														Total (79)		χ^2 P
		C. Albicans (33)		C. parapsilosos (33)		C. tropicalis (18)		C. glabrata (4)		C. dublinie nsis (2)		C. femata (1)		C. krusei (3)				
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
Amphotericin B	Susceptible	33	100	16	88.9	16	88.9	3	75	2	100	1	100	2	66.7	73	92.4	8.152
	Resistant	0	0	2	11.1	21	11.1	1	25	0	0	0	0	1	33.3	6	7.6	0.227
Fluconazole	Susceptible	27	81.8	15	83.3	15	83.3	1	25	2	100	1	100	0	0	61	77.2	28.48
	SDD	4	12.1	1	5.6	0	0	1	25	0	0	0	0	0	0	6	7.6	0.005*
	Resistant	2	6.1	2	11.1	3	16.7	2	50	0	0	0	0	3	100	12	15.2	
Voriconazole	Susceptible	31	93.9	16	88.9	17	94.4	3	75	21	100	1	100	2	66.7	72	91.1	4.483
	SDD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.612
	Resistant	2	6.1	2	11.1	1	5.6	1	25	0	0	0	0	1	33.3	7	8.9	

SDD: susceptible dose dependent.

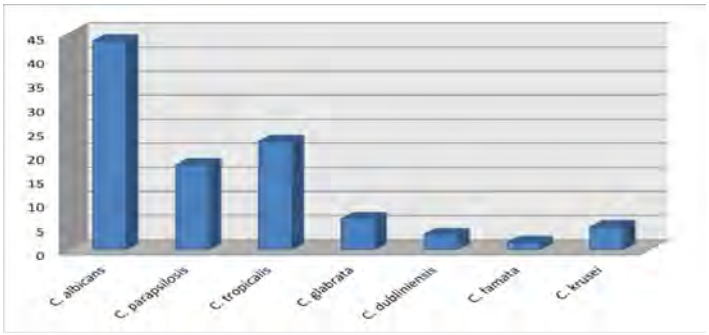

Fig. 1: Distribution of Candida species isolated from different patient samples.

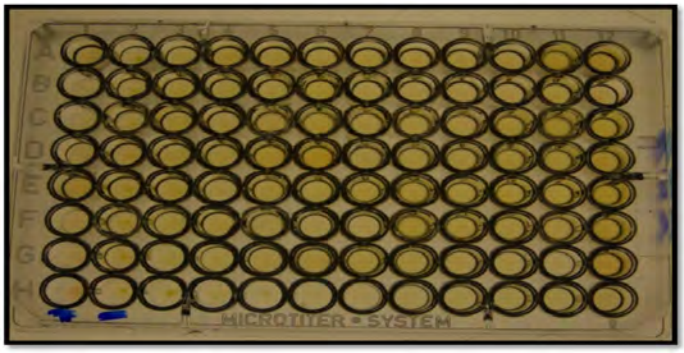

Fig. 2: Antifungal susceptibility pattern according to M 27A2.

Discussion

As noted, rates of Candida infection have increased significantly, and these infections are a special concern in PICUs and NICUs. C. albicans remains the most frequently isolated Candida species, but there has also been a notable rise in infections caused by non-albicans Candida species[7,8].

For appropriate antifungal therapy, accurate identification of the isolates at the species level is necessary[9-11].

During a period of one year, 320 samples were collected from patients in neonatal and pediatric ICU suspected for Candida infection and 95 personnel. Of the 320 patient samples, 62 yielded Candida species (19.4%). Candida isolation rate was 17.9% among personnel group.

Predisposition to fungal infection among PICU and NICU patients was not related to the age or sex in the previous studies[12,13]. In our study we found no significant difference in sex (p=0.909) and age distribution between patients.

Prematurity and LBW were the commonest risk factors for candidemia[14]. In our study all the neonates infected with Candida were preterm and 60% of them were VLBW. Mortality was significantly higher in patients with Candida infection (56.5%). This was in agreement with Figueras, et al., 2011[15].

Potential predisposing factors in our study included, length of ICU stay >1 week (90.3%), surgery (12.9%), urinary catheterization (87.1%), MV (69.4%), CVC (40.3%), TPN (33.9%) and VPS was found in only one patient, Corticosteroids (48.4%), vancomycin (46.8%), broad spectrum Caphalosporins (74.2%) and empirical Fluconazole was administered in (33.9%). These a re well known risk factors to Candida infection[14-26].

Amphotericin B was reported to be the most effective against all Candida isolates and resistance to azoles varies[19,27]. This may be explained by resistance by some Non-albicans Candida species specially C. glabrata[28]. Voriconazole also is effective therapy for candidiasis. In a study of 422 pa-

tients with invasive candidiasis, approximately 40% of patients were successfully treated with voriconazole. While there were a small number of children enrolled in both of these studies, there are no pediatric-specific outcomes reported from these trials[29].

In this study, Amphotericin B was the drug of choice; 92.4% of Candida isolates showed susceptibility to it, Voriconazole comes next as it was active against 91.1% of Candida isolates and Fluconazole was active against 77.2%. Amphotericin B has excellent antifungal activity against C. albicans, C. dubliniensis and C. famata, C. parapsilosis and C. tropicalis and less active against C.glabrata and C. krusei. It was found that all C. dubliniensis and C. famata isolates were susceptible to Fluconazole, while all C. krusei (100%) isolates were resistant. In vitro activity of Fluconazole was significantly lower in C. glabrata isolates than in other Candida species (P value =0.005). Voriconazole was active against most Candida species with low activity against C. glabrata.

Conclusion

Candida albicans is still the commonest Candida species isolated from patient population. Non-albicans Candida is increasing especially C. tropicalis and C. parapsilosis in pediatric and neonatal population that's why amphotericin B was the most active antifungal. Candida was isolated from health care personnel indicating the need for strict infection control measures.

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SPECIES DISTRIBUTION AND ANTIFUNGAL SUSCEPTIBILITY TESTING OF CANDIDA ISOLATES FROM PATIENTS IN PEDIATRIC AND NEONATAL INTENSIVE CARE UNITS

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INSULIN RESISTANCE AND TREATMENT OUTCOME IN PATIENTS WITH CHRONIC HEPATITIS C GENOTYPE 4

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Abstract

Background: *HCV infection is a major health problem in Egypt. The prevalence of persons infected with HCV in Egypt is 22%. HCV infection is strongly associated with development of insulin resistance (IR). IR is a specific feature of chronic HCV, associated with genotype 1 and 4. A factor of clinical importance is the negative impact of IR on response to antiviral treatment. It has been observed in a number of studies that IR interferes with the standard antiviral treatment. Several studies proved significant improvement in insulin resistance was observed in patients who achieved sustained virologic response (SVR). The objective of the present study was to study the inter-relationship between insulin resistance and treatment outcome in patients with chronic hepatitis C (CHC) genotype 4.* ***Patients and methods:*** *Two groups of patients (43 insulin resistant patients and 43 insulin sensitive patients) prepared for antiviral therapy. IR was measured by calculation of HOMA-IR. HOMA-IR was also calculated after 12 weeks therapy for the early responders. The statistical methods used were independent T test, one way ANOVA and ROC curve.* ***Results:*** *The early virologic response (EVR) was higher in insulin sensitive patients (91%) than the insulin resistant patients (74%). We found HOMA-IR levels were significantly higher in the nonresponders than the responders within the insulin resistant patients. ROC curve showed that HOMA-IR could be considered as an independent predictor of failure of EVR. We found that insulin resisant patients*

who achieved EVR showed significant decrease in HOMA-IR levels after 12 weeks antiviral therapy. ***Conclusion:*** *IR could be considered as a predictor of treatment failure in patients with CHC genotype 4. EVR is associated with improvement of IR.*

Introduction

HCV infection is a major health problem in Egypt. The prevalence of persons infected with HCV in Egypt is 22%[1].

HCV infection is strongly associated with development of insulin resistance and type-2 diabetes, however molecular mechanism of these associations is not known[2]. Experimental data suggest a direct interference of HCV with the insulin cascade via proteosomal degradation of the insulin substrate receptor-1 &2[3]. In addition functional impairement of signaling pathway may occur via proinflammatory cytokines such as TNFα[4] or other post receptor defects[5]. In patients with genotype 3, HCV may alter the intrahepatic insulin signaling through down-regulation of PPARα and γ[6].

IR is a specific feature of chronic HCV, associated with genotype 1 and 4[7]. A factor of clinical importance is the negative impact of IR on response to antiviral treatment. It has been observed in a number of studies that IR interferes with the standard antiviral treatment, pegIFN and ribavirin[8].

The effect of successful antiviral therapy was studied by Romero-Gomez et al., 2005[8]. They stated that significant improvement in insulin resistance was observed in patients who achieved sustained virologic response (SVR).

The objective of the present study was to study the interrelationship between insulin resistance and treatment outcome in Egyptian patients with chronic HCV genotype 4.

Patients and Methods

The present study was conducted on 86 Egyptian patients with chronic HCV genotype 4 proved by HCV RNA using PCR techniques. The age of the studied patients was between 18-60 years. Their body mass indices were less than

30. Diabetic patients and hepatitis B surface antigen positive patients were excluded. All patients were prepared for antiviral therapy (peginterferon plus ribavirin). The patients were screened for insulin resistance by calculation of HOMA-IR [FPG (mg/dl) x Fasting plasma insulin (μU/ml) ÷ 405].

The patients were recruited into 2 groups, group (A) representing the 43 insulin resistant patients (HOMA-IR >2). Group (B) represents 43 randomly selected with HOMA-IR ≤2. Both groups had matched age and sex. HOMA-IR was done after 12 weeks therapy for the early responders.

Parametric independent T test and one way ANOVA were used to compare the median quantitative values. ROC curve was done to detect a cut off value of HOMA-IR for failure of antiviral therapy.

Results

No significant variation was observed in demographic data between insulin resistant patients and the insulin sensitive patients except that total body fat (TBF) was significantly higher in insulin resistant group than insulin sensitive group as shown in table 1.

We found that HOMA-IR levels were significantly higher in insulin resistant patients than the insulin sensitive patients. The early virologic response was higher in insulin sensitive patients than the insulin resistant patients as shown in table 2.

No siginificant demographic variation between the early responders and nonresponders was observed as shown in table 3.

HOMA-IR levels were significantly higher in the nonresponders than the responders within the insulin resistant patients as shown in table 4.

ROC curve showed that Higher levels of HOMA-IR than 3.7 were associated with failure of early virologic response to 12 weeks antiviral therapy as shown in figure 2 and table 5.

Insulin resistant patients who achieved EVR showed significant decrease in HOMA-IR levels after 12 weeks antiviral therapy as shown in table 5.

Table (1): Demographic data of groups (A) and (B).

Parameter	Group A (n = 43)	Group B (n = 43)	P value
Age	36.47 ± 7.17	36.02 ± 8.36	0.109
Sex ♂ ♀	 37 (86%) 6 (14%)	 34 (79%) 9 (20.9%)	0.394
BMI	26.29 ± 2	25.57 ± 1.74	0.081
TBF	26.84 ± 4.13	23.36 ± 3.53	‹0.001

♂ = male ♀ = female BMI = body mass index TBF = total body fat.

Table (2): Comparison of HOMA-IR & EVR between group A and group B.

Parameter	Group (A) (n = 43)		Group (B) (n = 43)		P value P
HOMA-IR	3.19 ± 1.45		0.97 ± 0.48		‹0.001*
EVR	Responders	Nonresponders	Responders	Nonresponders	
	32 (74%)	11 (26%)	39 (91%)	4 (9%)	0.05*

EVR = early virologic response. *Group (A) = insulin resistant group*
Group (B) = insulin sensitive group *P value is significant if ≤ 0.05.*

Table (3): Demographic data of the early responders and nonresponders.

	Responders (n = 71)	Non-responders (n = 15)	P value
Age	35.94 ± 7.47	37.67 ± 9.11	0.437
Sex ♂ ♀	 58 (81.7%) 13 (18.3%)	 13 (86.7%) 2 (13.3%)	0.644
BMI	25.86 ± 1.77	26.27 ± 2.47	0.548
TBF	24.95 ± 4.06	25.93 ± 4.70	0.411

Table (4): Comparison of HOMA-IR between the early responders and the nonresponders.

Parameter	Responders (n = 32)	Nonrsponders (n = 11)	P value
HOMA-IR	2.62 ±0.40	5.33 ± 1.98	0.004*

Table (5): Cut off point of HOMA-IR (threshold for response failure) & sensitivity, specificity and significance of the ROC curve.

Area under the curve	Cut off	P value	Sensitivity	Specificity
1.0	3.7	0.000	100%	100%

Table (6): Comparison of HOMA-IR levels before therapy and after 12 weeks therapy.

Parameter	Before therapy	After therapy	P value
HOMA-IR	3.19 ± 1.45	2.79 ± 1.34	0.002*

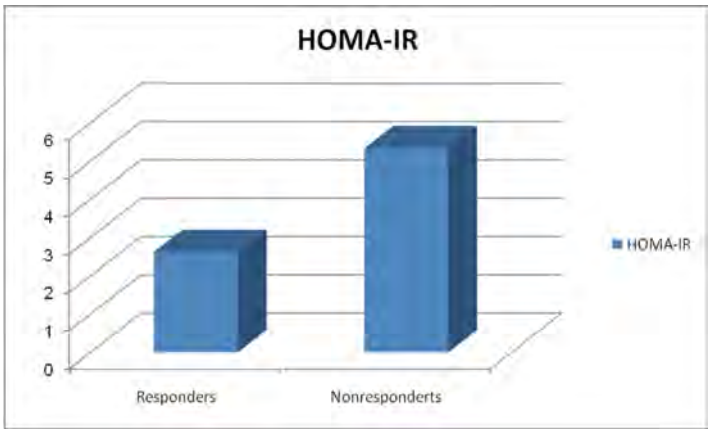

Fig.1: (comparison of HOMA-IR between the early responders and the nonresponders).

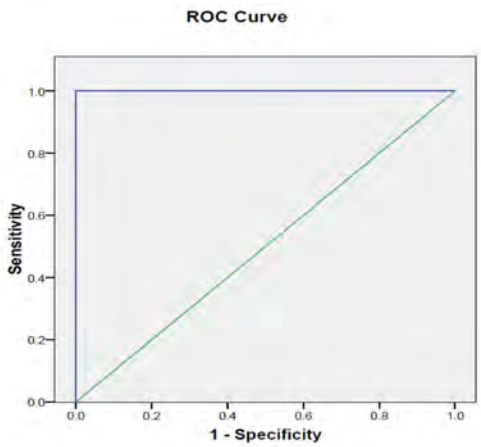

Fig.2: ROC curve illustrating the effect of HOMA-IR on early virologic response.

Discussion

In our study we found that the percentage of early responders to 12 weeks antiviral therapy within the insulin resistant patients was 74% and the percentage of responders within insulin sensitive patients was 91%. HOMA-IR was found to be significantly higher in the nonresponders of the insulin resistant patients than the responders (P=0.004). From the ROC curve which illustrated the relationship between HOMA-IR and response to 12 weeks antiviral therapy (EVR), we found that HOMA-IR can be considered as a reliable independent predictor of the response to antiviral therapy. Higher levels of HOMA-IR above cut off point which was 3.7 indicated failure of response to antiviral therapy. The sensitivity of the curve was 100%, specificity was 100% and P value was <0.0001.

Romero-Gómez et al.,[8] showed marked differences in the rates of SVR in HCV infected patients with IR (32.8%) and without IR (60.5%). Also, Carter[9] found that the presence of insulin resistance has been associated with poor six month response to anti-hepatitis C treatment with pegylated interferon and ribavirin in patients infected with hepatitis C.

An Egyptian study searching for predictors of failure of antiviral therapy confirmed that one of these predictors in genotype 4 is insulin resistance measured by HOMA-IR>2.99[10].

Khattab et al.,[11] proved that IR is a major determinant of both RVR and SVR in genotype 4 CHC patients. They suggested that HOMA-IR is a useful tool for predicting the response to therapy. In addition, Danielle et al.,[12] also found that IR was associated with lower rates of SVR.

In our study the effect of 12 weeks antiviral therapy on insulin resistance was evaluated. We found that there was significant decrease of HOMA-IR in the early responders of the insulin resistant patients (3.19±1.45 decreased to be 2.79±1.34 & P=0.002).

A study of 89 Japanese patients found that eradication of HCV led to improved HOMA scores and intrahepatic expres-

sion of IRS-1 and IRS-2[3]. In a prospective study of 6 months follow up of HOMA-IR of HCV genotype 1 patients during antiviral therapy. There was significant decrease in the number of insulin resistant cases after SVR[13].

Delgado et al.,[14] confirmed that HCV suppression correlates with IR improvement. Interestingly, Conjeevaram et al.,[15] found that only SVR-achieving patients had significant decreases in HOMA-IR during and after therapy. Improvement in IR persisted even after patients regained weight following treatment.

Conclusion

Insulin resistance could be considered as an independent predictor of failure of the early response to 12 weeks antiviral therapy.

Early virologic response is associated with significant decrease in insulin resistance.

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INSULIN RESISTANCE AND TREATMENT OUTCOME IN PATIENTS WITH CHRONIC HEPATITIS C GENOTYPE 4

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PHASE II STUDY OF VINORELBINE AND CAPECITABINE AS FIRST LINE TREATMENT FOR METASTATIC BREAST CANCER: FINAL RESULTS AFTER 4 YEARS FOLLOW-UP

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Abstract

Background: *Vinorelbine (V) and Capecitabine (C) are likely to have a synergistic interaction. V up-regulates thymidine phosphorylase, a key enzyme in the conversion of C to active 5-FU in the tumor tissue. Available Phase II clinical data reports response rates ranging from 48-70% in first-line MBC for this combination. We evaluated the efficacy and safety of V + C in pts with MBC relapsing after adjuvant anthracycline-based treatment.*

Patients & Methods: *40 pts were enrolled between Oct 2004 and Feb 2005. All pts had measurable MBC (WHO) recurrent after adjuvant anthracycline treatment,*

WHO PS ≤ 1, adequate bone marrow, renal and hepatic functions. Pts received i.v. V 25 mg/m 2on days 1 and 8 + oral C 825 mg/m2 bid on days 1 to 14. Cycles were repeated every 3 weeks. Pts with PD went off study while those with CR, PR, or SD continued treatment for a maximum of 8 cycles.

Results: *Median age 54 years (range 35-67); median WHO PS 0 (range 0-1). Previous adjuvant therapy: anthracycline (100%), hormone therapy (45%). Median disease free interval: 5 months. Main metastatic sites: lung (40%), skin (35%), lymph nodes (35%), bone (25%), liver (23%). 25% of pts had one metastatic site, 60% had 2, 12.5% had 3 and 2.5% had more than 3 sites. The total number of cycles delivered was 312 with a median number of cycles per patient of 8 (range 3-8). The RDI was 100% for both drugs. An objective tumor response was achieved in 36 pts (90%) (OR = CR + PR), complete response CR in 20*

(50%) pts. 2 (5%) pts had stable disease. After 4 years follow-up, median time to progression & overall survival were 22 & 6 months respectively. No WHO grade 4 toxicities were noted.2 (5%) pts developed Gr 3 neutropenia. Gr 2 anemia and neutropenia were reported in 1 (3%) and 5 (13%) pts respectively. 1 patient (3%) developed Gr 2 oral stomatitis.

Conclusions: *The combination of vinorelbine and capecitabine showed significant efficacy & mild toxicity as first line treatment for patients with metastatic breast cancer after failure of adjuvant anthracyclinebased therapy.*

Introduction

BREAST cancer is the most frequent malignancy in women and the second most common cause of cancer relate death. It is a global problem and a leading cause of cancer mortality[1]. Despite early diagnosis of breast cancer patients (40%) will develop metastatic disease that is incurable[2]. The median survival from diagnosis of metastatic disease is 2 to 3 years, with 5-10% survival beyond 5 years[3]. Currently, there is no standard treatment for metastatic breast cancer after failure of anthracycline and taxane containing treatment and consequently, there is a need to find an effective schedule that is non-cross resistant with these drugs[4]. The treatment goal in women with metastatic breast cancer include prolongation of life, control of tumor burden, reduction in cancer related symptoms or complication and maintenance of quality of life and function[5].

Cytotoxic chemotherapy remains a mainstay of treatment for women with breast cancer irrespective of hormonal receptor status and is the backbone of many novel treatments incorporating biological therapy[6].

Vinorelbine; a semisynthetic Vinca alkaloid inhibits microtubules assembly and thus its activity is cell cycle specific, this compound blocks formation of the mitotic spindle apparatus at metaphase and prevents cell division. It has a high therapeutic index and less neurotoxicity than other vinca alkaloids due to the lower degree of damage of axonal microtubules[7].

Clinical resistance to taxanes

results from the decreased stability of tubulin complexes in tumor cells. Vinorelbine also acts on tubulin but via another cellular mechanism to destabilize microtubules, with the result that a patient resistant to taxanes is not necessarily resistant to vinorelbine[8]. The response rates (RRs) of vinorelbine in management of breast carcinoma ranged from 40-60% in previously untreated patients and of about 30% when used as a second or third line therapy[9].

Vinorelbine combined with 5-FU administered either as a bolus or in continuous infusion is able to achieve impressive results, with RRs of 50-64% in first line treatment and overall survival up to 23 months. This level of activity is observed even in anthracycline pretreated patients as well as in patients with visceral metastasis[10,11].

Capecitabine is an orally administered prodrug that is activated in the liver and at the tumor sites by a series of enzymatic reaction that converts it into its active form, 5-FU. It requires thymidine phosphorylase, an enzyme that is expressed significantly higher in tumors than in healthy tissue. The antimetabolite 5-FU exerts its antitumor effects through several mechanisms including inhibition of RNA synthesis and function, inhibition of thymidy late synthase activity and incorporation into DNA, leading to DNA strand breaks and subsequent cell death[12]. Capecitabine generates 5-FU predominantly within tumor tissue through the exploitation of high intratumoral concentrations of the enzyme thymidine phosphorylase. The preferntial generation of 5-FU of the tumor its reduces systemic exposure to 5-FU, thereby potentially reducing the risk of significant toxicity, also patients can receive capecitabine at home, thus fulfilling important requirements of palliative therapy for refractory metastatic tumors[13].

In phase I/II studies capecitabine gave RRs of 20-30% in patients with paclitaxel refractory MBC, along with minimal bone marrow suppression[14].

Capecitabine and navelbine are among the drugs of choice for anthracycline and taxane resistant

breast cancer because they have shown a high level of antitumor activity and are well tolerated in this setting. The combination of xeloda and navelbine would be a reasonable choice for chemotherapy of MBC, due to their different toxicity profile and synergistic effect of both drugs. The preliminary data of this combination from phase I & II studies in second line therapy have shown RRs of 40-55%[15,16,17].

The aim of the study was to evaluate the efficacy, tolerability and safety of capecitabine and navelbine combination and its implication on survival in patients with MBC relapsing after adjuvant anthracycline based treatment. The Primary Endpoint was Overall response rate (WHO criteria). The Secondary Endpoints were Time to progression, Overall Survival, Safety profile.

Patients and Methods

This is a prospective study done on 40 patients with metastatic breast cancer who attended at Mansoura University Hospital, Department of Clinical Oncology & Nuclear Medicine in the period between October 2004 and Feb. 2005. Eligibility criteria were female patients with metastatic breast cancer with measurable disease (clinical and/or radiological), bone metastasis or malignant pleural effusion as only site of metastasis were excluded. Adequate bone marrow (white blood cell count >35000/mm3, hemoglobin >10gm/dL and platelet count >100000/mm 3, liver (total bilirubin <1.5 upper normal limit and AST, ALT <2 upper normal limit) and renal function (serum creatinin <1.2) WHO performance status <1, age >18 years and <70 years, life expectancy >3 months, prior adjuvant chemotherapy with anthracyclines and adjuvant hormonal therapy were allowed, no prior treatment with chemotherapy for metastatic breast cancer, and oral informed consent was obtained prior treatment. Exclusion criteria were brain metastasis. Patients known brain or leptomeningial infiltration are excluded Pregnant or lactating women & male patients are excluded.

Pretreatment evaluation:

The initial evaluation included: Accurate full history, thorough clinical examination including

weight and height, performance status, general loco regional and systemic examination. Any mass whether at the primary or metastatic site was carefully recorded and measured in two perpendicular dimension, complete blood picture, renal & liver function tests were done and repeated before each cycle of chemotherapy, chest X-ray, computed tomography of abdomen, pelvis and/or chest and bone scan (as clinically indicated).

Treatment protocol:

Chemotherapy protocol was given as a 3 weekly schedule of capecitabine 850mg/m 2 twice daily on days 1-14, administered orally within 30 minutes after a meal (ideally after breakfast and dinner)with 200ml of water. Vinorelbine was given in a dose of 25mg/m 2 diluted in 50ml of normal saline and infused over 5-10 minutes D1 & D8 followed by 100ml of normal saline + dexamethasone to wash the vein treatment evaluation.

Tumor response was assessed according to WHO criteria.

Complete response (CR): Disappearance of all known disease determined by 2 observations not less than 4 weeks a part.

Partial response (PR): Decrease of 50% or more in the sum of the product of diameters of all measurable lesions determined by 2 observation not less than 4 weeks a part with no appearance of new lesions or progression of any lesions.

Stable disease (SD): Defined as less than 50% reduction or less than 25% increase in tumor size.

Progressive disease (PD): Was an increase of at least 25% of measurable lesion or the appearance of new lesions.

Tumor response was assessed by clinical examination and the same imaging method used to establish baseline tumor measurement every 2 cycles and then every 2 months after completion of treatment, while toxicity was assessed according to WHO criteria[20] before each cycle by clinical and laboratory examination, patients showing an objective response CR, PR or SD continued the treatment for a maximum of 8 cycles.

Statistical analysis:

The primary efficacy end points included objective response rate and time to disease progression. The overall patients' survival rate and the safety profile were also determined.

Overall survival (OS) and time to progression (TTP) were assessed from the date of diagnosis to the date of death and the date of objective disease progression respectively. Survival rates were calculated by using the Kaplan-Meire method[18].

Results

Forty women entered this prospective study and all of then were evaluable for efficacy and toxicity. The main clinical characteristics of the recruited patients were reported in Table (1). The median age was 54 years (range 35-67 years), median WHO PS was 1 (range 0-1), 40% of patients were premenopausal and 60% were postmenopausal 18 patients (45%) were hormone receptors positive.

All patients received anthracycline based chemotherapy as adjuvant; sites of metastases include liver (23%) lungs (40%). Nodal 35%, bone 25% and skin 35% 25% of patients had one metastatic site, 60% had 2 metastatic site 12.5% had 3 metastatic site and 2.5% had more than 3 sites.

Response to treatment:

All patients were evaluable for responses the objective response rate was 75% (CR 25%, PR 50%).

Toxicity:

Toxicity was manageable, hematological and non-hematological toxicities were obtained in Table (3).

The intensity of treatment related adverse events was mild to moderate (G1-2) in the majority of patients. There were no treatment related deaths reported. G3 neutropenia was occurred in 2 patients (5%). As regards the non haematological toxicities, the most frequent adverse events were grade 1 & 2 diarrhea (15%), hand foot syndrome (10%), alopecia (15%). Table (4) shows the univariate analysis of prognostic factors for response. Performance status (P=0.001), grade (p=0.03), number of metastatic site p=0.02)

and disease free interval (p=0.002) were significant prognostic factors for response.

Survival:

At a median followed up of 30 months (6-48 months), the median survival was 36 months (95% CI, 34.5 to 40.5) Fig. (1) and the median TTP was 22 months (95% CI = 20.7 to 25.3) Fig. (2).

Table (1): Patients & Tumor Characteristics.

	No. of Pts	%
Number of patients	40	
Median age [range]	54 35-67	
Median PS (WHO) [range] Ps0 Ps1	0 0-1 16 36	 40% 60%
Anthracyclin pretreated: Prior neoadjuvant therapy Prior adjuvant therapy	 0 40	 0 100
Prior adjuvant hormone therapy	18	45
Main metastatic sites Lung Skin Lymph nodes Bone Liver	 16 14 14 10 9	 40 35 35 25 23
Number of metastatic sites 1 2 3 >3	 10 24 5 1	 25 60 12.5 2.5

Table (2): Response To Treatment.

Evaluable Patients: n = 40	No. of Pts	%
Complete Response (CR	10	25%
Partial Response (PR)	20	5%
Objective response (OR+PR)	30	75%
Stable Disease (SD)	2	5%
Disease Control (OR+SD)	32	80%
Progressive Disease (PD)	8	20%

Table (3): Safety profile.

	Grade 1	Grade 2	Grade 3
Hematological:			
Anaemia	1 (3%)	1 (3%)	(5%)
Neutropenia	2 (5%)	5 (13%)	2 (6%)
Thrombocytopenia			
Non hematological:			
Diarrhoea	4(10%)	2(5%)	
Hand & Foot syndrome	3 (7.5%)	1(3%)	
Constipation	2(5%)		
Stomatitis	4 (10%)	1 (3%)	
Alopecia	4 (10%)	2 (5%)	
Fatigu	2 (5%)		

Table (4): Univariate analysis for prognostic factors for response to navelbine plus xeloda as first line regimen for metastatic breast cancer.

Prognostic factors	**Response**				
	CR	**PR**	**SD**	**PD**	**p-value**
Performance status:					
0	7	5	-	1	0.001
2	3	15	2	7	
3	-	-	-	-	
Grade:					
II	3	15	-	1	0.03
III	2	5	2	7	
# of metastatic sites:					
1	7	18	-	1	0.02
2	2	11	2	2	
3	-	-	-	5	
Disease free interval:					
>2y	8	18	-	2	0.002
<2y	2	2	2	6	

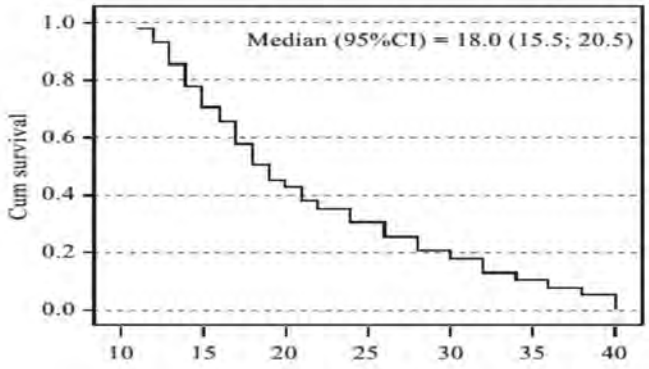

Fig. 1: Overall survival (months)

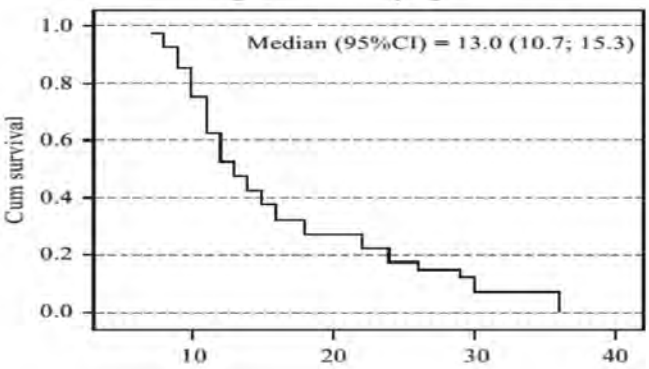

Fig. 2: Disease-free survival (months)

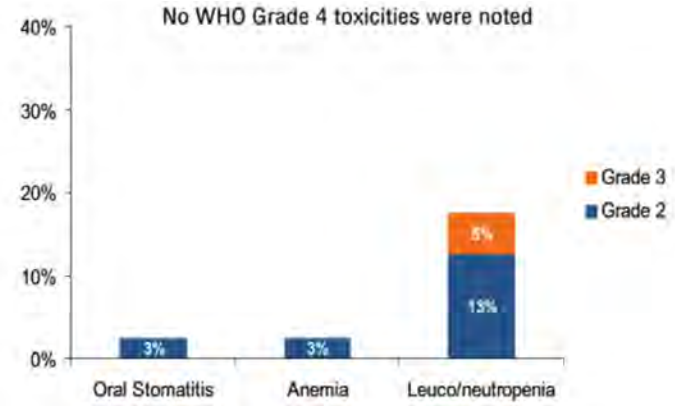

Fig. 3: Toxicity profile.

Discussion

Metastatic breast cancer is a highly heterogeneous disease where particular criteria must be considered taking into account not only the clinical & biological parameters but also patients' expectation and preference. The objectives of an optimal chemotherapy for MBC are to prolong survival and improve the quality of life with minimal toxicity[19]. Combination chemotherapy regimens are generally through to be more effective than single agent chemotherapy in advanced breast cancer. The high remission rates observed with navelbine as a single agent as well as its low toxicity profile makes it a very attractive proposition for combination with other active compounds. Since xeloda mimics continuous infusion of 5-fluorouracil without the inconvenience and complications associated with this method of administration, this makes it a good candidate to combine with navelbine[20].

In the current study the combination of xeloda and navelbine showed an overall RR of 75% (CR 25% & PR 50%). This result is within the range (33%-70%) that reported by phase II studies used combination xeloda & Navelbine in patients with metastatic breast cancer. These values are consistent with the present study results and further confirm the benefit of using this combination of drugs. In 2 of these studies in which higher efficacy rate were observed, with on ORR of 78%, a median TTP and median OS of 23 & 35.4 months respectively, the treatment was first line therapy in all case and 77% of the patients had previously received adjuvant anthracycline[21,22].

In a recent phase II study done by Fan et al.,[23] on 72 patients with MBC pretreated with anthracycline and taxanes, treated by navelbine/xeloda, the response rate was 52.8% with a median survival of 26.1 months, the variability betweenresults obtained would most probably be influenced by difference in the number and type of treatment previously administered to these patients.

In terms of safety, in the current study, treatment was well tolerated, the majority of reported

adverse events were mild to moderate in intensity no grade 4 toxicity, 2 patients (5%) developed G3 neutropenia. Granulocyte colony stimulating factor was given to these 2 patients who developed G3 neutropenia as prophylactic in the subsequent cycles.This result is similar to that obtained by Ghosn et al.,[21] and Lorusso et al.,[24].

Non hematological toxicity in the present study were G2 Diarrhea (5%), hand-foot syndrome (3%) and stomatitis (3%) these results are comparable to those reported by Ghosn et al.,[21] and Fan et al.,[23].

There were no treatment related deaths; however, these side effects are generally manageable and consistent with the known toxicities of individual agents.

For the prognostic factors: PS, number of metastatic site, grade and disease free interval were statistically prognostic factors for response, these results are comparable to that reported by and Fan et al.,[23].

In the current study the median TTP and median OS were 22 & 36 months, respectively. These results are similar to that obtained by Estevezl et al.,[24] who used capecitabine/navelbine therapy as first line treatment in anthracycline pretreated patients with MBC and they reported median TTP of 20 months and a median OS of 34 months. The interest of oral drugs in the management of cancer patients in the palliative setting is growing parallel to the preference of the patients for oral chemotherapy provided that the efficacy and toxicity of these agents are comparable to that of their IV counterparts[24]. These are other advantages of oral initial as it allows outpatients treatment avoid inconvenience and problems associated with IV infusions and potentially reduces the cost of parenteral treatment, also it is well to tolerated in elderly patients [25,26].

Conclusion

• This study shows high antitumoral activity for the combination Vinorelbine + Capecitabine, with an objective tumor response of 80%.

• This regimen demonstrates a

very good safety profile with no WHOgrade 4 toxicities. It compares favorably with other combination chemotherapies commonly used in MBC; in particular, it does not cause alopecia.

• Compliance to treatment was excellent with an RDI of 100%.

• After 4 years follow-up, the TTP is 22 months and the Overall Survival is 36 months.

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PHASE II STUDY OF VINORELBINE AND CAPECITABINE AS FIRST LINE TREATMENT FOR METASTATIC BREAST CANCER: FINAL RESULTS AFTER 4 YEARS FOLLOW-UP

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ROLE OF MR PERFUSION IMAGING IN: POST IRRADIATION NECROSIS VERSUS BRAIN TUMORS RECURRENCE

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Abstract

Aim: *to evaluate the value & diagnostic effectiveness of perfusion weighted images (PWI) in the differentiation of the tumor recurrence from irradiation related necrosis.*

Patients and methods: *Thirty-six patients with known intra-axial SOL of the brain underwent surgical resection followed by radiotherapy on 3 months interval. On follow up they have developed enlarging regions of enhancement within the radiation field. Conventional MRI was done followed by PWI study.*

Results: *Normalized mean relative cerebral blood volume (rCBV) was significantly higher in tumor recurrence / progression group (1.9±0.63) than in post treatment necrosis (0.76±0.09). Inspection of rCBV, relative cerebral blood flow (rCBF) and percentage of baseline at peak (PBP) was more beneficial than relative time to peak (rTTP) & relative mean transit time (rMTT).*

Conclusion: *Normalized mean rCBV is a good differentiating factor and Inspection of rCBV, rCBF & PBP maps is more efficient than rTTP & rMTT in the evaluation of recurrent brain tumors.*

Keywords: *Perfusion; tumor recurrence; glioblastoma multiforme; radiation necrosis.*

Introduction

Differentiating post treatment necrosis (PTN) from recurrent tumor in brain tumor patients using conventional morphologic imaging features is a very challenging task. Delayed radiation necrosis can manifest as progressive contrast enhancement on follow-up serial imaging[1]. A patient's clinical course, biopsy, and serial imaging for several months have tradition-

ally been used to distinguish tumor recurrence and radiation necrosis[2]. Functional imaging techniques also offer moderate success due to the complexity of the tissue microenvironment and the inherent limitation of the various modalities and techniques.

MR perfusion can overcome the limitations of conventional MRI and can be used to derive information on tumor hypoxia, necrosis, proliferative activity, or vasculature[3].

Patients and Methods

This study was performed in radio-diagnosis department Mansoura university hospital from August 2012 till January 2014.

Selection criteria:

Patients with known intra-axial malignant SOL of the brain underwent surgical resection followed by radiotherapy on 3 months interval were referred from radiotherapy, neurology and neurosurgery departments to do MRI brain. Whenever enlarging regions of abnormal SI were discovered within the radiation field, functional imaging (Perfusion) were applied and patient is included in this study.

MR examination:

Magnetic resonance imaging of the brain was performed on a 1.5T at MRI Unit, Mansoura University Hospital. All patients were examined in the same position used for routine MRI examination of the brain using head coil. Conventional MRI sequences as well as functional MRI techniques were used for all patients. Total examination time did not exceed 60 min.

The standard imaging protocol consisted of the following sequences: localizing sagittal T1-weighted (TR/TE/NEX: 300/14ms/1), axial T1-weighted (TR/TE/NEX: 500/15ms/1), fast spin-echo axial T2-weighted (TR/TE/NEX: 4490/85ms/1). For all the above sequences, the slice thickness was 5mm with interslice gap of 1mm, FOV was 220-240 mm, and the matrix was 128x256.

After that, perfusion weighted MR imaging (PWI) and contrast enhanced T1-weighted in the axial, sagittal & coronal planes (TR/TE/NEX: 500/15ms/1) were performed.

Perfusion weighted MR imaging:

Perfusion weighted images were obtained using dynamic post-contrast T2* gradient-echo EPI sequence (TR/TE/NEX: 4200/47ms/1, flip angle 20 degree, slice thickness of 5mm, interslice gap of 1mm, FOV: 240mm, matrix 128x256). A series of 50 multisection acquisitions was acquired at one-second intervals. The first ten acquisitions were performed before contrast agent injection to establish a precontrast baseline. At the tenth acquisition, a bolus of gadopentate dimeglumine (in a double dose of 0.2 mmol/kg) was injected using an automated injector at a rate of 5ml/sec through a 18-20 gauge intravenous catheter inserted in an antecubital vein. This was followed by a bolus injection of saline (total of 20ml at 5ml/sec). So the images were acquired before, during, and after injection of gadopentate. The data acquisition time was 120 seconds. The temporal resolution was sufficient to monitor the passage of bolus through the whole brain.

Mean rCBV values of each contrast-enhancing lesion were calculated as follows:

rCBVmean: region of interest (ROI) covered the contrast-enhancing lesion except necrotic part. These values were normalized to normal appearing white matter.

rCBVnawm: ROI in normal appearing white matter (nawm) was placed within normal appearing white matter in the contralateral hemisphere.

Mean normalized rCBV = rCBVmean/ rCBVnawm were analysed.

ROIs area ranged from 2 - 4 mm. The ROIs were located completely within the lesion by manual trace, avoiding major cortical vessels and cystic parts of the lesion by an observer unaware of the clinical data.

Perfusion maps were evaluated by 2 experienced radiologists. Lesion localization was done using T2 and T1post contrast images, then altered signals were evaluated either: decreased (d), increased (i) or no significant change (n). TTP, PBP, MTT, CBV & CBF were evaluated.

Time to Peak (TTP): is the time

interval to the bolus peak. In the TTP map, the signal intensity of each pixel shows in grey or in color-encoding the regional distribution of the time from the injection of the contrast agent to the bolus peak.

The Percentage of Baseline at Peak (PBP) determines the relative amount of signal loss due to bolus passage through the capillary bed. Reduced perfusion, that is, less contrast agent, is shown as bright pixels.

rel**MTT:** the relative mean transient time is the mean duration of a bolus passage through a voxel. Its pixel by pixel display results in rel MTT map

Pathological analysis:

Twenty patients underwent second operation which revealed 14 tumor recurrence and six cases of radiation necrosis. Time interval between MR imaging and re-op ranged from 1-3 months. No radiation therapy was taken during that interval.

Clinico-radiologic follow up:

Six lesions were not histologically confirmed. Post treatment necrosis (PTN) was diagnosed when progressively enhancing areas decreased definitively in size or remained unchanged on serial follow-up MR imaging for a minimum of 3 months. The length of enhancement stability on follow-up imaging in patients clinically diagnosed with radiation necrosis ranged from 9 to 18 months.

Recurrent/progressive tumor (RPT) was clinic - radiologically diagnosed when the enhancing lesion progressively increased in size on serial examinations, accompanied by neurologic deterioration during a minimum follow-up period of 3 months. The duration of progressive enhancement on follow-up imaging in patients clinically diagnosed with recurrent metastatic tumor ranged from 6 to 12 months.

Statistical analysis:

Descriptive statistics for each property were computed. Statistical analysis was performed using commercially available software (SPSS21).

The values of rCBV ratio for tumor recurrence and post treat-

ment necrosis were compared by use of the Mann-Whitney nonparametric test. Significance was defined as a probability value of less than 0.05. Sensitivity was defined as the proportion of correctly identified recurrent tumors, and specificity was defined as the proportion of correctly identified post treatment necrosis.

Receiver-operating characteristic (ROC) curve analysis was used to determine the cut-off values for the differential diagnosis of tumor recurrence and post treatment necrosis.

Results

This study included 36 patients (16 females and 20 males), their age ranged from 10 years to 68 years with mean 42 years.

All patients had known intra-axial SOL of the brain. They underwent surgical resection followed by radiotherapy on 3 months interval. Subsequently they have developed enlarging regions of enhancement within the radiation field. Post therapy surgical resection was done for 24 patients. While the rest 12 patients underwent clinic-radiologic follow-up, on which progressive increase established a diagnosis of recurrent tumor or radiation necrosis.

Eighteen patients had GBM as original pathological diagnosis. Most of recurrent tumors were of large size (18 lesions were > 3cm) and majority of lesions showed peripheral enhancement (20/24) as shown in (table 1).

Perfusion MR imaging data sets were processed, and regions of interest were drawn around the entire contrast-enhancing region.

Mean normalized rCBV values are presented in (Table 2). Mean normalized rCBVmean were significantly higher in the recurrent tumor group than in the radiation necrosis one. Normalized rCVBmean: 1.9 ±0.63 for tumor reccurence/pregression versus 0.76 ±0.09 for post treatment necrosis; p value = 0.045 (table 4).

ROC curve analysis was used to assess the diagnostic utility of the metrics to discriminate recurrence from radiation effects. The ROC curve for the rCBV ratio is

shown in (Fig. 3). The optimum rCBV value for differentiating tumor recurrence from radiation effect was 0.9, giving an accuracy profile of the best sensitivity and specificity for recurrent brain tumors, 100%. The area under the ROC curve of 1 indicates high sensitivity and specificity.

Direct inspection of perfusion maps revealed that rCBV and rCBF maps were most accurate with sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) equals 100%, 80%, 95% & 100% respectively. It was followed by PBP which showed sensitivity of 90%, specificity 80%, PPV 95% and NPV 67%. TTP showed sensitivity of 75%, specificity 100%, PPV 100% and NPV 44%. rMTT showed sensitivity of 70%, specificity 100%, PPV 100% and NPV 40%. These results are represented in Table 3.

Table (1): different types of SOLs included in this study.

	Tumor recurrent/residual	**Post treatment necrosis**
Original pathological diagnosis		
GBM	18	2
Anaplastic glioma	4	2
Astrocytoma	6	2
Meningioma	1	
metastasis	1	
Location		
Frontal	6	1
Parietal	8	2
Temporal	9	2
Occipital	4	1
More than one lobe	3	-
Size		
<1cm	2	2
1-3 cm	2	2
3-5cm	18	2
> 5cm	8	-
Pattern of enhancement		
Peripheral	26	5
heterogenous	4	1
Time elapsed from RT till appearance of enhancing lesion		
3m	12	2
6m	8	2
12m	6	1
18 m	4	1

Table (2): Normalized mean rCBV values obtained in both groups of patients.

	Tumor recurrence/ progression	Post therapy necrosis
1	2.25	0.85
2	1.72	0.82
3	0.98	0.72
4	2.41	0.66
5	1.82	0.64
6	0.96	0.58
7	2.16	
8	1.81	
9	2.24	
10	2.73	
11	1.83	
12	1.61	
13	1.52	
14	0.97	
15	1.64	
16	3.15	
17	1.28	
18	2.55	
19	1.86	
20	2.94	
21	1.84	
22	2.53	
23	2.62	
24	3.18	
25	2.70	
26	1.55	
27	1.65	
28	1.73	
29	2.82	
30	3.22	
Mean	1.9 ±0.63	0.76 ±0.09

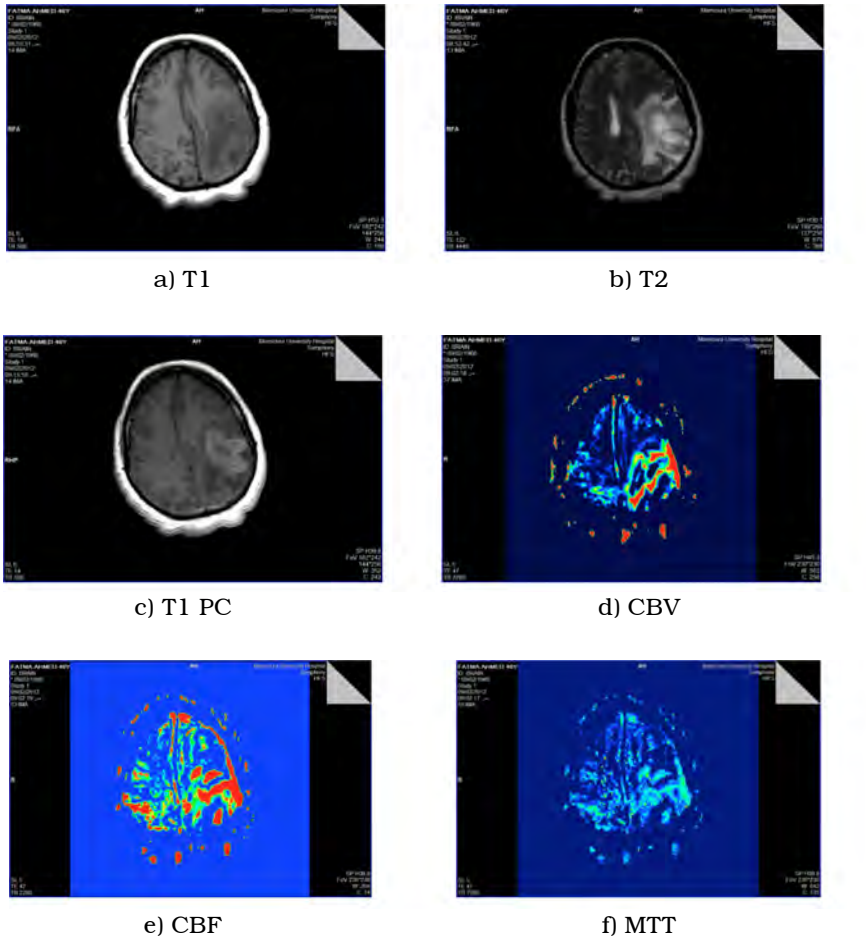
Table (3): Perfusion maps in 18 cases of tumor recurrence.

	TTP	relMTT	rCBV	rCBF	PBP
sensitivity	75%	70%	100%	100%	90%
specificity	100%	100%	80%	80%	80%
+ve predictive value (PPV)	100%	100%	95%	95%	95%
-ve predictive value (NPP)	44%	40%	100%	100%	67%

Table (4): ***P*** value of significance for normalized rCBVmean in tumor recurrence/ progression versus post therapy necrosis.

	***P* value of significance**
Normalized rCBVmean	0.045

NB: P value is significant if < 0.05.



a) T1 b) T2

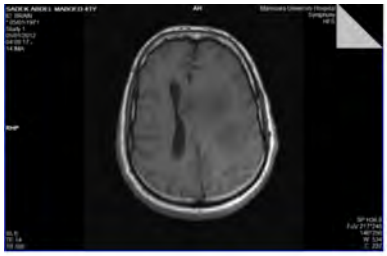
c) T1 PC d) CBV

e) CBF f) MTT

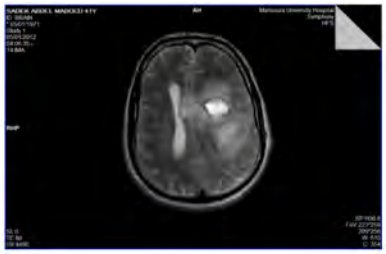
Fig.1: Female patient aged 46 years, she had left parieto-occipital glioblastoma multiforme (GBM). Surgical excision was done followed by 25 cycles of radiotherapy. Perfusion MRI was done.

(a, b & c): A large intra-axial SOL located at the left parieto-occipital region. It displays low SI on T1WI, high SI on T2WI and surrounding by mild perifocal vasogenic edema. It exerts mass effect in the form of effacement of the related cortical sulci, compression on ipsilateral lateral ventricle and contralateral shift of midline structures. After IV contrast injection (image c) showed irregular heterogenous enhancement.

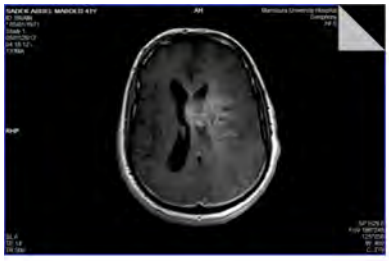
(d, e & f) rCBV, CBF & MTT maps respectively revealed: increased vascularity...final diagnosis was recurrent tumor (GBM) proved by pathological analysis after re-surgical excision.



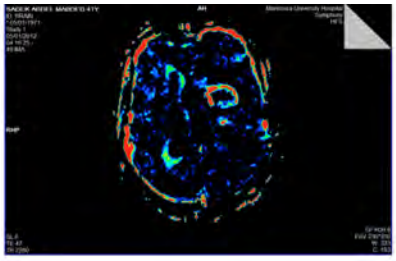
a) T1



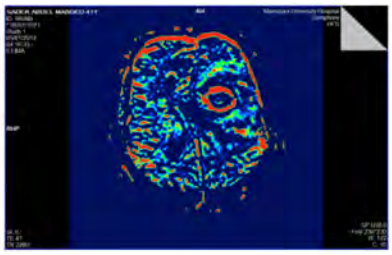
b) T2



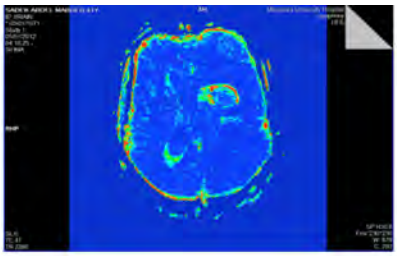
c) T1 PC



d) CBV

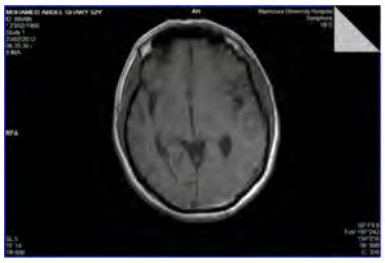


e) CBF

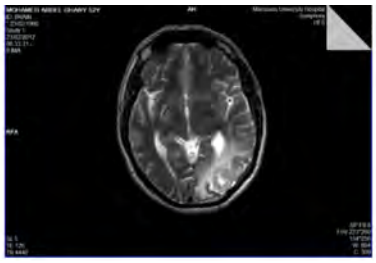


f) MTT

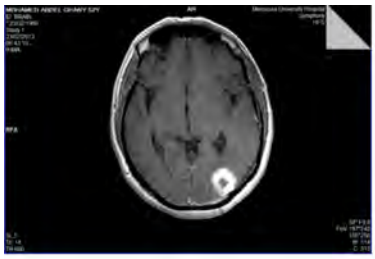
Fig.2: Male patient aged 41 years. He had left parietal glioblastoma multiforme. He underwent surgical excision followed by radiotherapy. MR perfusion was done.
(a, b & c): T1WI, T2WI & T1 post contrast respectively revealed: An area of abnormal signal intensity low on T1 & high on T2WI is seen in the left parietal region. It is seen surrounded by mild perifocal vasogenic edema. It exerts mass effect in the form of effacement of the related cortical sulci, compression on ipsilateral lateral ventricle & midline shift to opposite side. It showed heterogeneous enhancement after IV contrast administration.
(d, e & f) CBV, CBF & MTT respectively revealed: showed increase vascularity in the site of the lesion.
Final diagnosis: recurrent high grade glioma proved by clinic-radiologic follow up.



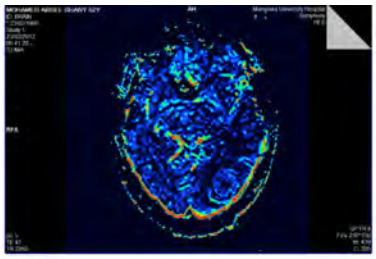
a) T1



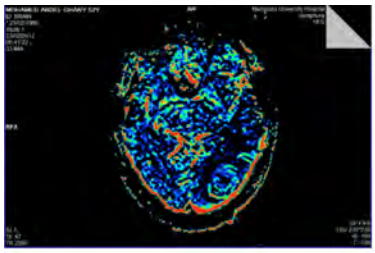
b) T2



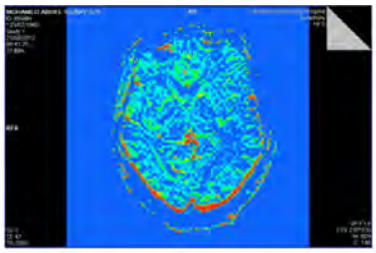
c) T1 PC



d) CBV



e) CBF



f) MTT

Fig.3: Male patient aged 52 years, He had left occipital glioblastoma multiforme (GBM). Surgical excision was done followed by 20 cycles of radiotherapy. Perfusion MRI was done.

(a, b &c): T1WI, T2WI & T1 post contrast images respectively revealed: A well defined intra-axial area of abnormal signal intensity is seen at the left occipital region. It displays low SI on T1WI, high SI on T2WI. It is seen surrounded by mild perifocal vasogenic edema with no significant mass effect. After IV contrast injection it showed mild enhancement.

(d, e & f) CBV, CBF & MTT maps respectively revealed: no increased vascularity ...final diagnosis was post radiation necrosis proved by clinic-radiologic follow up.

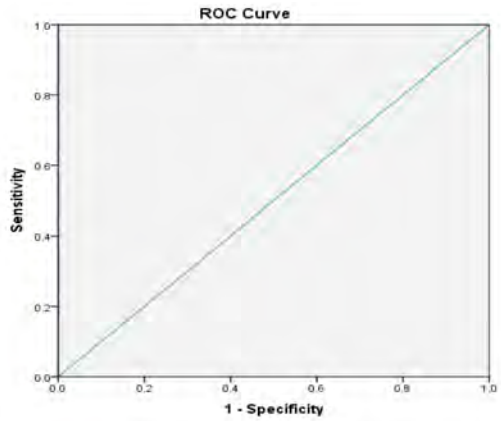


Fig.4: ROC curve.

Discussion

Postoperative radiotherapy in patients with gliomas improves the results of treatment, but it brings some side effects to the brain[4]. Among those, radionecrosis as the end point of radiation injury is the worst. Its development depends on irradiated brain volume, dose of the radiotherapy and concomitant chemotherapy[5].

Although radiological features commonly seen in radionecrosis has been described (location in periventricular white matter, corpus callosum and distant from the site of primary tumor, soap bubbles, Swiss cheese pattern), differential diagnosis from tumor recurrence based on conventional MR is still impossible[6,7]. Contrast-enhancing lesions occur within residual tumor or tumor recurrence and also within radiation injuries with or without necrosis[8]. Both lesions (tumor and radionecrosis) are heterogeneous, mainly hyperintense on T2-weighted images and show strong, often heterogeneeous contrast enhancement with surrounding edema and mass effect. Both entities can increase in size or be stable over serial examinations. It is essential to remember that from clinical point of view the differentiation between tumor recurrence and radionecrosis has a pivotal role, because of the further treatment implications[9]. Diagnosis of a post-

treatment lesion as glioma recurrence rather than radiochemotherapy or immunotherapy treatment effect is critical[10].

In comparison to healthy brain tissue, high-grade gliomas develop an increased macro- and microvasculature. The relative cerebral blood volume (rCBV) is increased, predominantly as a result, and correlates with aggressive tumor growth[3]. Tumor vessels in comparison to normal ones are characterized by increased tortuosity, lack of maturity and increased permeability[1,2]. On the other hand rCBV is decreased in radiation necrosis which mainly consists of ischemic changes because radiotherapy induces endothelial cell damage and small vessel injury[11,12].

rCBV is the most efficient among parametres obtained in PWI, in assessing brain tumors or treatment effectiveness[6,7].

MR perfusion imaging provides only semiquantitative data because of a non-linear relationship between signal and contrast concentration[13]. Nevertheless, our results suggest rCBV enables invivo assessment of microvasculature in lesions after SRS, which provides important information for differential diagnosis for better clinical decisions.

It should be noted that cases of pure tumor recurrence and pure radiation necrosis occur in only a minority of cases. The majority of cases fall within a spectrum containing a mixture of both tumor and necrosis. This adds further complexity to reaching a correct diagnosis[14]. The areas of tumor recurrence are comprised of mixture of neoplastic and necrotic tissue in at least 33% of cases[15]. Because of this overlap of the rCBV between tumor recurrence and radiation injury is predictable. Not only irradiated brain tissue but also irradiated tumor is not the same as before treatment. Normalized rCBV ratios (rCBV[tumor]/rCBV[contralateral tissue]) in the recurrent glioblastoma are significantly lower than those in the initial glioblastoma in the same patient[16].

In the current study we found significant difference in normal-

ized rCBV mean between tumor recurrence/progression (TRP) & post treatment necrosis (PTN) lesions. A cutoff value of 0.9 would have sensitivity and specificity of 100%. These results are close to Hu et al[12] who presented a threshold of 0.71 of rCBV with a sensitivity of 91.7% and a specificity of 100% for the best differential diagnosis.

Other researchers[17-24] reported significant difference between these two groups of lesions but presented different cutoff values from 0.5 - 0.93. So it is postulated that cutoff value is specific to each machine and its software and must be determined individually.

Direct inspection of perfusion maps revealed that rCBV and rCBF maps were most accurate with sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) equals 100%, 80%, 95% & 100% respectively. They were followed by PBP maps also showed sensitivity of 90%, specificity 80%, PPV 95% and NPV 67%.

We had one false positive lesion which showed high vascularity on rCBV and rCBF maps. It could be explained by the fact that within irradiated brain tissue many vessels are occluded but there are aneurysmal formations, teleangiectasias and profliferation of endothelial cells that can lead to high perfusion[22].

Limitations to this study include small number of cases as larger sample size would be recommended for more valuable statistical results specially for the post treatment necrosis group. Post treatment necrosis lesions included in this study were all of small size (1-3cm), while on the other hand most tumor recurrence/ progression lesions were large (3-5 & < 5cm) which may have contributed to overall results. We believe it would be more accurate if same sizes were compared.

Conclusion

PWI seems to be most reliable in differentiation between tumor recurrence and post iradiation necrosis. In these results normalized mean rCBV is the best differentiating factor Inspection of rCBV,

rCBF & PBP maps is more efficient than rTTP & rel MTT in the evaluation of recurrent brain tumor.

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BENHA MEDICAL JOURNAL

ROLE OF MR PERFUSION IMAGING IN: POST IRRADIATION NECROSIS VERSUS BRAIN TUMORS RECURRENCE

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INTERLEUKIN-28B POLYMORPHISM AND RESPONSE TO INTERFERON THERAPY IN PATIENTS WITH CHRONIC HEPATITIS C

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Abstract

Introduction: *Although HCV is endemic worldwide with overall prevalence of 3.3% of the world's population, there is a unique exception in Egypt, with estimated an overall anti-HCV antibody prevalence of 14.7% and the number of Egyptians estimated to be chronically infected was 9.8% up to 50% in certain rural areas. Genetic variation in the interleukin-28B (IL-28B) genes has been associated with the response to pegelated interferon (PEG-INF) and ribavirin (RBV) therapy in patients chronically infected with HCV.* ***Aim of the work:*** *assessing IL-28B single nucleotide polymorphism (SNP) at (rs12979860), as a genetic pretreatment predictor of therapy-induced viral clearance in chronic hepatitis C (CHC) Egyptian patients. Also, studying the possible potential association between IL-28B polymorphism and the other factors that could influence treatment response such as age, gender, severity of fibrosis, body mass index, ALT levels, and HCV RNA load.* ***Patients and Methods:*** *One hundred of CHC patients were selected and submitted to combined INF/RBV therapy, The genotypes of the patients were determined through detection of the SNP in locus rs 12979860 by the use of real time PCR (Rotor gene 6000 system). on purified genomic DNA extracted from all patients before therapy.* ***Results:*** *Sustained virological response (SVR) was achieved in 61% of the studied patients, while the remaining 39% were non responders. The CC genotype of rs12979860 was identified in 28 patients, 25 of them (89.3%) achieved SVR, while the heterozygous CT genotype was detected in 54 patients, 31 of them achieved SVR (57.4%) and the TT genotype was found in 18 patients*

and only 5 of them (27.8%) was responder. SVR was significantly associated with CC genotype as compared to non CC genotype (p<0.001), and CC genotype is independent predictor of response with 65% diagnostic accuracy, specificity (92%), PPV of 89% and 50% NPV. ***Conclusion:*** *Host genetics are useful for the prediction of treatment outcomes in CHC patients. IL-28B SNP (rs12979860) could be an important pretreatment predictor of SVR in CHC Egyptian patients subjected to anti-*

Introduction

Chronic hepatitis C (CHC) infects more than 170 million individuals worldwide, and HCV genotype 4 is the cause of 20% of this number (Khattab et al., 2011). CHC is number one indication for liver transplantation and one of the most common causes of chronic liver disease worldwide (Douglas et al., 2007); Therefore, CHC is comparable to a 'viral time bomb' (Derbala et al., 2006). In Egypt, HCV is currently the most significant public health problem with reported overall anti-HCV antibody prevalence of 14.7% and the number of Egyptians estimated to be chronically infected was 9.8% up to 50% in some rural areas (El-Zanaty and Way, 2009). In countries in which genotype 4 HCV is most commonly found, like Egypt, cost represents a considerable hurdle to patients seeking health care. Many patients are required to fund their own treatment, and failure to complete treatment regimens because of financial constraints is common (Kamal et al., 2008).

Recent biological models for the prediction of response include a variety of host and viral parameters, unfortunately, these models explains only between 40-50% of response outcome, which indicates a strong underlying genetic basis of the response to current therapy (Holmes et al., 2012). An accurate ability to predict response would allow both patients and clinicians to make more informed decisions as regard the risk-benefit of treatment (Mc Hutchison et al., 2009).

IL-28B genes are located on chromosome 19 in humans, and it comes in two isoforms: IL-28A and IL-28B, both isoforms are 96% homologous, and although differences in the functions between the

two forms remains unclear, both plays a role in immune defense against viruses (Kempuraj et al., 2004). IL-28A and IL-28B belong to the type III interferon family of cytokines and are highly similar in amino acid sequence, their classification as interferons is due to their ability to induce an antiviral state, while their additional classification as cytokines is due to their chromosomal location as well as the fact that they are encoded by multiple exons (Sheppard et al., 2003).

IL-28B genotyping among Australian subjects infected with CHC genotypes 1 and 4 who carry CC alleles at rs12979860 (the most favorable genotype) had a greater decline in HCV-RNA within 24 hours after the first injection of PEG INF than did subjects who carried T alleles either TT or CT, this provides more evidence that IL-28B polimorphism (rs12979860) genotype is a strong pre-treatment predictor of SVR (Stattermayer et al., 2011).

Aim of The Work

The aim of the present study is to assess IL-28B SNP at (rs12979860), as a genetic pre-treatment predictor of therapy-induced viral clearance in CHC Egyptian patients. Also, study the possible potential association between IL -28B polymorphism and the other factors that could influence treatment response such as age, gender, severity of fibrosis, body mass index, ALT levels, and HCV RNA load.

Patients and Methods

One hundred Egyptian patients with CHC infection (HBsAg seronegative and anti-HCV seropositive with detectable HCV-RNA) were randomly selected from attendents of the outpatient clinics in Internal Medicine Specialized Hospital, Mansoura University, in the period between 1/8/2011 and 25/7/2013. This is a case control study that conforms to the Medical Sciences Ethics Committee of Mansoura Faculty of Medicine and all the included patients have their written informed consent.

The study included both male and female patients, aged between 18-60 years with documented CHC infection, compensated liver disease (total serum bilirubin <1.5 mg/dL; INR <1.5; serum albumin

>3.5mg/dl, platelet count >75,000 /mm3 and no evidence of hepatic decompensation (hepatic encephalopathy or ascites), acceptable hematological and biochemical indices (Hemoglobin >13 g/dL for men and >12 g/dL for women; neutrophil count >1500 /mm3 and serum creatinine <1.5 mg/dL), Liver biopsy showing chronic hepatitis with significant fibrosis, no contraindications to therapy and all the included patients were willing to be treated and committed to treatment requirements (Ghany et al., 2009).

We excluded patients with advanced cirrhosis (signs of liver cell failure), viral infection other than HCV, those with any other liver disease, autoimmune disease, thyroid disease, major uncontrolled depressive illness, pregnant or unwilling to comply with adequate contraception, severe concurrent medical disease such as severe hypertension, heart failure, significant coronary heart disease, poorly controlled diabetes, chronic obstructive pulmonary disease, and patients less than 18 years (Ghany et al., 2009).

All the studied patients were submitted to the standard of care (SOC) therapy of PEG-INFα2a 180 mcg per week or PEG INFα2b 1.5 mcg/kg per day in combination with RBV1000-1200 mg per day according to body weight for 48 weeks. For subsequent analysis, patients are classified according to response to SOC into two groups; The first group (responders group) includes patients who have shown negative HCV-RNA after 24 weeks following completion of a 48 weeks treatment course. The second group (non-responders) includes null-responders (no disappearance of HCV RNA after the first 12 weeks of therapy), breakthrough (reappearance of HCV RNA after being negative during therapy), and patients who relapse within 24 weeks after the end of treatment.

Following the medical history and clinical examination, pathological examination of the liver biopsies was performed in the Pathology Department, Mansoura Faculty of Medicine by pathologists. Study of IL-28B polymorphism and the other laboratory investigations were conducted in the laboratories of the Clinical Pathology Department, Mansoura Faculty of Medicine.

The following investigations were performed before SOC therapy:

a- Detection of anti-HCV antibodies, hepatitis B virus surface antigen and core antibodies by EIA (COBAS Amplicore, Germany).

b- Detection of HCV-RNA by PCR at 0, 12, 24, 48, and 72 weeks using a commercial kit (Roche Diagnostic, Branchburg, NJ) according to the manufacturer's instructions.

c- Liver function tests were done on Cobas Integra-400 (Roche diagnostics -Germany).

d- Complete blood counts were done on Sysmix KX-21 automatic cell counter (Japan).

e- Detection of ANA, TSH, AFP and IHA for Schistosomiasis.

f- Abdominal ultrasound.

g- Ultrasound guided true-cut needle liver biopsy according to the consensus recommendations of the Asian Pacific Association for the Study of the Liver (APASL) (Shiha et al., 2008). The fibrosis and necro-inflammatory activity were staged according to the METAVIR Scoring System (Bedossa and Poynard, 1996).

h- Ocular fundus examination and electrocardiography for patients above 40 years.

Sample collection and DNA extraction:

By molecular biology technique, Genomic DNA is isolated from 2 ml of peripheral blood mixed with EDTA, treated whole blood by sodium dodecyl sulfate lysis and proteinase K digestion, extraction with saturated sodium chloride solution and chloroform, and precipitation with ice-cold absolute ethanol. The DNA was washed twice in 70% ethanol and dried at 37 °C before being resuspended in sterile water. Genomic DNA was extracted by means of the QIAamp genomic DNA blood extraction mini kit (Qiagen, Milan, Italy) according to the manufacturer's instructions.

Genotyping for the IL-28B rs12979860 Polymorphism:

The genotypes of the patients were determined through detection of the SNP in locus rs 12979860 by the use of real time PCR (Rotor gene 6000 system). The real time PCR included a two-step PCR (15 s at 95°C; 60 s at 60°C) on an ABI 7000 instrument using primers rs12979860_F,GTGCCTGTCGTGT

ACTGAACCA and rs12979860_R, AGCGCGGAGTGCAATTCA and the Taqman MGB-probes rs12979860-C_P, FAM-CCTGGTTCGCGCCTT-MGB and rs12979860-T_P, VIC-CCTGGTTCACGCCT-MGB. Allelic discrimination was obtained by post-PCR read of fluorescence intensity from each of the fluorophores.

Statistical analysis:

Statistical package for SPSS (version 18.0, Echosoft Corp., USA, 2008) was used for data analysis. Quantitative variables were summarized using Mean ± SD and both number and percentage for categorized data. Il-28B will be determined by counting. Odds ratios (ORs) will be estimated according to the Woolf formula. The level of significance will be assessed in univariate analysis by using the x2 or Fisher's exact test for comparisons of qualitative values or the unpaired Student's t test for quantitative values. P≤0.05 was considered to be significant, and the Bonferroni correction will be applied for multiple tests by multiplying the P value by the number of alleles compared (corrected P value). Differences in the means of continuous variables will be assessed by the Student's paired t test. The Mann-Whitney U test will be used to compare non-parametric variables in independent samples. Multivariate analysis will be done by logistic regression. Adjusted ORs and 95% confidence intervals were derived from the coefficient of the final multivariate logistic model.

Results

The included patients have age range from 18 to 60 years; 42% of patients >40 years and 58% are ≤40 years. Females represented 27%, of which 16 patients aged >40 years and 11patients are are ≤40 years; meanwhile males represented 73%, of which 26 patients >40 years and 47 are are ≤40 years. Body mass index (BMI) of the included patients ranged from 21 to 29 kg/m^2, 46 patients have BMI >25 kg/m^2, while 54 patients has BMI are ≤25 kg/m^2.

Pretreatment ALT ranged from 18 to 188 IU/L, 22% of the studied patients have normal ALT (≤30 IU/L for males and ≤19 IU/L for females), and 78% have elevated ALT.

fasting blood glucose (FBG) in the studied patients ranged from 67 to160 mg/dl, 39% of patients have FBG are >100 mg/dl and 61% are are ≤100 mg/dl. 44% of the studied patients have low viremia (≤600,000 IU/ml) and 56% with high viremia (>600,000 IU/ml). 55% of the studied patients have F1, 29% have F2, and 16% have F3 according to Metavir scoring system for liver fibrosis.

There are significantly lower ages and BMI in responders compared to non responders ($p<0.0001$, <0.0001 respectively). There is a significant decrease in FBG levels in responders group compared to non responders ($p<0.0001$). There are insignificant difference between responders and non responders as regards gender, pretreatment ALT, the presence of bilharzial co-infection, and grade of activity ($p>0.05$). There is significantly higher reporting of low viremia (≤600000 IU/ml) and early fibrosis stages (F1) among responders compared to non responders ($p<0.0001$ and $p<0.0001$ respectively). There is significantly lower reporting of fibrosis stages F2 and F3 among responders compared to non responders ($p<0.01$and $p<0.001$ respectively). There is a highly significant difference between responders and non responders as regards IL-28B polymorphism genotypes distribution (CC, CT, and TT) with $p<0.0001$, $p<0.001$, and $p<0.0001$ respectively. There is a highly significant difference in early virological response (EVR) and end of therapy (EOT) between responders and non responders ($p<0.0001$ and <0.001 respectively), Table (1).

There are a significantly lower age, FBG in CC genotype patients compared to non CC genotype ($p<0.04$, $p<0.002$ respectively). In CC genotype, high viremia (>600000 IU/ml) is significantly lower than non CC genotype ($p<0.021$). There is a significantly difference in pretreatment fibrosis stages between CC genotype and non CC genotype, there is a significantly higher reporting of F1stage in CC genotype compared to non CC genotype ($p<0.041$), with significantly lower F2 and F3 in CC genotype compared to non CC genotype ($p<0.001$ and $p<0.021$). There is a highly significant differ-

ence between CC genotype and non CC genotype as regards EOT (p<0.003), SVR (p<0.001), and relapsers (p<0.001), table (2).

There is a significantly higher EVR, EOT, and SVR, in CC genotype compared to CT and TT genotypes (p<0.015, p<0.0001, and p<0.0001 respectively), and significantly higher non response in TT genotype compared to CC and CT genotypes (p<0.0001), table (3).

The predictive odds ratio for age ≤ 40 years versus >40 years was 2.32 (P<0.0001), BMI≤ 25 kg/m2 versus >25 kg/m2 was 2.78 (P<0.0001), pretreatment viral load ≤600,000IU/ml versus >600,000IU/ml was 7.95 (P<0.0001), FBG ≤100 mg/dl versus >100 mg/dl was 2.56 (P<0.0001), IL-B28 CC genotype versus non CC genotype was 8.33(P<0.001), fibrosis score F0-2 versus F3 was 3.75 (P<0.0001), table (4) and figure (1).

There is a highly significant negative correlation between SVR and age with (r=-0.488) (P<0.0001), BMI with (r=-0.700) (P<0.0001), FBG with (r= -0.602) (P<0.0001), Fibrosis stage with (r=-0.747) (P<0.0001), IL-B28 genotype with (r=-0.425) (P<0.0001), and pre treatment viral load with (r=-0.778) (P<0.0001). There is no significant correlation between SVR and gender (r=0.114) (p>0.05), table (5).

CC genotype is independent predictor of response; CC has high specificity (92%) with PPV of 89%, 65% accuracy, and 50% NPV. BMI carries the highest sensitivity (98%) and accuracy (92%) compared to the other parameters. followed by pretreatment viral load (87%) and fibrosis stage (85%). Pre treatment viral load has the highest PPV (95%), specificity (92%) and high sensitivity (87%). Fibrosis stage has high PPV (92%), accuracy (87%), specificity (87%), and 85% sensitivity. Patient's age has 38% accuracy, 87% PPV, 82% specificity, and 84% sensitivity, table (6).

CC genotype is dependant predictor and the highest accuracy is obtained when combining CC genotype with age ≤40 years or combining CC genotype with low fibrosis stage (F1-F2). CC genotype with age ≤40 years offers 100% specificity and 100% PPV with 92% sensitivity, table (7).

Table (1): Comparison between responders and non responders in the studied patients:

Variable	Responders (n=61)	Non-responders (n=39)	p-value
Demographic and history data:			
-Age (years)*	35.6±7.65	44.04±7.12	**0.0001**
-Gender:			
Male	47	26	0.363
female	14	13	0.152
Clinico-Laboratory data:			
-BMI (kg/m²)*	24.38±1.3	27±1.69	**0.0001**
-FBG (mg/dl)*	85.43±15.89	99.13±19.79	**0.0001**
-ALT:			
Normal (≤30 IU/L)	17	5	0.127
Elevated (>30 IU/L)	44	34	0.116
-Pre-treatment viral load:			
Low (≤600000 IU/ml)	53	3	**0.0001**
High (>600000 IU/ml)	8	36	**0.0001**
-HCV & Bilharziasis:			
Co-infection	20	20	0.103
Non-Bilharzial	41	19	0.320
-IL28B polymorphism:			
CC	25	3	**0.0001**
CT	31	23	**0.001**
TT	5	13	**0.0001**
Histopathological data:			
-Fibrosis stage			
F1	52	3	**0.0001**
F2	8	21	**0.01**
F3	1	15	**0.001**
Activity:			
A1	35	30	0.104
A2	10	14	0.125
A3	4	7	0.164
EVR	85/100	15/100	**0.0001**
EOT	69/28	31/100	**0.001**

(*) values expressed as (Mean±SD), difference tested with independent sample t-test, Other variables tested with Chi square statistics P<0.05 significant P>0.05 non significant BMI: body mass index, FBG: fasting blood glucose, ALT: alanine aminotransferase, IL-28B: Interleukin -28. EVR: early virologic response, EOT: end of therapy.

Table (2): Comparing CC genotype and non CC genotype (CT and TT) in the studied patients:

	rs 12979860 genotype		p-value
	CC genotype (n=28)	Non-CC genotype (n=72)	
Demographic and history data:			
Age (years)*	36.1±8.84	39.99±8.16	**0.04**
Laboratory Data:			
FBG (mg/dl)*	88.64±12.67	99.18±15.96	**0.002**
Baseline viral load			
Low (≤600000 IU/ml)	21	35	**0.031**
High (>600000 IU/ml)	7	37	**0.021**
Histopathological data:			
Fibrosis score (METAVIR)			
F1	21(75%)	34 (47.2%)	**0.041**
F2	4 (14.3%)	25 (34.7%)	**0.001**
F3	3(10.7%)	13(18%)	**0.021**
Response:			
EVR	26/28 (92.8%)	59/72 (81.9%)	0.289
EOT	26/28 (92.8%)	43/72 (59.7%)	**0.003**
SVR	25/28 (89.3%)	36/72 (50%)	**0.001**
Relapsers	1 (3.6%)	7 (9.7%)	**0.001**

(*) values expressed as (Mean±SD), difference tested with independent sample t-test, other variables tested using Chi square statistics, P<0.05 significant, P>0.05 non significant. FBG: fasting blood glucose, EVR: early virologic response, EOT: end of therapy, SVR: sustained virologic response.

Table (3): Comparing of response of treatment between IL-28B genotypes in the studied patients:

	rs 12979860 genotype			p-value
	CC genotype (n=28)	CT genotype (n=54)	TT genotype (n=18)	
EVR	26/28 (92.8%)	48/54 (88.9%)	11/18 (61.1%)	**0.015**
EOT	26/28 (92.8%)	38/54 (70.4%)	5/18 (27.8%)	**0.0001**
SVR	25/28 (89.3%)	31/54 (57.4%)	5/18 (27.8%)	**0.0001**
Non responders	3/28 (10.7%)	23/54 (42.6%)	13/18 (72.2%)	**0.0001**

P<0.05 significant, P>0.05 non significant. EVR: early virologic response, EOT: end of therapy, SVR: sustained virologic response.

Table (4): Pretreatment patient and viral predictors of SVR:

	Odds ratio	95% CI	p-value
Age (≤ 40 years vs. >40 years)	2.32	1-5.8	**0.0001**
BMI (≤25 kg/m² vs. >25 kg/m²)	2.78	1.8-5.9	**0.0001**
Pre treatment Viral load (≤600,000 IU /ml vs. >600000 IU/ml)	7.95	6.8-10	**0.0001**
Fasting blood sugar (≤100 mg/dl vs. >100 mg/dl)	2.56	1.5-4.2	**0.0001**
IL B-28 genotype (CC vs. non CC)	8.33	5.9-9.8	**0.001**
Fibrosis stage (F0-F2 vs. F3)	3.75	2.8-5.4	**0.0001**

Table (5): Correlation between SVR and different patient and viral factors:

	Age	BMI	FBG	Gender	Fibrosis stage	IL –28 B genotype	Pre treatment Viral load
Correlation coefficient (r)	-0.488	-0.700	-0.602	0.114	-0.747	-0.425	-0.778
p-value	0.0001	0.0001	0.0001	0.285	0.0001	0.0001	0.0001

BMI: body mass index, FBG: fasting blood glucose, IL: interleukin. P<0.05 significant P>0.05 non significant. Fibrosis stage (coded as 0, 1, and 2 for F1, F2, and F3 respectively), pretreatment viral load (coded as 0 and 1 for low viremia and high viremia respectively), IL-28B genotype (coded as 0, 1, and 2 for CC, CT, and TT respectively).

Table (6): Performance characteristics of pretreatment predictors of SVR:

	Sensitivity	Specificity	PPV	NPV	Accuracy
CC- genotype	41%	92%	89%	50%	65%
TT-genotype	33%	92%	72%	68%	69%
Age	84%	82%	87%	76%	83%
BMI	98%	82%	90%	97%	92%
Fibrosis stage	85%	87%	92%	80%	87%
Viral load	87%	92%	95%	81%	89%

BMI: body mass index, PPV: positive predictive value, NPV: negative predictive value.

Table (7): Performance characteristics of CC genotype combined with other significant pretreatment predictors of SVR:

	Sensitivity	Specificity	PPV	NPV	Accuracy
CC genotype	41%	92%	89%	50%	65%
CC- genotype *and* Age ≤40 years	92%	100%	100%	60%	93%
CC- genotype *and* BMI ≤25 kg/m²	84%	67%	95%	33%	82%
CC- genotype *and* METAVIR (F1-F2)	96%	67%	96%	67%	93%
CC- genotype *and* Viral load ≤600,000 IU/ml	80%	67%	95%	29%	79%

BMI: body mass index, PPV: positive predictive value, NPV: negative predictive value.

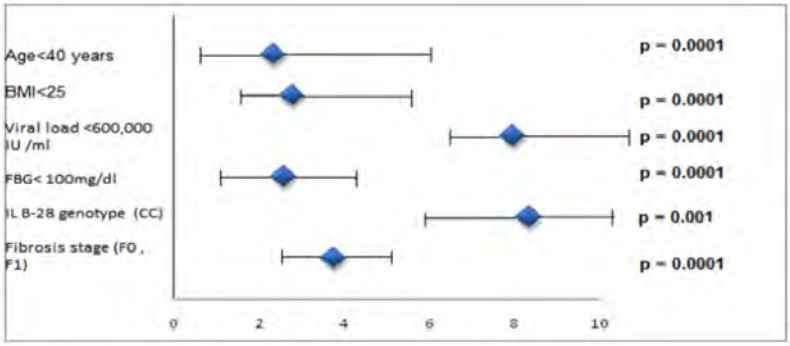

Fig. 1: Relative predictivity of patients and viral characteristics for SVR.

Discussion

In the current study, the responders have a significantly lower BMI compared to non responders group (p<0.0001), table (1). A finding that is in accordance with Bressler et al. (2003) who reported that BMI was inversely correlated with SVR, and with Berg et al. (2006) who showed that a lower pretreatment BMI was significantly associated with achieving a higher SVR across all genotypes subjected to SOC therapy. Obesity and high BMI are well established predictors of disease progression in patients with CHC; this is logic, because a higher BMI is associated with more hepatic steatosis. Moreover, CHC frequently cause hepatic steatosis which augment more liver damage and subse-

quently lower SVR rates. This conclusion is in concordance with Esmat et al. (2009) who reported that steatosis appears to be related to high BMI, rather than viral load or the degree of liver injury, and steatosis is common in patients with genotype 4 HCV and significantly improves after HCV eradication. Also, De Careaga (2006) reported that obesity reduces treatment efficacy and hepatic steatosis increases the necro-inflammatory process of HCV and accelerates the development of fibrosis. These published data encouraged Stern et al. (2010) to classify obese patients with CHC as hard-to-treat with high failure to achieve SVR and a greater likelihood of relapse after treatment, and Khattab et al. in 2011 to consider BMI as one of the predictors for non alcoholic steatohepatitis in HCV-4 patients.

In the present study, responders were found to have lower level of pretreatment viremia (≤600,000 IU/ml) than non responders ($p<0.0001$), table (1). This is in agreement with the results of Ghany et al. (2009) who reported low pretreatment RNA viral load as a predictor of SVR. On the other hand, a high pretreatment HCV RNA level is considered as a negative predictive factor for treatment outcome (Hasan et al., 2004; Kamal et al., 2005). In our study, lower pretreatment HCV viral load in responders than non responders could be considered as a new cumulative data added to the previously well established knowledge supporting the consideration of this parameter to be good prognostic indicator for better response outcome.

In our study, a significantly higher number of patients with early fibrosis stages (F1, F2) were found in responders than non responders ($p<0.0001$), table (1). A finding that agree with Gad et al. (2008) who reported that significant fibrosis (> F2 Metavir) is negatively associated with SVR, and those patients with advanced fibrosis are less likely to achieve SVR. Fibrosis is suggested to be one of the independent predictive factors associated with therapy-induced viral eradication in genotype-4 CHC patients, these data were further proved by Ghany et al. (2009), Thompson et al. (2010),

and Poordad et al. (2011). Still the debate about the necessicity of liver biopsy in the pretreatment requirements of antiviral therapy of patients with HCV an unresolved issue. During conduction of this study liver biopsy is planned for all the included patients aiming to get valuable information about both the necro-inflammation and stage of fibrosis which is considered as gold standard tool in many countries including Egypt. Fortunately, patients with advanced fibrosis had lower SVR despite non significant value of necro-inflammation parameter. Of interest here is to mention the new-advancements of many non-invasive tools and different algorithms in a trial to surrogate liver biopsy, but none of them could achieve the diagnostic accuracy of liver biopsy, so, they can be considered as complementary rather than surrogate for liver biopsy.

During conduction of this study, patients who achieved SVR expressed significantly higher rates of EVR ($p<0.0001$), and EOT ($p<0.001$) than non responders, table (1). These findings were parallel to the results of Berg et al. (2003) who stated that failure to achieve EVR predicts treatment failure and could be considered as indication to cease therapy. Indeed, it was stated that 0-3% of patients with a decline of less than 2 log HCV-RNA IU/ml (partial responders) at week 12 have the chance of achieving SVR, and this has led to the implementation of a stopping rule for patients without EVR irrespective of genotype (Davis et al., 2003). In more recent studies, patients with a complete EVR achieved higher SVR rates (68-84%) with SOC therapy, unlike patients with a partial EVR who achieved SVR of only 17-29% (Fried et al., 2008; Ghany et al. 2009). Although, EVR is used to be the mainstay of HCV on-treatment decision making. These datum should be revised and each patient should be individualized and dealt with as case by case, evidenced by the frequent studies that proved encouraging SVR response in partial responders. Patients lacking good adherence and compliance with treatment in the first 12 weeks of therapy, particularly those with either dose reduction or transient stoppage of treatment should be

searched for and are candidates for completion of treatment as some may achieve negativity of HCV-PCR by the week 24, and in such situation, extended courses of treatment to 72 weeks were recommended.

In our study, IL-28B polymorphism distribution among our studied CHC Egyptian patients was 28% for CC genotype, 54% for CT genotype, and 18% for TT genotype. Many previous studies have addressed IL28-B distribution in HCV patients; Olfat et al. (2011) reported the frequency of IL-28B polymorphism at rs12979860 in the studied Egyptian CHC patients, and found that CC genotype was present in 39%, CT genotype 51%, and TT 10%. And, Mangia et al. (2011) who found the frequency of IL28B CC genotype in 29%, CT in 53%, and TT in 18% of their studied patients.

Studying the ILB-28 polymorphism distribution globally among our patients proved CC genotype in little more than one fourth of our patients, while CT genotype represented 54% of the studied patients. The a high frequency of heterozygous carriers CT (rs12979860) among CHC Egyptian patients could provides a vague predictive results and will not be useful in more than half of the patients, which by turn makes a major difficulty in decision-making at the start of treatment; therefore, the search for additional predictive factors for response is mandatory either by studying another loci on the IL-28B gene or adding one or more of the established predictive parameters targeting optimum decision making.

In our study, there was a significant difference in the distribution of IL-28B genotypes CC, CT, and TT between responders and non responders group ($p<0.0001$, $p<0.001$, and $p<0.0001$ respectively), table (1). Nearly, 92.8% of CC genotype achieved EVR in comparison to 81.9% in non CC genotype ($p<0.289$). By the end of therapy, 92.8% of CC genotype were still maintaining viral clearance compared to 59.7% in non CC genotype ($p<0.003$). 89.3% of CC genotype achieved SVR compared to 50% in non CC genotype ($p<0.001$). Non CC genotype expressed nearly three folds increase

in relapsers than CC genotype ($p<0.001$), table (2).

In this study, TT genotype has lower rates of EVR compared to CC and CT genotypes (61.1%, 92.8, and 88.9% respectively) ($p<0.015$), and lower rates of EOT compared to CC and CT genotypes (27.7%, 92.8%, and 70.4% respectively) ($p<0.0001$), and the highest percentage of non response which was 72.2% compared to in CC and CT genotypes (10.7% and 42.6% respectively) ($p<0.0001$), meanwhile, CT genotype has a midway percentages of EVR, EOT, and SVR compared to the most favorable CC genotype and the least favorable TT genotype, table (3).

These results agree with those of Asselah et al. (2012) who showed that the IL-28B rs12979860 CC genotype was associated with a better treatment response; the SVR was 81.8%, 46.5%, and 29.4% for genotypes CC, CT, and TT respectively. Similarly, Chevaliez and Hezode, (2010) reported that, CC genotype was associated with a two-fold greater SVR rate than the TT genotype, CT being closer to TT than to CC. Furthermore, Bitetto et al. (2010) considered that CC pattern have higher rates of RVR and SVR, people with the homozygous TT pattern have the least favorable response, while those with the heterozygous or mixed CT pattern fall in between. Chen et al. (2012) concluded that IL-28B rs12979860 CC is strong SVR predictor regardless of ethnicity. More recently, Rizk and Derbala (2013) suggested that patients with the C allele of rs12979860 exhibited an approximately eight folds higher chance of achieving SVR compared to patients with the T allele.

Derbala et al. (2013) stated that the CC genotype of rs12979860 was one of the most frequent genotypes among the responders at 12, 48 and 72 weeks of therapy, the SVR was acheived in 46.9%, 45.9% and 7.2% for CC, CT, and TT genotype respectively and multinomial logistic regression analysis revealed that the viral load and rs12979860 are the only significant factors involved in the efficacy of the treatment response. In 2012, Holmes et al. considered IL-28B genotype to be the strongest pretreatment predictor of SVR in

CHC patients receiving SOC. Several global studies revealed that the CC genotype is the major player in drug induced viral clearance and those patients are more likely to respond to current SOC, while both the CT and TT genotypes have a poor association with clearance rates (Ge et al., 2009; Fabris et al., 2011). Recent study conducted by El Awady et al. (2013) showed that 76% of CHC Egyptian patients carrying CC allele had a SVR while the genotypes CT and TT were associated with lower SVR rates, 50% and 48%, respectively. Therefore, concluded that IL-28B rs12979860 may have strong predictive value for the outcome of IFN-based therapy in the difficult to treat HCV genotype 4 patients, with the hope that IL-28B rs12979860 will improve future decision-making in the management of CHC in Egypt.

In this study, 3.5% of CC genotype patients relapsed within 24 weeks after the end of therapy compared to 9.7% in non CC patients ($p< 0.001$), table (2). Our results are similar to Thompson et al. (2010) who stated that CC genotype was associated with decreasing the rate of post treatment relapse. Given the strong association we observed between IL-28B genotype and breakthrough/ relapse, it is quite plausible that IL-28B genotype is associated with response to a PEG INF-α/ RBV regimen. Therefore, an IL-28B genotype-based model may identify patients who are at high risk for treatment failure (and selection of resistant HCV strains) when treated with this regimen. Furthermore, the results of our study could be valuable in furthering our understanding of the explanation for the higher chance of SVR which could be attributed to enhancement of early viral kinetics, increasing the rates of EVR and EOT, and decreasing the rate of post-treatment relapse.

In the current study, CC genotype reported a higher rates of F1 compared to non CC genotype (75% and 47.2% respectively) ($p<0.041$), with significantly lower reporting of F2 and F3 compared to non CC genotype ($p<0.001$ and $p<0.021$ respectively), table (2). This result agree Fabris et al. (2011) who reported that in patients with CHC infections, TT

genotype occurred more frequently in those patients affected by end stage liver disease, and both CT and TT allele is more prevalent in patients with cirrhosis due to HCV. Also according to Bitetto et al. (2010), IL-28B polymorphism at (rs12979860) appears to influence the natural history of CHC as carrying the TT genotype favors the evolution of the disease until the development of HCC. A recent Egyptian study found entire absence of the protective CC genotype in chronic HCV patients with end stage liver disease, leading to complete inversion of the C:T ratio to become 40%:60% instead of 67%:33% found in healthy subject and 50%:60% in CHC patients (El-Awady et al., 2012). Moreover, Rizk and Derbala (2013) found in a more recent study that patients with the T allele exhibited an approximately 13 times higher risk of severe fibrosis compared to patients with the C allele. While, this result disagree with Asselah et al. (2012) who studied IL-28B polymorphisms relation to the severity of liver disease and failed to find a significant relationship between rs12979860 and the severity of disease.

The findings in our study may raise the possibility that patients with CC genotype may be naturally protected and have a slower rate of fibrosis progression compared to non CC genotype which has nearly two folds higher tendency towards fibrosis progression at all stages of disease progression, and this means that patients with CT and TT alleles are more likely to have a higher fibrosis scores than CC genotype and advance more rapidly with aggressive course towards cirrhosis and end stage liver disease.

In our study, we performed a multivariate logistic regression analysis to detect relative predictivity of different host and viral characteristics of SVR and found that low pretreatment viral load ≤600,000 IU/ml is a viral predictor of SVR ($p<0.0001$); whereas host predictors of SVR included, age ≤40 years ($p<0.0001$), BMI ≤25 kg/m2 ($p<0.0001$), FBG ≤100mg/dl ($p<0.0001$), early fibrosis stage (F1, F2) ($p<0.0001$), and favorable IL-28B CC genotype ($p<0.001$), table (4) and figure (1). Although advanced fibrosis stage has been strongly associated with a lower

rate of SVR, IL-28B genotype has a higher predictive odds ratio (8.33) than fibrosis stage (3.75), and our results are nearly similar to that of lin et al., (2011) suggested that CC genotype was the only critical predictor of SVR. And Lai and Afdhal (2012) concluded that IL-28B CC genotype was the stronger pretreatment predictor than all the other significant pretreatment predictors of response (viral load, ethnic background, fibrosis stage, and FBG).

These results were confirmed more when a correlation study was performed between these predictors and SVR, and our results proved that there was a strong significant negative correlation between SVR and age ($r=-0.488$, $p<0.0001$), BMI ($r=-0.700$, $p<0.0001$), FBG ($r=-0.602$, $p<0.0001$), fibrosis stage ($r=-0.747$, $p<0.0001$), pretreatment viral load ($r=-0.778$, $p<0.0001$), and IL-28B genotype ($r=-0.425$, $p<0.0001$), while gender showed no significant correlation with SVR ($r = 114$, $p<0.285$), table (5). These results are in accordance with several previous studies that considered rs12979860 CC genotype, lower age, lower BMI, lower viremia, and early fibrosis stages are associated with higher SVR; while, age >40 years, advanced stage of liver fibrosis, higher BMI, high viral load, and carrying CT or TT genotypes have been negatively reported to affect likelihood of SVR (Barbara, 2009; Sarrazin et al., 2011; Lai and Afdhal, 2012).

Study of the performance characteristics of pretreatment predictors of SVR revealed that CC genotype is an independent predictor of higher SVR with diagnostic accuracy of 65%, 41% sensitivity, 92% specificity, 89% PPV, and 50% NPV, table (6), this result agree Mangia et al. (2011) who found that CC type was independently associated with higher SVR and with Lagginget al. (2011) who reported 32% sensitivity, 81% specificity, 66% PPV, and 52% NPV for CC genotyping; and with Thompson et al. (2010) who reported 35% sensitivity, 91% specificity; Mangia et al. (2009) who reported 40% sensitivity, 83% specificity, 82% PPV, and 29% NPV.

It is not astonishing during conduction of this study to find that IL-28B polymorphism

(rs12979860) is strong predictor of SVR and comparable with all other previously established baseline host and viral variables, and this knowledge could changes the "cost /benefit" of treatment and may help to encourage patients to commence treatment and to reassure them during a prolonged and occasionally difficult treatment course. Patients with unfavorable responder genotype (CT, TT) could probably be categorized as "difficult to treat" as little number of those patients offered response. For those patients, it may be prudent to defer treatment until future regimens are available. These data encouraged some to recommend pretreatment identification of IL-28B genotypes in patients infected with relatively difficult-to-treat genotype 1 or 4 HCV (Jia et al. 2012).

In our study, addition of each of the other well-known pretreatment predictors of SVR to IL-28B genotyping significantly improved its accuracy in predicting SVR. High accuracy (93%), with the highest specificity (100%) and the highest PPV (100%) were obtained in patients with favorable CC genotype and age less than 40 years. Also, high accuracy (93%) with the highest sensitivity (96%) was obtained in CC genotype patients with low fibrosis score, table (7). This finding is in agree with Bitetto et al., (2011) who studied the biological models for the prediction of SVR should include a variety of parameters besides IL-28B genotyping such as viral load, fibrosis stage, BMI, and age. This would offer better accuracy compared with IL28B typing alone. Furthermore, Paul et al. (2011) considered advanced fibrosis as a negative treatment prognostic criterion besides unfavorable IL-28B genotype.

A goal of genomic research is to yield information that leads to treatment decisions based on a patient's genetic makeup. Personalized clinical decision-making for treatment of patients with CHC requires estimates of the probability that a patient will achieve SVR which consider not only IL-28B genotype, but also other factors that are associated with treatment response. Genetic variation in IL-28B in addition to other host- and viral-related factors was associat-

ed with high SVR after SOC treatment for CHC. Thus, a clinical prediction model based on IL-28B genotype adjuncted to the other clinical variables can yield useful individualized predictions of the probability of treatment success which could be positively reflected on the outcome of SOC therapy by increasing SVR rates and decreasing the frequency of futile treatment among patients with CHC.

In conclusion: Our study could provide an additive critical evidence for usefulness of application of genetic data in clinical settings for predicting the treatment response in chronic hepatitis C patients. In Egyptians, patients carrying unfavorable IL-28B genotypes (non-CC) could be identified for waiting the directly acting antivirals. However, sustained virological response of HCV infection is determined by multiple genetic loci. However, the practical utility of genetic data in treatment choice remains to be completely elucidated for IFN-based therapy.

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INTERLEUKIN-28B POLYMORPHISM AND RESPONSE TO INTERFERON THERAPY IN PATIENTS WITH CHRONIC HEPATITIS C

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SAFETY AND EFFICACY OF ENDOSCOPIC SKULL BASE SURGERYIN RESECTION OF EXTRADURAL ANTERIOR SKULL BASE TUMORS

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Abstract

Objective: *To evaluate the role of endoscopic endonasal approaches in surgical management of extradural anterior skull base tumors as regard the extent of anatomic exposure, feasibility, efficacy and complications of these approaches will be ascertained.*

Materials & Methods: *Twenty patients with anterior skull base tumors was extradural location were treated through endoscopic endonasal approaches. Patients were preoperatively assessed through MRI and CT scan and postoperative radiology was compared with preoperative one and the complication and clinical outcome is monitored and analyzed.*

Results: *The extent of resection included "GTR" in 16 cases (80% of total cases), while subtotal resection "STR" were in 4 cases (20% of total cases). The postoperative CSF Leakage (10%) which is the main complication in this study.*

Conclusions: *A completely endoscopic endonasal resection of the extradural skull base tumors can be safely performed for benign and malignant tumors. The endoscope provides superior visualization and allows for more precise dissection. Oncologic principles are preserved with en bloc excision of involved skull base structures. Early oncologic results are promising but continued experience is needed. There are many potential benefits for patients with endonasal surgery.*

Keywords: *Endoscopic skull base, anterior skull base tumors, safety, efficacy.*

Introduction

The base of the skull is one of the most fascinating and complex anatomical areas from the anatomical and surgical perspectives. It can be involved in a variety of lesions[1]. The skull base regions are composed of complex tissue structures that give rise to histogenetically and biologically heterogeneous neoplasms of ectodermal, endodermal, and mesodermal origins. Accurate diagnosis and understanding of the clinical and pathologic presentations of the varied tumor entities in this region are essential for proper management.

Traditional approaches to the skull base tumors have included complex transcranial or transfacial operations. These procedures, facilitated by progress in the designs of surgical instruments and advances in perioperative intensive care, have afforded excellent exposure, allowing for complete removal of massive tumors. However, these open procedures have also been associated with significant morbidity and long term convalescence; the burden on the patient has been great[2]. As a result, the evolution of skull base surgery over the past decade has been characterized by an emphasis on the development of minimally invasive techniques that do not compromise surgical outcomes but do significantly diminish the perioperative burden on the patient.

Skull base surgery, like other subspecialties, has evolved to include minimally invasive procedures, such as endoscopic endonasal skull base surgery[3]. As with other evolutions in surgery, acceptance of a new procedure requires proof of its feasibility, safety, and efficacy[4].

"Pure" endoscopic endonasal transsphenoidal surgery has been described in detail by Jho et al.[5]. Extended transsphenoidal approach was originally described by Weiss in 1987. Extended approaches are essential for reaching the area from lamina cribrosa to the cranio cervical junction[6].

Materials and Methods

This is a prospective study which was conducted on twenty patients diagnosed with anterior

skull base tumors in extradural location; they were managed through endoscopic endonasal approaches at the Neurosurgical and/or Otorhinolaryngology Departments, Mansoura University Hospitals during period between March 2011 and September 2013.

Patients were selected for the study based on inclusion criteria including: skull base tumors extradurally located. Or intradural skull base tumors with extradural extension (invasive pituitary adenomas). While, excluded patients were those with medical co-morbidities making them anesthetically unfit for surgery and those with Intradural skull base tumors without extradural extension or invasion.

Preoperative evaluation:

All patients who are selected for the study were subjected to the following preoperative assessment protocol:

• **Informed consent** is obtained from all patients in our study.

• **Rigorous history taking** form the patient or his attendants.

• **Clinical assessment:** Typically done through General physical examination and full neurological examination, E.N.T. and ophthalmologic evaluation and endocrinological evaluation through pituitary hormonal assay.

• **Neuroradiological evaluation** was done through 1.5-Tesla contrast-enhanced magnetic resonance imaging MRI of the brain and skull base and paranasal sinuses and computerized tomography (CT) scan of the paranasal sinus and brain in a coronal and axial cuts.

Procedures and Operative technique:

Surgeons: The operations were performed by a team of neurosurgeons and otolaryngologists of neurosurgery and otorhinolaryngology departments of Mansoura University.

Equipment: The instruments used to execute the fully endoscopic endonasal approach successfully include an endoscopic tower (Karl Storz Endoscopy, Tuttlingen, Germany), containing a high definition digital camera, a xenon or halogen light source, 0°, 30° and 45° rigid endoscopes 4

mm in diameter.

Approaches: two categories of EEA were used, either standard approach or extended approaches selected according to the location of the tumor.

Standard EEA was used in two patients (10% of total) with invasive pituitary adenoma: two patients without lateral extension nor invasion of the pterygopalatine and/or infratemporal fossae.

Extended endoscopic endonasal approaches were used in 18 patients: Transethmoidal approach was used in large sinonasal tumours (with involvement of the cribriform plate/ethmoid roof). In four patients of olfactory neuroblastoma (20% of total). Transmaxillary Transpterygoid approach was used in tumours with exclusive involvement of the pterygopalatine and/or infratemporal fossae. In seven patients (35% of total). Three patients of juvenile nasopharyngeal angiofibroma, two patients of fibrous dysplasia, one with adenoid cystic carcinoma and one patient with left trigeminal nerve schwannoma. Transclival approach in seven patients (35% of total). Four clival chordoma, one clival chondromsarcoma, one clival fibroma, one clival histocytosis X.

Outcome assessment:

Evaluation of the outcome of endoscopic endonasal surgery in all patients is based on:

• **Intraoperative record** including the approach used operative time in minutes. Intraoperative complication such as; bleeding (severity and quantity in cc).

• **Clinical assessment** by comparing both preoperative and postoperative neurological status, endocrinological and ophthalmologic conditions and complications related to the surgical approach especially CSF leakage, pneumocephalus, meningitis.

• **Extent of resection** is categorized into gross total resection, subtotal resection and partial resection and was evaluated on the obtained CT scan and/or MRI 3 months postoperative.

• **Endoscopic follow up control was done at** 3 weeks postoperative.

Results

Demographic Data/Clinical Presentation:

Twenty consecutive patients underwent endoscopic skull base surgery of anterior skull base tumors. The age of patients ranged from 8 to 61 years (mean±SD) 36.07±16.51 years. 70% of the patients were males (14 patients) and 30% were female (6 patients). Headache was the most common presenting symptom (66.6%), followed by nasal airway obstruction (50%), epistaxis (40%), cranial nerve dysfunction (30%). Visual disturbance constitutes (26.6%). Endocraniologically symptoms (13.3%) as shown in fig. (1).

Tumor Characteristics:

There were 11 malignant and 9 benign lesions. Olfactory neuroblastoma was the most common pathology, occurring in 4 patients (20%). Followed by invasive pituitary adenoma 3 patients and chordoma 3 patients. All patients with malignant disease, received postoperative radiotherapy and 2 patients of functioning pituitary adenoma received postoperative hormone replacement therapy.

Operative and Perioperative Data:

The extent of resection included resection was "GTR" in 16 cases (80% of total cases) (see fig. 2 & 3), while subtotal resection "STR" were in 4 cases (20% of total cases) (see fig. 4). A residual tumor of invasive pituitary adenomas had not been resected because it was encircling left internal carotid artery. In one chordoma a residual was left on the anterior surface brainstem as it was adherent and in other chordoma a remnant is left behind right carotid.

The reconstruction of the resulting anterior skull base defects mainly depend on allografts like fascia lata and fat graft and sealant like duraseal and fibrin glue followed by foley's catheter balloon as a tamponade. The mean operative time was 165.9 minutes (range 83-280 minutes). The mean blood loss was 657.6 ml (range 250-1650 ml). The average hospital stay, which included the day of surgery, was 4.7 days (range 2-11 days). Only one patient required a period of observation in the ICU, which lasted two days.

Complications:

Two patients developed postoperative CSF Leakage (10%) which is the main complication in this study both of them were treated with lumbar drain only. Only one of them complicated by tension pneumocephalus followed by meningitis and improved on lumbar drainage and conservative treatment and pneumocephalus resolved completely.

One patient developed Permanent anosmia presumably due to avulsion of the olfactory nerves due to aggressive superior dissection of the nasal mucosa or infiltration of olfactory nerves (esthesioneuroblastoma). One patient developed atrophic rhinitis and prolonged crusting, which were treated with transnasal irrigation, and topical and oral antibiotics with improvement. Two patients were presented preoperatively with ophthalmoplegia showed postoperative no improvement in their complaint and normal ocular motility did not restorated.

Epistaxis was postoperative complication in only one patient, it occurred at one month postoperatively requiring endoscopic cauterization and it was successfully controlled. One patient developed behavioral and mental status changes secondary to pneumocephalus and frontal lobe compression and he was managed conservatively and we had no mortality.

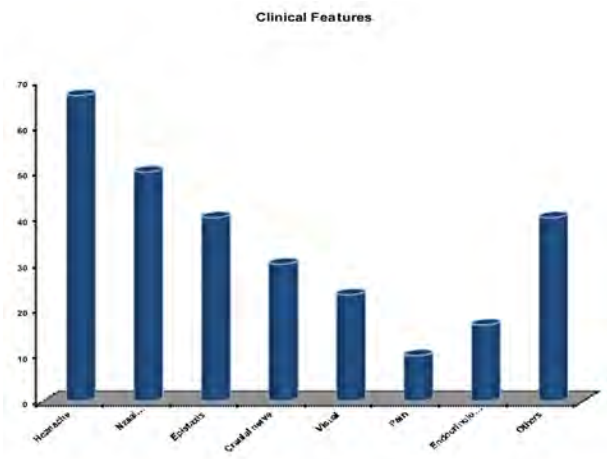
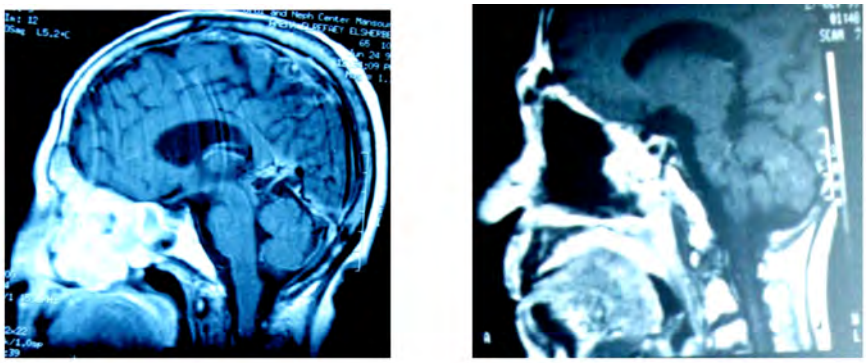
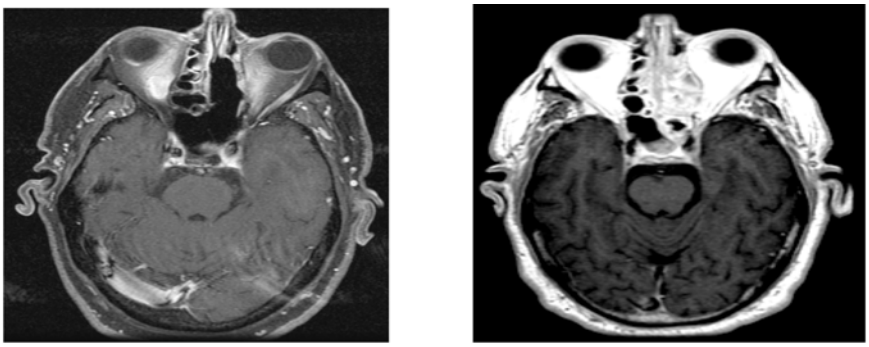


Fig. 1: The prevalence of clinical feature in the study group.



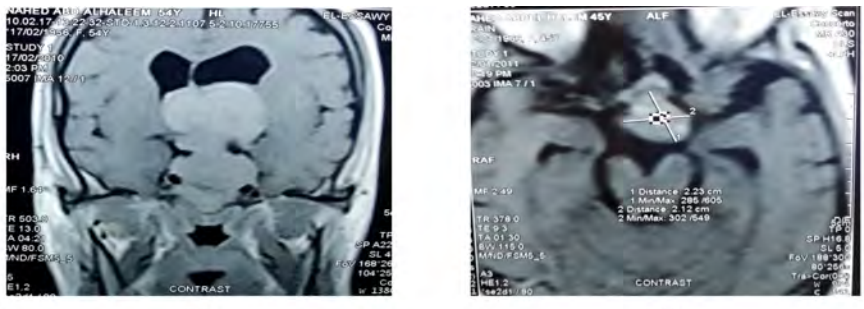
(A) (B)

Fig. 2: A- Preoperative MRI; esthioneuroblastoma, B- Total resection of the tumor.



(A) (B)

Fig. 3: A- Preoperative MRI; left ethmoidal esthioneuroblastoma, B-Total resection.



(A) (B)

Fig. 4: A- Preoperative MRI; dumbbell-shaped pituitary adenoma, B- Small residual of the tumor.

Discussion

The field of endoscopic cranial base surgery has made significant advances in the past few years. However, the principles of endoscopic endonasal approaches to the cranial base find their roots in the evolution of transsphenoidal pituitary surgery and minimally invasive sinus surgery. In the early 1900s, Hirsch in 1910 and Cushing in 1914 described the transnasal transsphenoidal approach to the sella. Over the years, this approach has been expanded to remove lesions above and below the sella; however, the use of a microscope and retractors limited its versatility and applicability[7]. Simultaneously, the field of functional endoscopic sinus surgery evolved, and it became clear that straight and angled endoscopes could provide full visualization of the entire midline cranial base as well as aspects of the lateral cranial base through an endonasal approach[8].

In this evolutionary context of change in the treatment of tumours affecting the skull base, in the present study with the endoscopic endonasal approach (EEA) for the treatment of tumours involving the skull base. Twenty patients were selected with extradural skull base tumors, cases selection is based on selection criteria and patient data is collected and gathered for analysis as previously described. Although the number of cases in our study is not very high, but it contain a wide variety of tumors as regard anatomical distribution (area of skull base involved), a wide range of histologic subtypes and different approaches used in their management. Thus, we attempt to analyze the results of usage of EEA as a whole not to analyse its value in each pathological subtype.

The purpose of this study was not to recommend EEA versus open techniques, which are still useful and, in most cases, do not cause significant aesthetic or functional sequelae. However, this study, along with the rest of those published, provides evidence of the usefulness of EEA for the management of tumors affecting the skull base, being the technique of choice in selected cases.

Several series have attested to

the efficacy of endoscopic endonasal approaches for resection of skull base neoplasms. In an early report, Stammberger et al.[9] reported on 43 sinonasal and skull base neoplasms managed strictly by endoscopic endonasal approaches. They noted that the initial outcomes were "at least equal to standard external approaches, however with excellent functional terms and significantly better overall quality of life." In a more recent series Lund et al.[10] reported a 49-patient prospective cohort that underwent endoscopic resection with 36 patients free of disease, seven patients with residual disease, and six patients' dead of disease. Nicolai et al. 2008 have reported the largest series to date, with 184 patients with nasoethmoidal malignancies involving the adjacent skull base. One hundred thirty-four patients (72.8%) were managed exclusively by an endoscopic approach. The overall rates of disease-free survival, alive with disease, died of disease, and died of other causes were 80.9%, 4.4%, 11.4%, and 2.2%, respectively. López et al.[11] published a retrospective analysis of 63 patients treated by an endoscopic endonasal approach (EEA). Tumor resection was complete 69% of cases.

The previous published series support our extent of tumor resection; Overall, gross total tumor resection was obtained in 16 cases (80% of our cases), while subtotal resection was achieved in 4 cases (20% of our cases). A lot of reasons stand against achieving total resection including; major cerebral vasculature encasement (one invasive pituitary adenoma encircling left internal carotid artery), large intracranial extension into the floor of middle cranial fossa (only one case of JNA) and one chordoma was not totally resected due to firm adherence to the anterior surface brainstem and only one case of mortality (5% of total).

Our follow-up period is short and the sample is small for survival calculations and the prognosis. At this time, no tumor has recurred, and the success of intraoperative endoscopic resection is encouraging. As in other series, this fact suggests a promising future for this approach.

Finally it is clear that surgical

management of extradural skull base tumors is undergoing a paradigm shift as endoscopic endonasal approaches become more widely practiced. However, in examining the limited published data and adding our own experience, it is clear that the early short-term results of endoscopic endonasal resection, provide encouraging data for high rates of tumor control and progression-free survival with limited morbidity for selected cases.

Given the preliminary nature of these data, there are several limitations to our study. The small number of patients limits the statistical power of any conclusions that one may draw from this report, Second, the short follow-up provides only short-term outcomes; further follow-up will be necessary to determine the durability of disease control in patients treated with this type of resection compared with more traditional open skull base approaches.

Conclusions

A completely endoscopic endonasal resection of the extradural skull base tumors can be safely performed for benign and malignant tumors. The endoscope provides superior visualization and allows for more precise dissection. Oncologic principles are preserved with en bloc excision of involved skull base structures. Early oncologic results are promising but continued experience is needed. There are many potential benefits for patients with endonasal surgery.

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SAFETY AND EFFICACY OF ENDOSCOPIC SKULL BASE SURGERYIN RESECTION OF EXTRADURAL ANTERIOR SKULL BASE TUMORS

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DETECTION OF THE ELECTRODE ARRAY POSITION INSIDE THE COCHLEA BY MULTISLICE CT SCAN

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Abstract

Introduction: *Cochlear implantation has become standard management for severe to profoundly deaf children, making the attainment of intelligible, age-appropriate spoken language an achievable goal for many. However, there remains unexplained variation in outcomes from implantation and the challenges of ensuring life-long use and benefit remain.Thus, there are multiple incentives to strive for an exact documentation of the position of the individual electrode in relation to cochlear structures and the insertion depth of the electrode array.*

Aim: *To find out if it is possible to detect exactly the position of the electrode array in the cochlea by MSCT scan and to measure the distance of this electrode from the modiolus of the cochlea.*

Material and methods: *Multislice CT scan was used to detect the electrode position in 15 cochlear implant patients.*

Results: *all electrodes were successfully detected with full insertion and the electrode modiolus distances were measured.*

Conclusion: *Multislice CT scan is a valuable tool for detection of intracochlear electrode array.*

Introduction

Cochlear implantation has become standard management for severe to profoundly deaf children, making the attainment of intelligible, age-appropriate spoken language an achievable goal for many. However, there remains unexplained variation in outcomes from implantation and the chal-

lenges of ensuring life-long use and benefit remain[1].

In cochlear implant patients, a large proportion of the success or failure depends on the transfer of stimulating signals from the electrode toward the auditory nerve fibers. An important aspect of the electrode design is electrical impedance, which depends on electrode surface area, morphological processes, and electrochemical processes initiated by electrical stimulation[2].

Thus, there are multiple incentives to strive for an exact documentation of the position of the individual electrode in relation to cochlear structures and the insertion depth of the electrode array[3].

In order to assess the electrode position in cochlear implant recipients, van Wermeskerken, et al.[4] considered the benefits of high-resolution computerized tomography (HRCT) scanning as a more exact method to visualize the structures of the temporal bone and assumed that locating the electrode will be possible with HRCT and that this visualization will allow to measure the distance to important landmarks in the cochlea, such as the modiolus.

Material and Methods

High-resolution multislice CT scanning of electrode position was performed on 15 pediatric cochlear implant candidates, 4 days after implantation, at Ain Shams Specialized Hospital using the Siemens Somatom Definition Flash 128-slice CT (Siemens Healthcare, Forchheim, Germany).

The patients were scanned with the following parameters (Table 1). 3-D reconstructions were created using multiplanar reformation (MPR), i.e., calculating slices along arbitrary sections. In our study, an MPR was made parallel to the basal turn of the cochlea and perpendicular to the modiolus and thus in the plane of the electrode array. Window width and window level were adjusted until both the cochlear tissues and the individual electrodes could be visualized. The electrode modiolar distance (EMD) was measured from the center of each electrode to the center of the modiolus (Figure 1).

We calculated the EMD of five electrodes (No.1, 6, 11, 16, and 22) in each patient; taking in consideration the electrodes are numbered from No. 1 (in basal turn) towards the electrode No. 22 (in apical turn).

Results

The study was conducted on 15 patients (6 male and 9 female) with range of age between 2 years and 14 year with mean age 5.07 years old.

All electrodes were fully inserted and successfully detected inside the cochlea, Individual electrodes were detected and the relation to the modiolus was measured.

After adjusting the bone window in the HRCT; the electrode modiolus distance (EMD) was measured between the electrodes No. 1, 6, 11, 16 and 22, and the center of the modiolus (Table 2). EMD ranged from 1.00um to 6.70um (mean 3.5107um, ± SD 1.51466).

Table (1): Imaging and reconstruction parameters.

Acquisition protocol	
Tube voltage	120 kV
Tube current	118 mA
Beam collimation	2 x 0.5 mm
Rotation time	0.5 seconds
Scan field of view FOV	240 mm
Reconstruction protocol	
Section thickness	0.6 mm
Section interval	0.3 mm
Filter	Kernel WEDGE_2

Table (2): The electrode modiolus distance measurements.

Electrode	No.	Range (um)		Mean (um)	± SD
		Minimum	Maximum		
1	15	5.00	6.70	5.8267	0.58611
6	15	3.20	5.30	4.3067	0.55865
11	15	1.80	4.00	2.6533	0.63793
16	15	2.00	4.00	2.7067	0.60647
22	15	1.00	3.30	2.0600	0.73173
Total	75	**1.00**	**6.70**	**3.5107**	**1.51466**

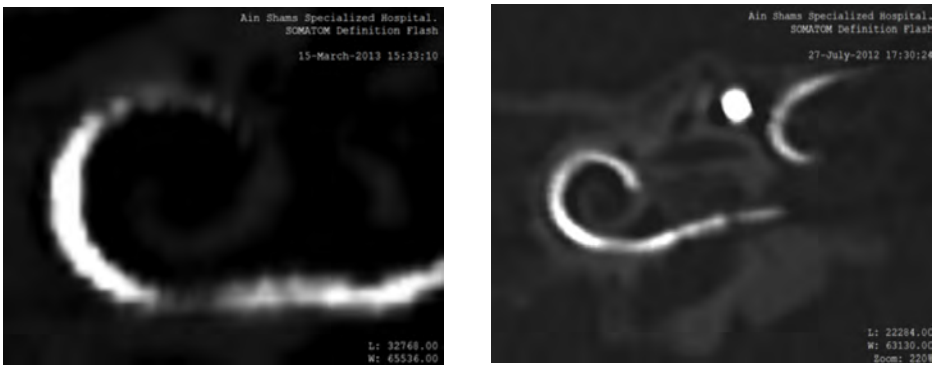

Fig. 1: HRCT image with an MPR in the plane of the electrode array showing complete insertion of the electrode inside the cochlea.

Discussion

The development of a new generation of cochlear implant devices has, to a great extent, been aimed at improving stimulus-transferring mechanisms. Thus, there is a growing interest in precisely documenting the position of the electrode array in relation to cochlear structures.

Multislice CT (MSCT) has proven its efficacy in the postoperative imaging of cochlear implant (CI) patients[5-9]. It confirms the intracochlear position of the implant. The positioning of an electrode array, as well as the individual electrode contact-to-modiolus distance, can be assessed. This yields objective measurements facilitating the evaluation of differences in outcome after implantation of different types of electrode arrays[7].

The aim of imaging cochlear implant patients in our study using MSCT scan was to find out if it is possible to detect exactly the position of electrode array in the cochlea by MSCT scan and measure the distance of this electrode from the modiolus;

Results of imaging in our study revealed that in all patients the electrode arrays were fully inserted, and the electrodes in all turns of the cochlea could be clearly distinguished. There was a decrease of the electrode modiolus distance measurements from the electrode No. 1 towards the electrode No.

22. This is in agreement with cochlear anatomy as the electrode No. 1 is in the wide basal turn, while the electrode No. 22 is in narrow apical turn.

Both Ketten[10] and Skinner[11] succeeded to visualize the electrode array and there depth in the cochlea using MSCT scan but they failed to detect the exact position of single electrode and its relation to cochlear structures due to imaging technology available at that time.

Multislice CT scanning can also play a role in the development of new electrodes, as the devices must not only allow for non traumatic insertion and positioning but also for safe explantation and reimplantation(4). These results provide a baseline for a subsequent study to verify the estimated electrode positioning.

Conclusion

Multislice CT scan is very useful in detection of the electrode position inside the cochlea and can be used in complications and research purposes.

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REPRINT

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DETECTION OF THE ELECTRODE ARRAY POSITION INSIDE THE COCHLEA BY MULTISLICE CT SCAN

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EFFECTS OF GREEN TEA ON HEPATIC FUNCTIONAL, HISTOLOGICAL AND ULTRASTRUCTURAL CHANGES OF HEPATOCYTE INDUCED BY HIGH SUCROSE DIET IN ALBINO RATS

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Abstract

Introduction: *High sucrose diet has various effects on hepatic function. In addition the obese persons are susceptible to develop fatty liver disease (FLD). Green tea contains powerful antioxidants, polyphenols, which help to remove free radicals from your body's cells. There is a little information about the effects of green tea extract on liver function and histolological changes in the liver of obese rats.*

Aim of the study: *The objective of this study was to assess the effects of high sucrose diet on body mass index, serum lipid profile, blood glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT) level and liver histology (light microscope using H&E; toluidine blue& transmission electron microscope) in rats and the role of green tea extract in minimizing these changes.*

Materials and Methods: *The rats included in this study were classified into 4 main groups; group I: control group received standard diet, group II: rats received standard diet and green tea, group III: High Sucrose rats, group IV: high Sucrose group received green tea extract.*

Results: *High sucrose diet group caused significant changes in the histology of liver where fatty infiltration is prominent in most hepatocytes while the remaining cells exhibited vacuolated cytoplasm with pyknotic nuclei. Ultra structurally, the most characteristic features observed in most hepatocytes were accumulation of fat droplets and the degenerative changes especially in mitochondria. Biochemically there*

was a significant increase in serum triglycerides, total cholesterol, LDL.C, blood glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as well as a significant decrease in serum HDL.C. All these histological & biochemical effects were improved by green tea.

Conclusion: *From this study we can conclude that, high sucrose diet caused significant changes in histological structure of the liver with a significant increase in body mass index, serum triglycerides, total cholesterol, LDL, blood glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as well as a significant decrease in serum HDL. All these effects were counteracted by green tea consumption.*

Key Words: *obesity, green tea, hepatocyte, albino rats.*

Introduction

Nonalcoholic fatty liver disease (NAFLD) covers a spectrum of liver disease ranging from simple hepatic steatosis (accumulation of triglyceride inside hepatocytes) to nonalcoholic steatohepatitis (necrosis and inflammation), with some people ultimately progressing to liver cirrhosis and failure. The prevalence of nonalcoholic fatty liver disease (NAFLD) is high and linked to obesity, diabetes mellitus, and hypertriglyceridemia[1].

High-fat and high-sucrose (HS) intakes were shown to contribute to syndromes such as hyperlipidemia, glucose intolerance, hypertension, and atherosclerosis[2,3].

Green tea is a rich source of polyphenol catechins. Epigallocatechin gallate (EGCG) is the most active form of the catechins responsible for green tea's antioxidant, anti-inflammatory, and metabolic effects. Green tea also contains caffeine, which appears to act synergistically with EGCG to assist metabolism[4].

Some studies show that substances in green tea may offer several weight-loss-promoting effects, such as speeding up your metabolism and suppressing your appetite[5]. Consumption of green tea may enhance health because it reduces the incidence of cancer in various experimental models, is a potent antioxidant, and modulates serum cholesterol concentrations[6]. Long-term consumption of green

tea may decrease the incidence of obesity and, perhaps, green tea components such as EGCG may be useful for treating obesity[7]. Dietary green tea extracts alleviated body weight gain and insulin resistance in diabetic and high-fat mice, thus ameliorating glucose intolerance[8].

Green tea may help your liver -- or it may not, depending on how you consume it and in what quantities. While drinking a moderate amount of green tea may reduce the risk of liver cancer and other liver disorders, taking large amounts of green tea supplements could have toxic effects on your liver[9]. Hepatoprotective effects of green tea against carbon tetrachloride, cholestasis and alcohol induced liver fibrosis were reported in many studies. Green tea may protect liver cells and reduce the deposition of collagen fibers in the liver. Green tea provides a safe and effective strategy for improving hepatic fibrosis[10].

The use of green tea extract appeared to be beneficial to rats in reducing lipid peroxidation products. These results support and substantiate traditional consumption of green tea as protection against lipid peroxidation in the liver, blood serum, and central nervous tissue[11].

Green tea, is a known cancer fighter, but it also has liver-protective properties. The catechins in green tea are powerful antioxidants that seem to protect against the damage that toxins wreak upon cells. Various animal studies indicate that green tea is helpful in the treatment of viral hepatitis and liver cancer. It has been found to reduce and prevent the growth of abnormal liver tissue in rodents[12].

Besides an obesogenic environment and reduced energy expenditure during work and less activities, one of the primary causes of the current epidemic of obesity and related metabolic disorders is related to the western-style diet, which includes excessive intake of high-fat and high-sucrose foods. Several studies have assessed the long-term (over 10 weeks ~ 2 years) effects of high-fat and/or high-sucrose diets on metabolic risk factors[13]. The rapid onset of

hepatosteatosis, adipose tissue hypertrophy and hyperinsulinemia by ingestion of a diet high in fat and sucrose may possibly be due to the rapid response of lipogenic, insulin signalling and inflammatory genes[14].

The aim of our study is to investigate the effect of high sucrose diet in deterioration of liver structure and function and the role of green tea in these changes in male albino rats.

Material and methods

Animals:

This study was carried in department of physiology, Benha faculty of medicine, where the animals were housed for the entire experimental period. Eight-week-old male rat were housed in a room at average temperature with a normal light-dark cycle.

Diets:

Standard chow diet: In this type of diet

- The fat represented 3.73% of the total caloric requirement.

- The carbohydrates represented 43.88% carbohydrate (40.75% starch and 3.13% sucrose) of the total caloric requirement.

- The protein represented 23.54% of the total caloric requirement [15].

High sucrose diet:

The fat represented 6.40% of the total caloric requirement.

- The carbohydrates 49.85% (4.5% starch and 47.35% sucrose) of the total caloric requirement.

- The protein represented 23.60% of the total caloric requirement.

- The fibers represent 9.15% of the total caloric requirement.

- The high-sucrose diet was obtained mixing 600 g sucrose and 60 g of soy oil to 1000 g of a previously triturated standard chow for four weeks. Casein was added to achieve the same protein content as the standard chow[16].

Green Tea Extract Administration:

Rats received 300mg/kg bwt. green tea extract (GTE) [Multi - treat Arab Co. for Pharmaceutical & Medicinal plants (MEPACO-MEDIFOOD) Enahas El Rami-Sharkeya- Egypt, each tablet contains 300 mg green tea dry extract, (30% polyphenols)] in 1 ml

distilled water/ rat by gavages daily for 14 weeks. In our present study we chose to use a moderate dose of green tea extract (GTE) to avoid adverse effects of GTE on many body organs, as there were evidence of deleterious effects of high doses of GTE including treatment-related mortality occurred in male and female mice receiving 1000 mg/kg bwt. Treatment dose which was likely related to liver necrosis, while using doses not exceeding 500mg/kg bwt. showed no adverse effects in males and females of both species sexes[17]. Humane care for rats was provided according to the guidelines of the National Institutes of Health (NIH) of animal Care and the local committee approved this study. All animals survived till the end of the experiment.

Experimental protocol:

The animals had free access to water and standard mouse chow for an acclimatization period of 1 week. Thereafter, animals weighing 200-225 g were randomly assigned to four groups for the feeding experiment. The control Group I: (n=10) was fed standard mouse chow, the green tea Group II: (n=10) was fed standard mouse chow with green tea by gavages', group III the HS Group (n=10) was fed the diet which was high in sucrose and Group IV: (n=10) was fed the diet which was high in sucrose and received green tea by gavages'. Body weight and food intake were monitored throughout the study. At the end of the experiment, all rats were anesthetized using diethyl ether inhalation, the body weight and body length were used to confirm the obesity through the obesity parameters body mass index (body weight g/ length cm2).

Blood samples were collected by intracardiac suction for biochemical analysis, Whole blood was collected into tubes, and serum was obtained by centrifugation at 3000 rpm for 15 min at 4°C and stored at -80°C until biochemical analysis. The determination of the activity of hepatic transaminases AST and ALT, glucose, triglyceride, total cholesterol, LDL, HDL and albumin were determined enzymatically using commercially available reagent kits in benha biochemistry analysis unit.

A histological study was performed following a midline laparotomy to remove the liver. The liver was dissected and fixed in 10% formalin solution at room temperature. An experienced pathologist evaluated all samples Liver portions were fixed in 10% formalin for histological examination.

Statistical Analysis:

All data were expressed as mean ± S.D; data were evaluated by the one way analysis of variance. The calculations were performed by SPSS program version 17. Difference between groups were compared by Student's t-test with $P<0.05$ selected as the level of statistical significance.

Results

Body weight index (BWI):

Mice in the HS diet group gained weight rapidly. As shown in table (1) and Fig.1 the HS diet in group II (Hs) increased the body weight ($P<0.01$), compared with the control group fed standard chow diet. Green tea administration had no significant decrease in body weight index in group II (standard diet & green tea)=Gt while, it significantly decreased BWI in group IV (high sucrose diet & green tea)=Hs Gt.

Plasma lipids profile and blood glucose:

As shown in table (2), the HS diet increased total cholesterol, LDL-C, triglyceride ($P<0.001$) & HDL-C was decreased ($P<0.01$) as compared with the control group. Green tea consumption decreased total cholesterol, LDL-C, triglycerides ($P<0.01$). HDL-C was increased ($P<0.01$) as compared with (HS group).

Plasma glucose, ALT and AST levels:

As shown in table (3), The HS diet significantly increased blood glucose, ALT and AST level ($P<0.01$) in group (III). Green tea consumption in group (IV) caused significant decrease of blood glucose, ALT and AST ($P<0.01$), as compared with the Hs group.

Histopathological study

Group 1 (control group):

The histological appearance of the liver in the control group was normal. Light microscopic examination of the liver of control rat group stained by H&E showed the normal characteristic hepatic ar-

chitecture. The hexagonal hepatic lobules were formed of hepatocytes arranged in cords radiating from the central veins. The hepatocytes appeared polyhydral in shape with large rounded or oval nuclei and sometime it contained two nuclei. The hepatic sinusoids were seen as narrow spaces in-between the hepatic cords (Fig. 1). The hepatocytes stained by toluidine blue appeared polyhydral in shape with large rounded or oval nuclei and enclosed thin walled blood sinusoids (Fig. 2). Ultrastructural examination of the liver specimen sections of this group showed normal polygonal hepatocytes with rounded or oval nuclei that had regular nuclear membrane, prominent nucleolus and clumps of chromatin (Fig.3). The cytoplasm showed different cell organelles. The mitochondria appeared rounded or elongated and had a homogenous matrix of moderate electron density. The rough endoplasmic reticulum appeared in the form of a group of flattened cisternae and commonly located in the perinuclear regions. Lysosomes appeared as heterogeneous organelles with extremely electron- dense matrix (Fig. 3).

Group 2 (non obese green tea received group):

Light microscopic examination of the liver in group 2, stained by H&E showed the normal characteristic hepatic architecture. The hexagonal hepatic lobules were formed of hepatocytes arranged in cords radiating from the central veins (Fig.4).

Group 3 (obese high sucrose diet group):

Examination of the liver sections of group (3) stained with H&E by the light microscope revealed a congested central vein & hepatic sinusoids by the blood elements surrounded by affected hepatocytes with multiple changes in their shapes. Many hepatocytes appeared polyhydral with large oval nuclei that showed a signet ring appearance and cytoplasmic lipid infiltration. Some hepatocytes had vacuolated cytoplasm and deeply stained nuclei. (Fig. 5) Many hepatocytes stained with toluidine blue appeared polyhydral in shape with large oval nuclei that showed a signet ring appearance and infiltration by many cytoplasmic lipid droplets allover the cytoplasm (Fig. 6).

Electron microscopic examination of group 2 showed hepatocytes with many vacuoles and multiple small lipid droplets that appeared as electron-lucent areas allover the cytoplasm. Some mitochondria appeared normal while others were degenerated. The indentation in the nuclear envelop was demonstrated with heterogeneous distribution of the nucleoplasm. The cytoplasm also includes some lysosomes and small amount of rough endoplasmic reticulum (Fig. 7).

Group 4 (High sucrose, green tea received group):

Light microscopic examination of a section in the liver of the adult rat group 4 stained by H&E clarified that, the liver tissue appeared more or less similar to the control group. The central vein was surrounded by cords of relatively normal hepatocytes and mild congestion of the hepatic blood sinusoids. Some hepatocytes were binucleated while others still had a vacuolated foamy cytoplasm with small dark nuclei, (Fig. 8). The semithin section in the liver of the adult rat group 3 stained by toluidine blue showed a group of polyhedral hepatocytes with rounded nuclei (N). Their cytoplasm had some lipid droplets (L) and the blood sinusoids (s) appeared slightly congested with blood elements (Fig.9). Ultrstructural examination of this group showed a relative improvement, where some hepatocytes had euchromatic nuclei and a prominent nucleoli. Their cytoplasm contained a mitochondria, rough endoplasmic reticulum, numerous glycogen granules and few vacuolization. Binucleated hepatocyte with euchromatic nuclei (mitotic figures) could also be observed (Fig. 10).

Table (1)

Group	Control	Gt	HS	Hs Gt
BWI (g/cm2)	0.527±0.030	0.545±0.03	0.818±0.05*	0.61+0.015#

*Significant changes compared with the control group.
Significant changes compared with the Hs group.

Table (2): Lipid profile (Triglycerides, Total cholesterol, and HDL.C and LDL.C mg/dl). Results are expressed as the Mean ± SE.

	Control	Gt	HS	HS Gt
Trigylc. (mg/dl)	86±2.5-64	92±1.73	144±2.04*	87.8±0.86#
T. choles. (mg/dl)	89±1.59	80±1.78	151±0.97*	92±1.34#
LDL (mg/dl)	18±1.69	17±1.63	19±0.56*	41±1.377#
HDL (mg/dl)	55±1.32	56±1.72	35±1.426*	58.8±0.71#

*Significant changes compared with the control group.
Significant changes compared with the Hs group.

Table (3): Serum glucose, ALT, AST. Results are expressed as the Mean ± SE.

	Control gp.	Gt gp.	HS gp.	HS Gt gp.
Glucose (mg/dl)	101±1.52	108±1.43	160±2.23*	110±0.701#
AST (u/l)	156.25±1.26	149.28±1.55	254.29±5.05*	152.78±2.16#
ALT (u/l)	43.75±1.12	47.24±0.69	65±0.57*	39.85±1.23#

*Significant changes compared with the control group.
Significant changes compared with the Hs group.

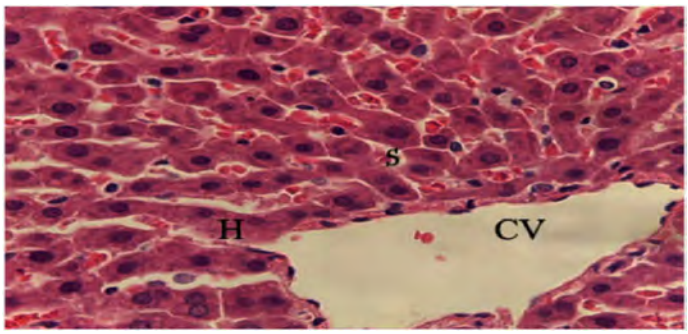

Fig.1: A photomicrograph of a section in the liver of an adult rat from G1 (control group) Showing a central vein (V) with radially arranged hepatocytes (H) & blood sinusoids (S) in between them. The hepatocytes appears polyhydral in shape with large rounded or oval nuclei and acidophilic cytoplasm. (H & E X 630).

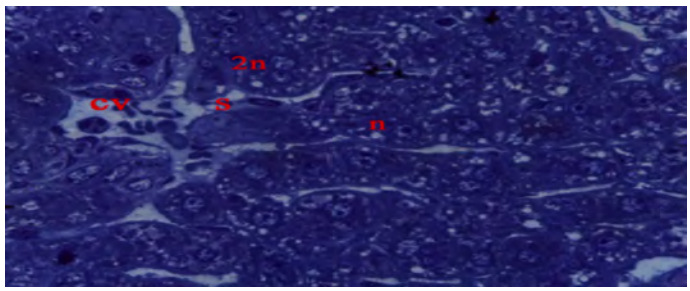

Fig.2: A photomicrograph of a semithin section in the liver of an adult rat from G1 (control group) showing a group of hepatocytes arranged in cords radiating from a central vein (cv). The hepatocytes appeared polyhydral in shape with large rounded or oval nuclei (n) and sometime it contained two nuclei (2 n). The hepatic blood sinusoids (s) were seen as narrow spaces inbetween the hepatic cords. (Toluidine Blue X l000).

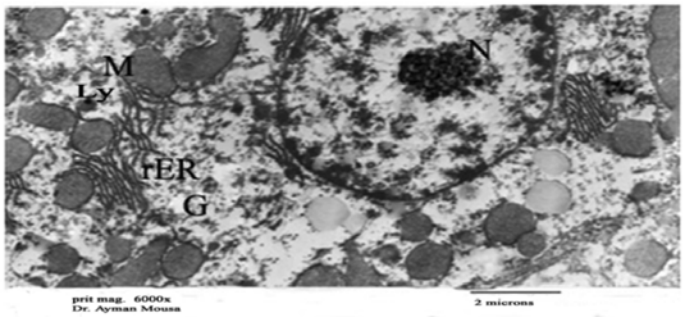

Fig.3: An electron micrograph of ultrathin section in the liver of an adult rat from G1 (control group) showing a part of normal hepatocyte with oval nucleus that has regular nuclear membrane, prominent nucleolus (N) and clumps of chromatin. The mitochondria (M) appears rounded or elongated with a homogenous matrix of moderate electron density while many free scattered glycogen particles (G) are seen inside the cytoplasm. The rough endoplasmic reticulum (rER) appears as a groups of flattened cisternae near the perinuclear regions and lysosomes have a heterogeneous electron- dense matrix (Ly). (Uranyl acetate and lead citrate X 6000).

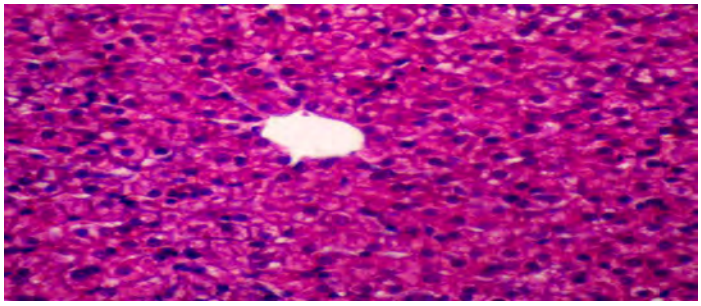

Fig.4: a section in the liver of an adult rat from G2 (non obese green tea receiving rats) Showing a central vein with radially arranged hepatocytes & blood sinusoids inbetween them. The hepatocytes appear polyhydral in shape with large rounded or oval nuclei and acidophilic cytoplasm.

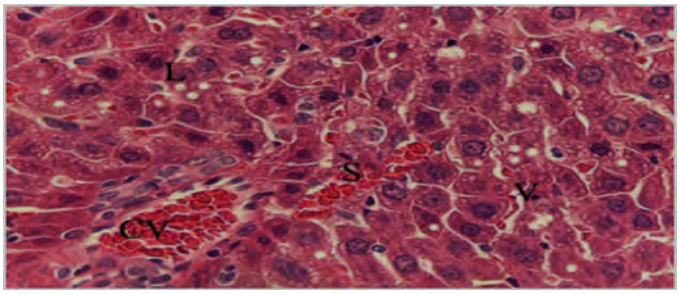

Fig.5: A photomicrograph of a section in the liver of an adult rat group 3 showing a central vein (CV) surrounded by affected hepatocytes with multiple changes in their shape. There are areas of lipid infiltration (L) and a signet ring appearance in the cytoplasm of hepatocytes while some hepatocytes have vacuolated cytoplasm (V) due to massive areas of degeneration with deeply stained nuclei. Moreover some areas revealed loss of hepatic architecture with dilatation and congestion of the blood sinusoids (s). (H & E X 630).

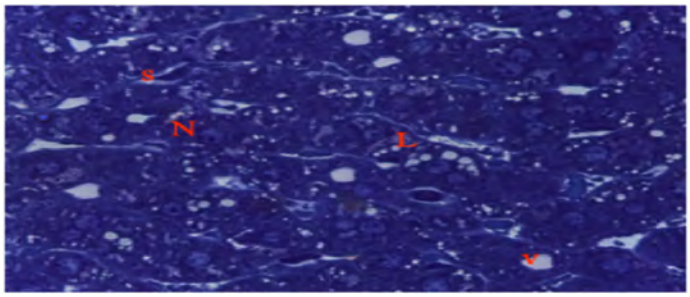

Fig.6: A photomicrograph of a semithin section in the liver of an adult rat group 3 showing many polyhydral hepatocytes with large oval nuclei that showes a signet ring appearance and infiltration by multiple small lipid droplets (L) allover the cytoplasm. A group of irregular hepatocytes appears with oval nuclei (N) and many vacuoles (v) in their cytoplasm. The blood sinusoids (s) have blood elements. (Toluidine Blue X l000).

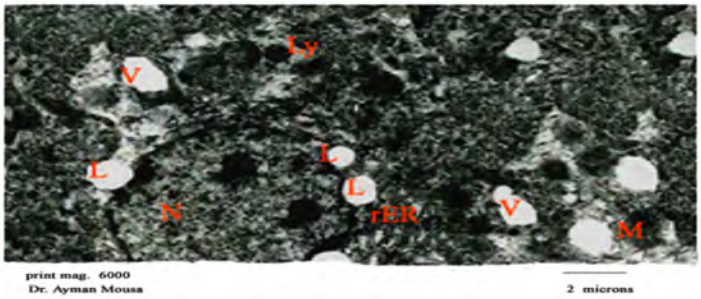

Fig.7: An electron micrograph of a hepatocyte of an adult rat group 3 showing a multiple small lipid droplets (L) all over the cytoplasm, polymorphic degenerated mitochondria (M), some lysosomes and little rough endoplasmic reticulum (rER). The nucleus (N) has an irregular indented envelop by three lipid droplets (Uranyl acetate and lead citrate X 6000).

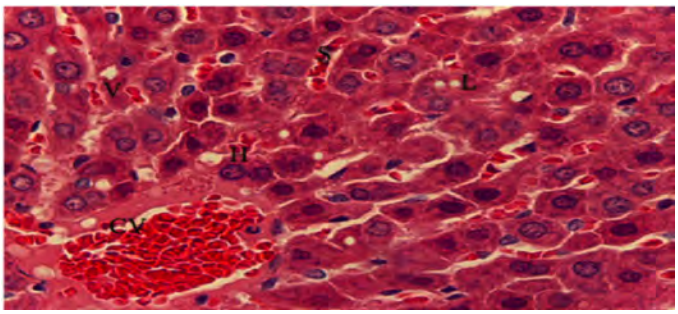

Fig.8: A photomicrograph of a section in the liver of an adult rat group 4 Showing a central vein (CV) with radially arranged relatively normal hepatocytes (H) and foamy appearance of some hepatocytes (L). Some hepatocytes have vacuolated cytoplasm (V) and small dark nuclei and mild congestion of the hepatic blood sinusoids (S) in between them. (H & E X 400).

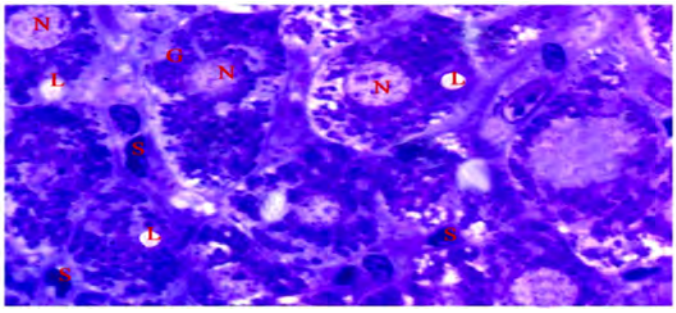

Fig.9: A photomicrograph of a semithin section in the liver of an adult rat group 4 showing a group of polyhedral hepatocytes with rounded nuclei (N), the cytoplasm contains numerous glycogen granules (G) and some lipid droplets (L). The blood sinusoids (S) appears slightly congested with blood elements. (Toludine Blue X 1000).

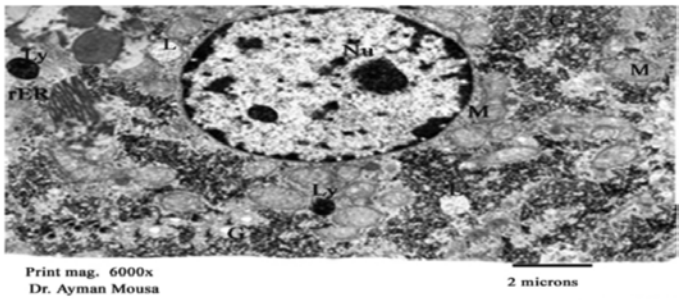

Fig.10: An electron micrograph of ultrathin section in a hepatocyte of an adult rat group 4 showing a rounded nucleus with nucleolus (Nu), heterochromatin and euchromatin. The cytoplasm has mitochondria (M), rough endoplasmic reticulum (rER), lysosomes with heterogeneous electron- dense matrix (Ly), some lipid droplets (L), and free scattered glycogen granules (G). (Uranyl acetate and lead citrate X 6000).

Discussion

In our study, we demonstrated that feeding of the HS diet caused gains in body weight and hepatic steatosis after 4 weeks. Thus, rapid onset of visceral obesity and fatty liver may occur with intake of a high-calorie diet that is high in fat and sucrose. These results were in agreement with[18] as they found that dietary fructose, but not glucose, increased de novo lipogenesis and promoted dyslipidemia, decreased insulin sensitivity, and increased visceral adiposity in overweight/obese adults. In addition Nagata R and colleagues revealed that adult male Sprague-Dawley rats fed a sucrose-rich diet (70% sucrose) for 2–3 wk that developed fatty livers and became obese. In addition they suggested that fructose, not glucose, is the primary cause of hepatic changes after chronic ingestion of a high-sucrose diet; diets enriched with a comparable amount of glucose, instead of sucrose or fructose, do not produce any overt hepatic abnormality. This finding may be mainly attributable to the unique metabolic properties of fructose, i.e. its rapid uptake by the liver and its entry into the glycolysis pathway after bypassing the phosphofructokinase regulatory step[19].

Our study revealed that green tea leads to significant decrease in the body weight index of rats. As well as significant decrease in the serum levels of glucose, ALT, AST, triglycerides, total cholesterol and LDL.C. With significant increase in serum level of HDL.C. These results were in agreement with[20] as they suggested that Green tea significantly decreased the BWI in high sucrose obese rats as green tea extract may boost metabolism and help burn fat.

Diet-induced obesity is largely caused by disorders of fat metabolism, resulting in a massive accumulation of fat in various tissues. Lipid and energy metabolism are regulated by a complex network of signaling processes, and therefore investigated mRNA expression of key genes regulating lipid metabolism, The HF-HS diet upregulated liver LPL mRNA expression. The lipolytic enzyme LPL mediates uptake of circulating lipid into peripheral organs, and it is the primary enzyme responsible for chylomicron- and very low-density lipo-

protein-triglyceride lipolysis[21].

Bioactive ingredients of green tea extract caused in the liver an increase in the activity of glutathione peroxidase and glutathione reductase and in the content of reduced glutathione as well as marked decrease in lipid hydroperoxides (LOOH), 4-hydroksynonenal (4-HNE) and malondialdehyde (MDA). The use of green tea extract appeared to be beneficial to rats in reducing lipid peroxidation products. These results support and substantiate traditional consumption of green tea as protection against lipid peroxidation in the liver, blood serum, and central nervous tissue[22].

In our results there was histological and ultastructural changes in obese high sucrose rat liver include degeneration and disruption of the hepatocytes, degeneration of the cells lining the bile ducts and occlusion of the central portal vein. Green tea consumption had greatly improved the hepatic structure and function. The liver dysfunction in obese rats where the serum liver enzymes (ALT & AST) increased was greatly normalized after green tea administration. In addition; there was a biochemical change as increased plasma triglycerides, total cholesterol, and LDL level and blood glucose in obese group and these changes became normal in green tea received obese rats. These results were in agreement with[23] as they showed that, the triglyceride content in the liver as well as the cholesterol content in the heart of rats fed sucrose-rich diet were elevated and were normalized by all types of tea drink tested. Although green and oolong tea extracts contained similar composition of catechin, their findings suggest green tea exerted greater antihyperlipidemic effect than oolong tea. Apparent fat absorption may be one of the mechanisms by which green tea reduced hyperlipidemia as well as fat storage in the liver and heart of rats consumed sucrose-rich diet.

Green tea contained very large amounts of catechins (173.1 mg/dl), including epigallocatechin gallate (61.8 mg/dl), which have potent antioxidant effects, in addition; Green tea contains 2% to 4% caffeine, and unlike black tea, green tea also contained ascorbic

acid (3.0 mg/dl) and may reduce the risk of liver cancer[24].

Eight studies showed a significant protective role of green tea against various liver diseases four studies showed a positive correlation between green tea intake and attenuation of liver disease. Moreover, the other two studies also presented the protective tendency of green tea against liver disease [25]. Research shows that green tea lowers total cholesterol and raises HDL ("good") cholesterol in both animals and people. One population-based clinical study found that men who drink green tea are more likely to have lower total cholesterol than those who do not drink green tea. Green tea also seems to protect the liver from the damaging effects of toxic substances such as alcohol. Animal studies have shown that green tea helps protect against liver tumors in mice[12].

From our current study we concluded that, high sucrose induced obesity resulted in structural and functional liver disturbance in adult rats, Green tea had a protective effect against these dysfunction. In addition; green tea had a weight lowering and anti lipidemic effect and could improve the fatty changes of the liver.

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EFFECTS OF GREEN TEA ON HEPATIC FUNCTIONAL, HISTOLOGICAL AND ULTRASTRUCTURAL CHANGES OF HEPATOCYTE INDUCED BY HIGH SUCROSE DIET IN ALBINO RATS

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ALVARADO SCORING SYSTEM TO AID IN DIAGNOSIS OF APPENDICITIS : A PROSPECTIVE STUDY

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Abstract

Hypothesis: *Decision making in cases of acute appendicitis poses clinical challenge especially in developing countries, where shortage in advanced radiological modality. Aim of this study is to evaluate the effect of Alvarado scoring system for diagnosis of acute appendicitis and correlate it with histopathological report.*

Patients and Methods: *all consecutive patients admitted in that period with pain in right iliac fossa were included. Data of the patients collected and divided the patients study into three categories according to Alvarado scoring system.*

Results: *positive predictive value, negative appendicectomy rate and complication rate were 86.7&, 13.3% and 20.8% respectively.*

Conclusions: *Alvarado score system is simple, easy and useful tool preoperatively in diagnosis of acute appendicitis and can be used effectively in routin practice.*

Keywords: *Duodenal injuries, associated trauma, delay, drainage.*

Introduction

Although acute appendicitis is the most common cause of an acute abdomen in Surgical Department, the diagnosis of this condition is still largely thought to be clinical one; a meaningful number of patients are found to have normal appendix at surgery (Russell, 2004). There are high negative appendicectomy rate (8 to 30%) reported in the literature (Dey, 2010; Jail, 2006; Ohmann, 1999). More observation

period may allow uncomplicated appendicitis to progress to gangrenous, perforated and peritonitis, even death due to septicaemia (Lancer, 1999). However some believe that it is better to remove normal appendix than to delay diagnosis, particularly in elderly patients. The erroneous diagnosis of acute appendicitis can be reduced by using different scoring systems (Abdeldaim, 2007; Dey, 2010 and Ohmann, 1999). Alvarado scoring system is one of various scoring systems; which is simple and based on history, clinical examination and leucocyte count.

The aim of this study was to evaluate the reliability of Alvarado score for diagnosis of acute appendicitis and correlate it with histopathological examination as a gold standard diagnosis.

Material and Method

This prospective study was carried out in the Surgical Department, El Thoura Teaching hospital, El Beida, Libya, for the period of 1st May to 31st October 2011.

All consecutive patients admitted in that period with pain in the right iliac fossa (RIF) were included. Patients with urological, gynaecological problem or surgical patients other than RIF pain, specially patient with RIF mass, were excluded from the study. Data of the patients collected and divided the patients study into three categories according to Alvarado scoreing system.

Alvarado scoring system: One point for each of RIF pain, nausea / vomiting, anorexia, rebound tenderness RIF, rise temperature (>37.50C) and shifting neutrophils to left (neutrophils more than 70% of leucocytosis). Two points for tenderness of RIF and Leucocytosis.

Score 1: (<5), Score 2: (5-6) and Score 3: (7-10) points. Patients belong to Score 1 and

Score 2 are kept under observation, for 24 hours of admission and appendicectomy performed for who worsen the symptoms . All the patients within Score 3, were underwent appendicectomy. The removal appendix sent for histological examination to confirm the appendicitis.

Results

Data of 180 patients admitted with history of RIF pain were collected and statistical analysis of the collecting data was done. There were 98 Female and 82 Male, with mean age 23.3 years (range 5-65 years), 40 (22.2%) of them belong to Score 1 and appendicectomy was performed for only 3 patients (2 Female and 1 Male) after 24 hours of admission. Acute appendicitis was confirmed histopathologically in only one patient and the remaining two (66.6%) patients had negative appendicectomy.

Score 2 : (5-6 points) 68 (37.8%) patients within this score, 45 of them (26 Female and 19 Male) underwent appendicectomy after 24 hours of admission, because of worsening of clinical features and shifted to last Score (7-10 points). Thirteen (28.9%) patients had negative appendicectomy. Score 3 : (7-10 points) 72 (40%) patients fitted to this score. 38 were Female and 34 Male. All of them underwent appendicectomy within 6-24 hours of admission. Only one (1.4%) patient had negative appendicectomy and in whom intra-operative diagnosis was acute diverticulitis.

Over all Positive Predictive value in the study was 86.7% and negative appendicectomy rate was 13.3% and complication rate was 20.8%. (Table 1).

Table (1): Shows detailed analysis.

Character Of patient	No. of patients	No & % of Operating pts.	No & % of +ve operation & histology	No & % of Complicated appendicitis	No & % of Negative appendicectomy
Sex					
Female	98(54.4%)	66(36.6%)	56(85%)	11(16.6%)	10(15%)
Male	82(45.6%)	54(30%)	48(88.8%)	14(25.9%)	6 (11%)
Age					
< 10	30	19	16 (87%)	3 (15.8%)	3 (15.8%)
11-20	64	47	43 (91.5%)	7 (14.9%)	5 (10.6%)
21-30	45	34	30 (88%)	6 (17.6%)	5 (14.7%)
31-40	28	17	12 (70.5%)	7 (41%)	3 (17.6%)
41-50	9	2	2 (100%)	1 (50%)	0
>51	4	1	1 (100%)	1 (100%)	0
Score					
Score 1	40	3(2F & 1M)	1 (33.3%)	0	2 (66.6%) 2F
Score 2	68	45(26F, 19M)	32 (71%)	6 (13.3%)	13(28.9%)8F, 5M
Score 3	72	72(38F, 34M)	71(98.6%)	19(26.4%)	1 (1.4%) 1M
			86.7%	20.8%	13.3%
Total No of patients	180	120 (66.7%)	104 (86.7%)	25 (20.8%)	16 (13.3%)

Discussion

The Vermiform appendix is considered by most to be a vestigial organ, responsible for the most common surgical emergency (Cuschieri, 1995 and Russell, 2004).

Surgical intervention in acute appendicitis is not without the risk of morbidity and mortality, even in a negative appendicectomy (Baidya, 2007). Hence, appendicitis has always been approached with two things in mind, minimizing of negative appendicectomy or preventing delayed complications (Gangrenous, perforation, peritonitis) (Russell, 2004).

In developing countries, where advanced radiological imaging and laparoscopic examination do not appear cost effective, the history, clinical examination with a few laboratory investigation remain the mainstay of diagnosis (Klein, 2007; Peterson, 1992 and Subhajeet, 2010). Various studies have shown a better outcome in the form of decreased negative appendicectomy rate by using diagnostic scoring system (Alvarado, 1986; Klein, 2007 and Teicher, 1983). In 1986, Alvarado published Score, consisting of ten clinical points for diagnosis of acute appendicitis (Alvarado, 1986). He recommended an operation for all patients with equivocal or more than 7 points, observation for patients who have score 5-6 points, while less than 5 points excluded from operation.

Many investigators conducting to evaluate the validity of this simple score system (Abdeldaim, 2007; Chan, 2001; Jawaid, 1999; Kalan, 1999 and Klein, 2007).

The positive predictive value of acute appendicitis in this series was 86.7%, which is comparable with other report (Chan, 2001, Jawaid, 1999; Kalan, 1999 and Klein, 2007). Over all negative appendicectomy rate was 13.3%, 2 (66.6%) patients in Score 1, 13 (28.9%) patients in Score 2 and only one (1.9%) patient belong to Score 3. There are a wide variation in rate of negative appendicectomy among the studies range from 8 to 30% (Jain, 2006; Ohmann, 1999 and Dey, 2010). The explanation may be due to the surgeon is operating on too few patients, hence increase risk of complications or

operating at a low score with increasing of the negative appendicectomy rate (Jain, 2006; Ohmann, 1999 and Dey, 2010).

This suggestion may be supported by a wide variation of appendicular complications, between previous reports. (Baruni, 1998; Ditillo, 2006; Ricci, 1998; Makael, 2009; Scher, 1980 and Wellmore, 2011). Over all complications rate in this series was 20.8%. There is an agreement with other investigators, the complications of appendicitis more in females and extreme age. These are explained by difficulty in diagnosis of the acute appendicitis in these age group. On the other hand, delay presentation of the patients or hospital organization as in general hospital leading to increase rate of complications (Makale, 2009 and Kochroo, 1984).

Conclusion

Alvarado score system is simple, easy, available and useful tool preoperatively in diagnosis of acute appendicitis and can be used effectively in routine practice. Score 5-6, doubtful cases have to be regularly examined clinically by surgeon to detect any worsening of their signs; in which case has to operate early. Score equivocal 7 or more, definitely confirmed diagnosis and early operation is indicated to avoid the complications.

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HUMAN ADIPOSE TISSUE MESENCHYMAL STEM CELLS REDUCE LIVER FIBROSIS IN IMMUNOCOMPETENT RATS

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Abstract

Objective: *Stem cell transplantation is a promising treatment for liver fibrosis. Mesenchymal stem cells (MSCs) can reduce liver fibrosis by different mechanisms. This study was conducted to assess the effect of human adipose tissue-derived mesenchymal stem cells (ASCs) on immunocompetent rats.*

Subjects and Methods: *Liver fibrosis was induced in 20 immunocompetent rats by intraperitoneal infusion of thioacetamide (TAA). Rats were divided into control group and stem cell group. The second group received human ASC infusion into the portal vein. One month later ALT, AST, serum albumin, serum bilirubin, and CBC were assessed. The whole liver was used for histopathological assessement and computed quantitation of the fibrous tissue by imajeJ program.*

Results: *Fibrosis score and amount of fibrous tissue were significantly lower while serum albumin was significantly higher in the stem cell group.*

Conclusion: *Human ASCs reduced liver fibrosis without the need of immunosuppression. Further research is needed to evaluate the mechanisms by which MSC reduce fibrosis.*

Introduction

Liver cirrhosis is a leading cause of morbidity and mortality worldwide. A wide variety of liver diseases lead to the impairment of liver function and require medical intervention. Liver transplantation is the primary treatment for end-stage hepatic diseases. Though effective, extensive clinical applica-

tion is limited by the lack of availability of donor organs. Other adverse factors such as rejection, problems associated with the long-term use of immunosuppressants, and perioperative morbidity and mortality contribute to additional complications[1].

Attention has been focused on the ability to use cellular resources to bridge patients until transplantation or to restore liver mass and function[2]. There have been significant advances in this field. Many groups reported improvement of toxin induced liver fibrosis in animal models with mesenchymal stem cells (MSCs) proved by histopathological evaluation, elevated serum albumin, and normalization of transaminases[3,4,5,6].

Though improving fibrosis, MSCs do not appear to contribute significantly in regeneration of recipient livers by transdifferentiation into hepatocytes[7]. The role of MSC in the reduction of fibrosis may be attributed to the secretion of certain cytokines and growth factors that favour regression of fibrosis and differentiation of hepatic oval cells[3].

An important limitation of most animal studies is the short duration, the use of carbon tetra-chloride alone which causes fibrosis but does not suppress hepatocyte replication, while retrosine, thioacetamide, and 2-acetylaminofluorane can do[8].

Aim of The Work

The aim of this study was to assess the effect of human ASCs on TAA-induced liver fibrosis in immunocompetent rats.

Methods

This study was conducted in Medical Experimental Research Center (MERC) .It was carried out on 20 male Sprague-Dawley rats with body weight ranged from 190 to 350 gm. Rats were purchased from the Holding Company for Biological Products & Vaccines (VACSERA). Thioacetamide was burchased from Sigma Aldrich company.

Liver fibrosis was induced in all rats by intarperitoneal infusion of thioacetamide 200mg/kg twice weekly for 8 weeks. Rats were divided into 2 groups:

1) Control group: This group in-

cluded 10 rats. One month after induction of fibrosis, blood samples were taken and rats were sacrificed to take specimens for histopathological evaluation.

2) Stem cell group: This group included 10 rats. 1 x $10^{(6)}$ hASC/ 1 ml were injected into the portal vein under anaesthesia in each rat. Blood samples were taken and rats were sacrificed to take specimens for histopathological evaluation one month later.

In all rats the body weight and weight of the whole liver were recorded. Blood samples were taken prior to sacrifice for CBC, ALT, AST, total bilirubin, and serum albumin analysis. The whole liver was extracted for histopathology. Hematoxylin and eosin stained slides were used to stage fibrosis using a modification of the staging criteria adopted by Zhao et al[9]. Masson trichrome slides were used for image analysis. Image analysis by ImageJ program was performed on 40X digital photos. Twenty photos were used for each specimen.

Statistical analysis was conducted by SPSS program. Shapiro-Wilk test was used as a test for normality. Mann-Whitney test was used to compare the two groups.

Results

A) Physical parametres: There was no significant difference between the control group and the stem cell group as regards body weigh, liver weight, and liver/body weight ratio.

B) Laboratory data: Serum albumin was significantly higher in stem cell group compared to the control group. No significant difference was detected between the two groups as regards other laboratory parameters.

C) Histopathology: The amount of fibrous tissue estimated by imageJ program was significantly lower in the stem cell group compared to the control group. Also, stage of liver fibrosis was significantly lower in the stem cell group compared to the control group.

Table (1):

Group / parmeters	Control (n = 10)	Stem Cells (n = 10)	P value
Wt (gm) median±V	262.5±29.5	271±23.4	.218
Liver wt (gm) median±V	12.62±7.33	13.41±6.5	.264
Liver wt/Wt median±V	.0454±.0025	.0473±.0017	.898

Wt: Body weight, **V:** Variance

Table (2):

Group / parmeters	Control (n = 10)	Stem Cells (n = 10)	P value
Alb. (gm/dl) median±V	2.31±.23	3.34±.08	<.0001
ALT (IU/L) median±V	113±107.3	118±54.64	.717
AST (IU/L) median±V	272±115.4	343±57.83	.091
AST/ALT median±V	2.34±1.27	2.66±.3	.442
T.bil. (mg/dl) median±V	.37±.006	.4±.002	.930
Hb. (gm/dl) median±V	10.55±4.03	11.7±3	.051
WBCs (n/cmm) median±V	9.2E3±2.08E3	6.4E3±3.7E3	.109
Plt. (n/cmm) median±V	753E3±73.72E3	625E3±34.04E3	.062

Table (3):

Group / parameters	Control (n = 10)	Stem Cells (n = 10)	P value
FS median±V	8	6±4.218	.002
IA (%) median±V	40.5±10.55	30±9.73	.012

FS: Fibrosis score, **IA:** Image analysis.

Discussion

Though improving liver fibrosis in animal models, several debates are still not solved concerning the use of MSCs in treating liver fibrosis. Some studies demonstrated transdifferentiation of these cells into myofibrobalsts with progression of liver fibrosis[10]. In our study human ASCs reduced the amount of fibrous tisssue estimated by image analysis and promoted hepatic regeneration proved by elevated serum albumin and improved fibrosis score.

A second debate is a bout the mechanism by which MSCs can reduce fibrosis. Different mechanisms may contribute to the therapeutic effects exerted by MSCs, which can differentiate into func-

tional hepatocytes and also produce a series of growth factors and cytokines that can suppress inflammatory responses, reduce hepatocytes apoptosis, regress liver fibrosis, and enhance hepatocytes functiona[11]. Most studies did not provide definitive evidence that MSCs have a capability to differentiate into functional hepatocytes in vivo[12]. Transdifferentiation of human ASCs into hepatocytes was not confirmed in our study.

A third debate is about the immunomodulatory effect of MSCs. MSCs express few HLA class I and no HLA class II molecules, allowing them to evade allogeneic immune response. This advantage makes these cells suitable for both autologous and allogeneic transplantation. MSCs also have suppressive effect on a wide variety of cells belonging to both adaptive and innate immunity, including T and B lymphocytes and natural killer cells[13]. The possible beneficial effects of MSC xenotransplants was not well investigated.

Human ASCs were used in our study because of its abundance and can be easely extracted in large amounts from liposuction. Also to assess if the immunological advantages of MSCs will help xenotransplantation without immunosuppression.

Trials on human hematopoietic stem cell transplantation into immunocompetent rats with liver cirrhosis showed that these cells were eliminated and did not engraft[14]. While similar trials on immunosuppressed rats resulted in improved hepatic regeneration[15]. The use of human amniotic membrane stem cells which are known by its immunotolerance could also improve liver fibrosis in immunocompetent mice[16]. Only few trials for xenotransplantation of ASCs were done. ASCs derived from porcine fat could improve acute on chronic liver failure in rabbits[17]. A single study used human ASCs in immunocompetent rats with liver cirrhosis. In this study stem cells were directly injected directly into the liver and could promote liver regeneration [18]. Our study had similar results although stem cells were infused in the portal vein. This result suggest that human ASCs may have immunoprevilege advantage even

with xenotransplantation.

More studies are needed to confirm the beneficial effect of human ASCs on liver fibrosis and to reveal the mechanisms behind that effect. Also studies are needed to assess the possibility of transdifferentiation of these cells into hepatocytes in vivo. This target may be reached by immunohistochemical stainig for human albumin and matching these stains with the detection of human Y chromosome by insitu hybridization after using male human ASCs in female rats.

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HUMAN ADIPOSE TISSUE MESENCHYMAL STEM CELLS REDUCE LIVER FIBROSIS IN IMMUNOCOMPETENT RATS

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ARMS TECHNIQUE FOR MOLECULAR SCREENING OF THE MOST COMMON ALPHA-ONE ANTITRYPSIN DEFICIENCY MUTATIONS AMONG CHILDREN WITH LIVER DISEASES

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Abstract

Objective: *To apply a molecular screening method for detection of the most common mutations (Z and S) associated with alpha-one antitrypsin deficiency in pediatric patients with liver diseases* ***Methods:*** *This study includes 45 pediatric patients with variable liver disease and 25 age and sex matched control. Blood sample was taken from the patients to measure serum AAT by radial immundiffusion (RID). DNA was extracted from peripheral leucocytes and followed by genotyping for S and Z mutation using ARMS-PCR. Statistical analysis was applied using SPSS 20.*

Results: *We found statistically significant difference between patients and control regarding serum AAT (1.506±0.512 vs. 1.867±0.212 g/L; P=0.001). Results of genotyping revealed predominant MM genotype in patients and control (64.5% vs. 96%). MS genotype was 4.4%, 4% in patients and control respectively. MZ and SZ genotypes were not detected in control but were present in 17.8%, 11.1% of patients respectively. Homozygous ZZ was observed in 2.2% of patients only. Results of serum AAT were concordant with the observed genotyping.* ***Conclusion:*** *the present work provides a simple, rapid, reliable molecular screening method, ARMS-PCR, for detection of AAT mutations associated with pediatric liver disease.*

Keywords: *Alpha-one antitrypsin deficiency, liver disease in children, ARMS.*

Introduction

Alpha-1-antitrypsin deficiency was first reported in 1963 by Carl-Bertil-Laurell and Sten Eriksson who noted a link between low plasma serum levels of alpha-1-antitrypsin and symptoms of pulmonary emphysema[1]. Since these first cases were described, an understanding of the biochemical mechanisms and genetic abnormalities involved has developed and alpha-1-antitrypsin deficiency is now thought to be one of the most common hereditary disorders worldwide, comparable in frequency to cystic fibrosis[2].

Alpha-1-antitrypsin is a member of the serine proteinase inhibitor or serpin superfamily and is referred as SERPINA1. The majority of this family are inhibitory proteins but some have other non-inhibitory functions[3]. Members of the serpin family have more than 30% sequence homology in primary structure with the archytypal AAT, that it is formed of three β sheets (A, B and C) and 9 α helices (termed A-I) and a mobile central reactive loop that exhibit a peptide sequence as a pseudosubstrate for the target proteinase[4]. Alpha-1-antitrypsin is a single-chain polypeptide with 394 amino acid, and three asparagine-linked complex carbohydrate side chains which are attached at residues 46, 83, 247[5]. It is mainly synthesized by the hepatocytes [6] and is also produced in smaller quantities by alveolar macrophages, circulating monocytes and possibly lung epithelial cells[7].

The main target proteases for AAT are neutrophil elastase, cathepsin G, and protinease 3 which are all produced by activated neutrophils. AAT constitutes more 90% of the inhibitory capacity of neutrophil elastase in one body fluid; pulmonary alveolar lavage[8].

A1-Antitrypsin functions by presenting its reactive center methionine residue at the 358 position (P1) on an exposed loop of the molecule such that it forms an ideal substrate for the enzyme neutrophil elastase. After docking the enzyme cleaves the peptide bond P1-P1' (methionine- serine) of AAT molecule and the proteinase is inactivated by a mousetrap action that, swings it from the upper to the lower pole of the

protein in association with the insertion of the reactive loop as an extra strand in b-sheet (s4A)[9,10]. This altered conformation of AAT bound to its target enzyme is then recognized by hepatic receptors and cleared from the circulation[11].

The AAT is encoded by the protease inhibitor (Pi) locus on chromosome 14q31-32.2. Pi locus is 12.2 kb in length with 4 coding exons (II, III, IV, V), 3 non coding exons (IA, IB, IC) and 6 introns. The region coding for the reactive loop and the active site Methionine 358 is located within exon V. It is a highly pleomorphic gene with approximately 125 single nucleotide polymorphisms (SNPs) reported in public databases[12]. Traditionally, each variant is identified by its speed of migration on polyacrylamide gel electrophoresis (IEF) in pH4 to pH5, the most common forms being F (fast), M (medium), S (slow) and Z (very slow). Alteration in the speed of movement through a gel occurs because of variation in protein charge, due to changes in amino acid composition[13].

There are different theories for the pathogenesis of liver injury in AAT deficiency but the accumulation theory is the most accepted one. Liver injury by the gain –of –toxic function is caused by accumulation of the mutant AAT molecules in endoplasmic reticulum of hepatocytes[8]. The Z mutation (Glu342Lys) distorts the relationship between the reactive center loop and β-sheet A, that causes reactive center loop of one AAT molecule to lock into the A sheet of a second to form a dimer, which then extends to form chains of loop-sheet polymers[4]. These polymers are then degraded[14] or accumulate within the endoplasmic reticulum of hepatocytes to form the PAS positive inclusions that are the hallmark of Z AAT liver disease[8].

Other variants that are known to cause rapid polymer formation are Siiyama (Ser53Phe) and Mmalton (52 phenylalanine deletion), while athers can polymerize at slower rates and include Pi S (Glu264Val) and the rare I variant (Arg39Cys)[13].

Cellular adaptive mechanisms are directed towards cell protection and degradation of the retained misfolded proteins in the ER. These mechanisms are subjected

to genetic variations that can explain the diversity of clinical presentation and susceptibility to liver disease in AATD patients[14]. The two main mechanisms are proteasome-associated degradation and lysosome- associated autophagy[15]. Soluble α1-ATZ is degraded by the proteasome while polymerized α1-ATZ is degraded by autophagy[8].

Rudnick and others[16] have studied the regeneration of hepatocytes in PiZ mice using bromodeoxyuridine (BrdU) to measure the degree of hepatocyte injury and quantify hepatocellular proliferation. The results indicate that there is increased proliferation but almost exclusively in globule-devoid hepatocytes. This hepatocellular proliferation in the presence of inflammation, creating a milieu that is prone to carcinogenesis[17].

Alpha-1-antitrypsin deficiency manifests clinically as pulmonary emphysema, liver cirrhosis and much less frequently as the skin disease panniculitis. It is considered the most common genetic cause of liver transplantation in children. Moreover, carrier subjects of deficiency alleles (MS and MZ) as well as homozygous and double heterozygous individuals for these deficiency alleles (SS, ZZ and SZ) are variably susceptible to a wide variety of other adverse health effects[18].

The prevalence of AAT deficiency is variable among different geographic areas and ethnic groups, where prevalence of the PI S allele is highest for countries in Southern Europe (Iberian Peninsula) and Sub-Sahara Africa. On the other hand, maximal PI Z frequencies (20-40 per 1000) are found in the southern regions of the Scandinavian countries as Denmark then Z prevalence steadily decreases from the west to the east of Europe, showing moderate values in some Central and Western Europe[19].

The aim of this work is to determine the frequency of S and Z mutations of AAT deficiency among children with variable presentation of liver diseases. This work also aims to adopt a simple molecular screening technique (ARMS) for diagnosis of AATD.

Subjects and Methods

The study included 45 patients (24 males and 21 females) who

were recruited from the hepatology outpatient clinic in Mansoura University Children's Hospital in the period between March 2012 to October 2013. Twenty-five aged and sex matched healthy controls were included in the study (11 males and 14 females), all have negative history of previous liver or chest diseases and negative family history. Signed informed consents were obtained from the legal gardeners of all children to be enrolled in the study. The research procedure was done in the Genetics unit, Mansoura University Children's Hospital.

Blood sample was taken from the patients and control to measure serum AAT by radial immune-diffusion (BINDARID; RN034.3) according to manufacturer's instructions. DNA was extracted from peripheral leucocytes for genotyping. Genotyping was performed by ARMS-PCR amplification of exon III (S allele) and exon V (Z allele). Specific primers were used with the following sequence: sense Z was 5' GCTGTGCTGACCATCGACA-3', sense non-Z was 5'-GCTGTGCTGACCATCGACG-3', anti- sense Z/non-Z was 5'-CCAGGGATTTACAGATCACATGC-3', anti-sense S was 5'-ATGATATCGTGGGTGAGTTCATTTA-3', anti-sense non-S was 5'-ATGATATCGTGGGTGAGTTCATTTT-3', sense-S/non-S was 5'-GAAGTCAAGGACACCGAGGA-3'. PCR was done with the following cycling conditions; 2 min at 96ºC, 30 cycles of 25 s at 96˚C, 60 s at 64.5˚C, 60 s at 72˚C and 2 min at 72˚C[20]. PCR products were then subjected to gel electrophoresis and photographed (figures 1, 2).

Statistical Analysis:

All statistical analyses were performed using SPSS version 20.0. Parametric data were expressed in mean ± standard deviation. Non parametric data were expressed in median, interquartile range. Normality of data was first tested by one sample K-S test. In addition, Mann-Whitney was used to compare continuous variables in two different groups. One way Anova and Kruskal Wallis test were used to compare continuous variables in three different groups. Chi-square tests were used to compare the categorical variables between both cases and controls. P value <0.05 was considered as statistically significant.

Results

There was a statistically significant difference in serum alpha-one antitrypsin between patients and control as shown in table (1).

Results of genotyping (table 2) revealed predominant MM genotype in both patient and control group (64.5% vs. 96%). Homozygous ZZ was detected in 2.2 of cases and not in control. Heterozygous MS genotype was present in 4.4% of cases and 4% of control, while heterozygous MZ was present in 17.8% of patients only. Double heterozygous SZ genotype was only present in patients (17.8%). Different allele frequency is listed in table (2).

Heterozygozity represented a risk for the development of liver disease as in table (3), with OR=12 (95% CI 1.5-97.4). The measured levels of serum alpha-one antitrypsin were concordant with the observed genotypes.

Table (1): Analysis of difference between patients and control as regard to serum level of Alpha-one Antitrypsin (Unpaired t-test).

	Patients Mean± sd	Control Mean± sd	T-value	P value
Serum AAT (g/L)	1.506± 0.512	1.867± 0.212	3.36	**0.001***

* p value is statistically significant if < 0.05.

Table (2): Genotypes and allele frequency in the studied groups.

Genotype	Patients N (%)	Control N (%)	P	OR	95% CI
MM	29 (64.5%)	24 (96%)	0.01*	0.07	0.01-0.6
MZ	8 (17.8%)	0	-	-	-
MS	2 (4.4%)	1 (4%)	0.9	1.1	0.09-12.9
ZZ	1 (2.2%)	0	-	-	-
SS	0 (0%)	0	-	-	-
SZ	5 (11.1%)	0	-	-	-
Total Heterozygous (MS+MZ)	10 (22.2%)	1 (4%)	0.07	6.8	0.8-57
Total Homozygous + compound heterozygous (SZ+ZZ)	6 (13.3%)	0	-	-	-
Allele Frequency					
M	68 (75.6%)	49 (98%)	0.3	0.8	0.5-1.2
S	7 (7.8%)	1 (2%)	0.2	3.8	0.5-32
Z	15 (16.6%)	0	-	-	-

Table (3): Odds of different genotype of AAT in the study groups.

	Patients N=45	Control N=25	OR	95% CI	X_2	P
Homozygous wild type (MM)	29 (64.5%)	24 (96%)	0.07	0.01-0.61	8.703	**0.006***
Heterozygous (MS, MZ, SZ)	15 (33.3%)	1 (4%)	12	1.5-97.4		
Homozygous mutant (ZZ)	1 (2.2%)	0	1.7	0.07-43		

* p value is statistically significant if < 0.05 (Continuity correction by Fisher's exact test).

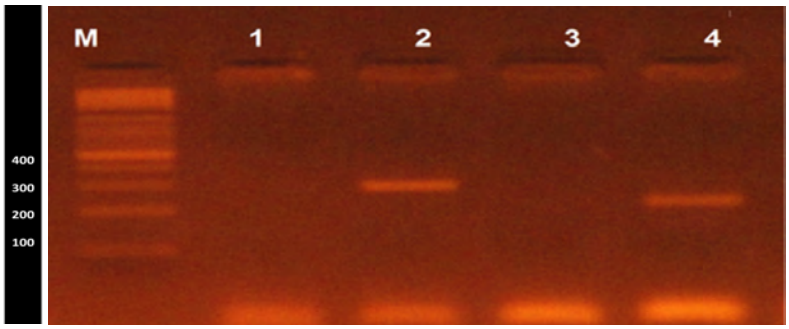

Fig. 1: AAT allele polymorphism by ARMS-PCR sowing non-S/non-Z genotype which is interpreted as wild type (MM) genotype.
- Lane M is DNA marker (100 bp ladder)
- Lane 1 and 2 represent Z allele
- Lane 3, 4 represent S allele
- Amplicon (band) at 288 bp is seen in lane 2 and stands for non Z genotype (absent Z allele)
- Amplicon at 221bp in lane 4 stand for non S genotype (absent s allele).Therefore, the genotype of this patient is non Z-non S genotype assuming MM genotype.

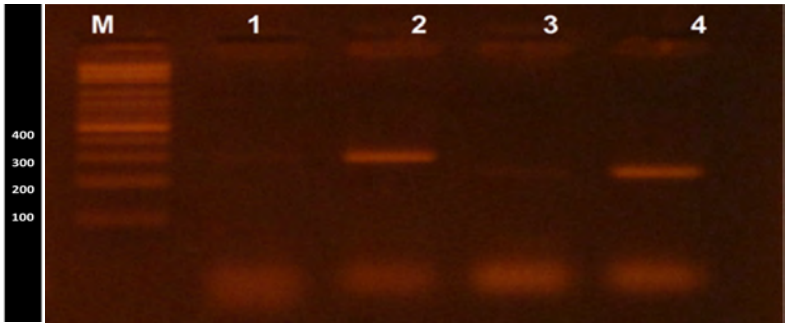

Fig. 2: AAT allele polymorphism by ARMS-PCR sowing SZ genotype.
- Amplicon at 288 bp are present in both lanes 1, 2 that represent heterozygosity at Z allele. Bands in lanes 3, 4 at 221 bp represent heterozygosity of S allele.

Discussion

In our study, we assessed the prevalence of AAT deficiency among children presented with variable liver affection after exclusion of contributing factors. The overall prevalence of disease-associated S mutation was 15.5% (7 cases), with two heterozygous (MS) patients (4.4%) and 5 compound heterozygotes SZ (11.1%) and was 4% in control group. The S-allele frequency was 7.8% in cases, 2% in control group, and this result is relatively lower than the previously reported results in Egypt. In the study conducted by Settin and colleagues among patients with HCV, the S-allele frequency was reported to be 26.04% vs 5.7% in control group[21]. Another study including pediatric and adult patients with cirrhosis revealed frequency of S allele to reach 24.1%, 27.1% in pediatric and adult cirrhotic patients respectively compared to 18.6% in the control group[18]. Interestingly, our study has documented no SS genotype either in patient or control group while it was higher in patients than control (10.4% vs 1.43%) in the studied children with cirrhosis[18].

The prevalence of homozygous Z genotype in the current study is estimated to be 2.2% and 0% in patients and control respectively. Heterozygous MZ genotype was present in 17.8% of cases. Z Allele frequency was estimated to reach 16.6% in patients and nil in control group. These findings are closely similar to the results observed previously as Z allele frequency was 14.8% in pediatric patients with cirrhosis and less frequent in adult cirrhotic patients (7.1%), and in healthy control subjects (8.6%)(18). In the current study, the prevalence of SZ compound heterozygosity was 11.1% in patients and absent in control group, while it was found in 7.4%, 0%, 11.4% in cirrhotic children, cirrhotic adults, and control respectively[18].

There were phenotypic-genotypic correlation between AAT genotype and severity of liver affection. The group of patients who had liver cirrhosis showed higher frequency of SZ compound heterozygosity. Moreover, the only ZZ patient in this study was assigned to have cryptogenic cirrhosis. These results are in agreement of the previously

published studies. In his prospective nationwide study, Sveger mentioned that 10-15% of Pi ZZ population developed clinically significant liver disease over the first 20 years of life[22]. There was a significant high relative risk noted in SZ and ZZ genotypes among patients with cirrhosis[18,23].

Conclusion

In conclusion, the present study supports screening of AATD in pediatric patients with unexplained liver diseases. Targeted mutation detection with ARMS-PCR technique is a simple reliable and relatively inexpensive diagnostic tool, together with measurement of serum AAT.

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ARMS TECHNIQUE FOR MOLECULAR SCREENING OF THE MOST COMMON ALPHA-ONE ANTITRYPSIN DEFICIENCY MUTATIONS AMONG CHILDREN WITH LIVER DISEASES

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THE ROLE OF LAPAROSCOPY IN DIAGNOSIS AND TREATMENT OF INTESTINAL MALROTATION

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Abstract

Aim of Work: *The aim of this study is to evaluate the laparoscopic diagnosis and treatment of intestinal malrotation.*

Patients and Methods: *This study included (30) patients who underwent definitive treatment for intestinal malrotation at the pediatric surgery units at Mansoura University Children Hospital and Alazhar University El-Hussein Hospital, Egypt during the period from October 2010 to September 2013. All patients were treated using laparoscopic Ladd's technique.*

Result: *In this study 25 cases were operated totally by laparoscope with operative time ranged from 123-150 minutes with a mean operative time 136 min. Seven patients (23%) developed intraoperative complications. There were five cases (17%) that converted to open method.*

Conclusion: *We believe that laparoscopic Ladd's procedure is a good choice for management of malrotation.*

Introduction

During intestinal embryological development, the midgut goes through the physiologic stages of: herniation, rotation, retraction of the herniated loops, and fixation. Disruption of these critical steps will lead to midgut rotational and fixation abnormalities[1]. The commonest presenting symptom, particularly in young patients, is bilious emesis; resulting from duodenal obstruction caused by peritoneal bands or from midgut

volvulus[2].

Exploratory laparotomy and the Ladd's procedure is the "gold standard" for treating malrotation with or without volvulus. The key steps of the procedure have not been changed since it was first described by Ladd and Gross in 1932[3]. Recent advances and experience in minimal access surgery have led to the development of techniques for successful correction of abnormalities of intestinal rotation and fixation. The first report of the laparoscopic management of malrotation was by Van Der Zee and Bax in 1995[4].

Patients and Methods

This study included (30) patients who underwent definitive treatment for intestinal malrotation at the Pediatric Surgery units at Mansoura University Children Hospital and Alazhar University El-Hussein Hospital, Egypt, during the period from October 2010 to September 2013. All patients were treated using laparoscopic Ladd's technique.

The present study included all patients who were diagnosed as intestinal incomplete malrotation without volvulus or with chronic volvulus; excluding cases with acute volvulus or those associated with major congenital anomalies.

In all patients the procedure was done using a laporoscopic approach under general anesthesia. The first port, 5-mm 30° scope was used for abdominal exploration to confirm the diagnosis of intestinal malrotation. This diagnosis was established by reporting the high and rather medial position of the cecum and appendix below the liver, to which they were attached by a peritoneal band. Furthermore, the second part of the duodenum was dilated, long and tortuous. In cases of malrotation without volvulus, there were ring like peritoneal bands encircling the duodenum, cecum and the ascending colon (Ladd's bands). These bands were exposed by traction of the duodenum to the right side and cecum and the ascending colon to the left side. Division of these bands was done along the whole length of the duodenum down to the duodenojejunal junction; by using the hook and the diathermy. The jejunum

is now in view. By pulling further and further on the duodenum and later on the jejunum, the whole small bowel obtained a right-sided position and the large intestine became on the left side. A last check was made to confirm whether the anterior mesentery had been widened enough. If not, the anterior leaf of the mesentery was further incised distally and the adjacent bowel further displaced to either side. Appendectomy was done either intracorporeal or extracorporeal.

In some cases, there was a volvulus of the mesenteric stalk in a clockwise direction. The volvulus had been maintained initially, as it provides good retraction on the bowel to the left side which facilitates dissection. All peritoneal bands between the liver, retroperitoneum, and bowel were identified then transected, and the duodenum was kocherized. Next the volvulus was undone in a counterclockwise direction. Care was taken not to open the mesentery of the ascending colon. At last, the whole duodenum as well as the jejunum became on the right side. A check for the final position of the intestine is performed at the end of the procedure to exclude kinked loop, bleeding or intestinal injury. An abdominal drain was inserted through the site of the left port in all cases. The pneumoperitoneum was evacuated and the ports were removed. The port sites were closed with fascial sutures and skin closure.

In this work, the technique of laparoscopic correction of malrotation had been evaluated as regard the operative technical point of view, post-operative outcome, early and late postoperative courses, complications and short term functional outcome, in order to assess its feasibility and safety.

Results

The present study included 30 cases diagnosed as intestinal malrotation with the age at time of operation ranging from 21 days to 8 years (mean age 21 months). Seventeen patients (57%) were males whereas thirteen patients (43%) were females with a male to female ratio 1.3:1. The body weight at time of operation ranged from 3.5 Kg to 26 kg with a mean of 9.3 kg. Five patients (17%) showed as-

sociated congenital anomalies in the form of congenital umbilical hernia in 2 children, hydrocele in 2 children and hypospadias in one child. Associated major life threatening congenital anomalies were excluded from this study.

Bilious vomiting was the presenting symptom in the majority of cases (87%), while only 2 patients (7%) presented by non bilious vomiting. Other presenting symptoms included malnutrition in 15 patients (50%), abdominal pain and failure to thrive in 9 patients each (30%), and abdominal distension in 3 patients (10%). Six patients (20%) had constipation, while 3 patients (10%) had diarrhea. No hematemesis or bleeding per rectum was reported in any case.

Upper GIT study was performed for all patients and it was found to be diagnostic in 28 patients (93%). In comparison, CT abdomen with intravenous (IV) and oral contrast, which also was performed for all patients, was found to be diagnostic in 25 patients (83%). All included cases also underwent abdominal doppler US examination. This study was diagnostic in 17 patients (57%). Barium enema was done for two patients with inconclusive results of the upper GIT study and it was diagnostic in only one case.

On the initial exploratory laparoscopy abnormal site of the cecum, Ladd's bands and dilated duodenum were reported in all cases. Narrow mesentery was found in 16 cases (53%), whereas chronic volvulus existed in 10 cases (33%). Furthermore, one case (3%) had chylous ascites.

In the current study 25 cases were operated totally by laparoscope. The operative time ranged from 123-150 minutes with a mean operative time 136 min.

Seven patients (23%) developed intraoperative complications in the form of intraoperative bleeding in 5 cases (17%), duodenal injury in one case (3%), and anesthetic complication with delayed recovery in another single case (3%). As regard duodenal injury, it occurred by the grasper teeth during duodenal kocherization and the duodenum was repaired after con-

version to open technique. There were five cases (17%) that converted to open method because of intraoperative bleeding in 4 cases in addition to the case of duodenal injury.

Oral feeding started between the 2nd and the 5th postoperative day with a mean time 3.3 days. Hospital stay ranged from 2 to 8 days with a mean hospitalization time 3.8 days.

Early postoperative complications were reported in nine patients (30%) (figure 1) with fibrinous adhesive intestinal obstruction being the commonest (10%) and without any reported mortalities.

Six patients (20%) developed late postoperative complications with 3% incidence of incomplete Ladd's procedure, intussusceptions, adhesive intestinal obstruction and recurrent volvulus, while two patients (7%) suffered from recurrent abdominal pain and distension.

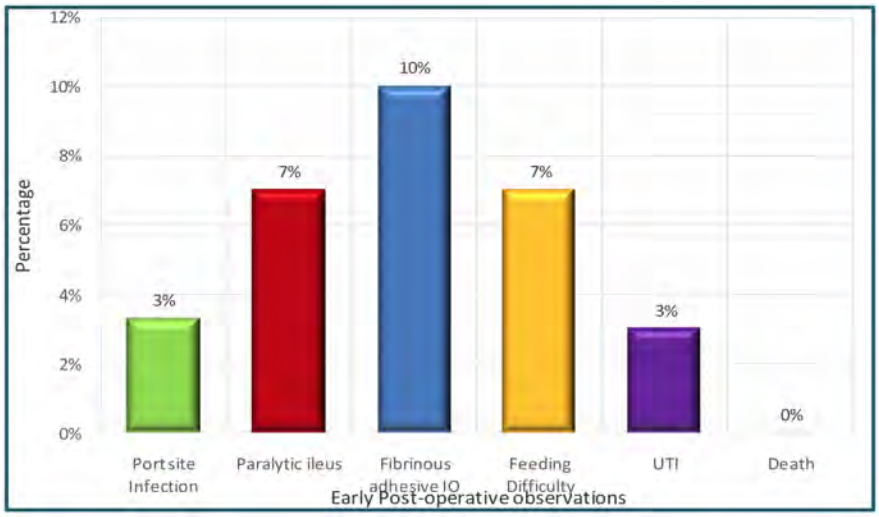

Fig. 1: Early post operative complications.

Discussion

In this study 25 cases were operated totally by laparoscope with operative time ranged from 123-150 minutes and a mean operative time of 136 minutes. These cases included 5 operations that started by laparoscope and converted to open method. Fraser et al., Bass et al., and Hagendoorn et al., reported a shorter mean operative time with average operating time (53±15 minutes, 58 minutes and 112±7.8 minutes) respectively[5,6,7]. Matzke et al., reported a longer mean operative time with average operating time 194 minutes for cases operated by laparoscope versus 143 minutes for cases operated by open method[8]. Longer operative time reoorted in this study is probably due to lack of experience in early cases, presence of chronic volvulus in some cases and also because of the very young age in the majority of cases with narrow working space.

In this study, there was one case (3%) with duodenal injury by grasper teeth. This is comparable with El-Gohary et al., who reported viscus injury in two cases (1.2%)[9]. Among the cases of the present study five cases (17%) were converted to open method. The causes of conversion included duodenal injury in one case in addition to intra-operative bleeding in the other 4 cases (13%). Intra-operative bleeding resulted from aberrant anatomy, marked dilated duodenum, difficult dissection and presence of volvulus. These consequences could not be managed by laparoscope due to lack of experience in early cases and so conversion to open method preferred. Draus et al., reported a lower conversion rate (12%) but Hagendoorn et al., reported a higher conversion rate (24%)[3,7].

In this study, nine patients (30%) developed early postoperative complications; this lie in the middle between the incidence of 24% reported by Murphy and Sparnon[11] and the 39% reported by Moldrem et al[12]. Early adhesive intestinal obstruction was the commonest early complication in this study; however this complication was not reported by by Moldrem et al[12]. On the other hand, Moldrem et al.[12] reported much higher incidence of paralytic ileus (27%) in comparison with the fig-

ure of 7% reported in this study. This may suggest possible misdiagnosis between early adhesive intestinal obstruction and paralytic ileus in either or both studies. Both Murphy and Sparnon[11] and by Moldrem et al[12] reported a similar incidence of port site infection (9%) which is higher than the 3% incidence that was reported in the current study. Murphy and Sparnon[11] also reported a 9% incidence of early postoperative feeding difficulties which is slightly higher than the present study (7%). There were no postoperative deaths in this study and this was also the same in other studies[11,12].

In the present study, six patients (20%) developed late postoperative complications; including incomplete Ladd's procedure, intussusceptions, adhesive intestinal obstruction and acute midgut volvulus with a similar incidence of 3% for each one of such complications. El-Gohary et al., reported a close incidence of adhesive small bowel obstruction (5.6%), and a lower incidence of recurrent volvulus (0.6%)[9]. However, Murphy and Sparnon reported a significantly higher incidence of small bowel obstruction (46%)[11]. Although this figure included both early and late cases, it remains much higher than the incidence of 13% reported in this study (10% early cases and 3% late cases).

In this study, reoperation was required for 3 cases (10%) (incomplete Ladd's, intussusceptions and volvulus). Fraser et al.[5], reported a lower reoperation rate (2.4%) while Kalfa et al.[10], reported higher reoperation rate (20%).

Conclusion

We believe that laparoscopic Ladd's procedure is a good choice for management of malrotation but we recommend more evaluation for long term results for these cases.

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THE ROLE OF LAPAROSCOPY IN DIAGNOSIS AND TREATMENT OF INTESTINAL MALROTATION

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NISSEN VERSUS THAL LAPAROSCOPIC FUNDOPLICATION FOR TREATMENT OF GASTROESOPHAGEAL REFLUX DISEASE IN CHILDREN

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Abstract

Introduction: *Gastroesophageal reflux disease (GERD) is a common condition in pediatric age group. Many surgeons believe that complete fundoplication provides better reflux control, yet results in more dysphagia and gas-bloat symptoms. On the other hand, a partial wrap is reported to have fewer adverse effects, but a higher failure rate in controlling reflux. Till now, there is no agreement and little evidence as to whether complete or partial fundoplication is the optimal procedure in this age group.*

Patients and Methods: *This is a prospective single blinded randomized comparative study that included 30 patients that were randomly managed laparoscopically by either of Nissen or Thal fundoplication.*

Results: *Operative time (minutes) was significantly longer in the Thal group (186±52) when compared to Nissen Group (150±48) (P 0.031). Intra operative complications showed no significant difference when comparing the two groups. Although the incidence of postoperative dysphagia was statistically insignificant, the duration of dysphagia did show statistically significant shorter duration in the Thal group (median 6 days), when compared to the Nissen group (median17 days). There were no recurrence in the Thal group versus one recurrence in the Nissen group, but this lead to no statistical significance*

Conclusion: *this study suggests that Thal fundoplication offers an effective alternative to Nissen fundoplication with apparently shorter duration of dysphagia and so earlier return to the normal eating pattern.*

Introduction

Gastroesophageal reflux disease (GERD) is a common condition in pediatric age group. It is defined as the pathologic consequences of the involuntary passage of gastric contents into the esophagus. Infants and children suffering from GERD may have growth retardation, esophagitis, reflux-associated reactive airway disease, aspiration pneumonia, and apnea which is sometimes prolonged and life-threatening. For these patients, surgical management is indicated[1].

Nissen fundoplication is the most popular laparoscopic operation for the management of GERD applying a complete wrap. Some surgeons believe that complete fundoplication provides better reflux control, yet results in more dysphagia and gas-bloat symptoms[2]. On the other hand, a partial wrap is reported to have fewer adverse effects, but a higher failure rate in controlling reflux. Fundoplication according to Thal is a commonly used procedure utilizing a partial wrap in the form of an anterior wrap for valve formation[3]. It is reported to achieve more effectiveness than the other fundoplication techniques[4]. Till now, there is no agreement and little evidence as to whether complete or partial fundoplication is the optimal procedure in this age group[5].

Patients and Methods

This is a prospective single blinded randomized comparative study that was conducted at the Pediatric Surgery Unit in Mansoura University Children Hospital, and included 30 patients who were admitted to the unit in the time period from October 2010 till October 2013 and diagnosed as having gastroesophageal reflux disease. Patients were managed laparoscopically either the Nissen fundoplication or Thal fundoplication procedures. All included patients were suffering from GERD and not responding to medical treatment or symptomatic Hiatus Hernia and the guardians consented to have a laparoscopic antireflux procedure. Patients with neurological impairment were excluded. Patients were divided into two groups of 15 patients each. Randomization was designed as assigning the first consecutive 15 cases to Nissen fundoplication[6] while the following 15 patients to Thal fundoplication randomly[7].

Patients of both groups were evaluated as regards operative time and complications as well as for post-operative Dysphagia, and its duration, bloating, vomiting and recurrence. Data was expressed as number and percent, median and range, or mean ± standard deviation as appropriate. Test of normalization was done using Shapiro-Wilk test of normality. Changes in qualitative data were compared using Chi square test, or Fisher Exact test if one value was less than five. Changes in quantitative date were tested using independent sample t test for parametric data, while, for non-parametric data, Mann Whitney U test was used.

Results

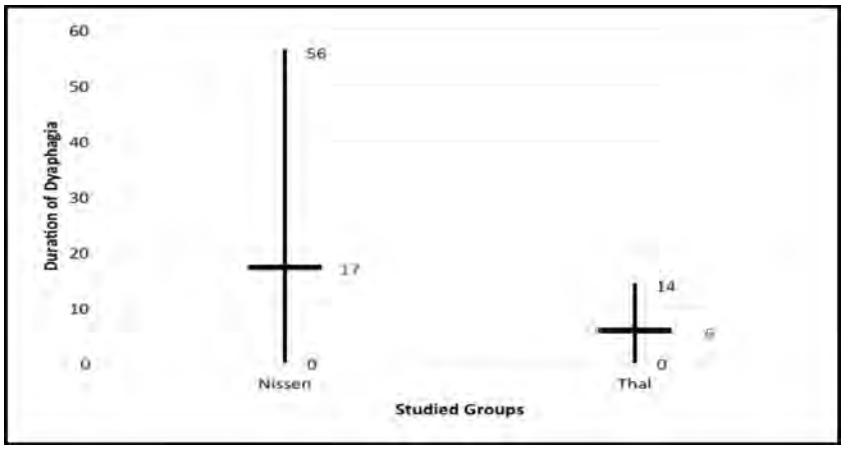
This study included 30 patients. The age (at time of operation) range was between 7 month and 6 years with the median age of 19 month. For gender distribution, 18 of them were males (60%) while the remaining 12 (40%) were females. The body weights (at time of operation) ranged from as low as 5 kilograms to as high as 21 kilograms with the mean weight of 10.7±4.2 kg. There was no statistical significance when the 2 groups were compared for demographic data.

Operative time (minutes) was significantly longer in the Thal group (186±52) when compared to Nissen Group (150±48) (P 0.031). Intra operative complications showed no significant difference when comparing the two groups.

In the Nissen group there were two cases with iatrogenic left pleural perforation which was managed by intercostal tube insertion with no effect on the procedure. In addition, one patient in the same group had bleeding during division of the short gastric vessels that couldn't be controlled laparoscopically, thus the operation was converted to the open approach and thus the patient excluded from the study. In the Thal group, one case had bleeding from an abrasion in the liver capsule that was controlled by mono-polar diathermy successfully. Another patient had iatrogenic tear in the anterior stomach wall during traction. That patient was converted to open approach and also excluded from the study.

Both dysphagia and bloating were found to be lower in the Thal group as in 5 (33.3%) and 3 cases (20%) respectively. Whilst, Nissen group was as high as 7 (46.7%) and 8 (53.3%) respectively. Meanwhile, early vomiting was similarly encountered in 4 patients of each group (26.7%). Despite this difference, no statistical significance was found between the study groups as regards either of early post-operative dysphagia, vomiting or bloating. Although the incidence of postoperative dysphagia was statistically insignificant, the duration of dysphagia did show statistically significant shorter duration in the Thal group, when compared to the Nissen group (P value 0.043). Dysphagia was present in 9 (60%) patients in the Thal group which is nearly equal to the Nissen group; 8 patients (56.7%). The duration of dysphagia in the Thal group showed a median duration of 6 days ranging from zero to 2 weeks, while in the Nissen group, the median duration was as high as 17 days ranging from zero to 56 days (Figure 1). The degree of dysphagia also varied. Most of patients was instructed to have soft food and used prokinetic drugs for their complaints. Only three cases required endoscopic evaluation, but none of them needed dilatation.

Vomiting was significantly lower in the Thal group (2 patients) compared to the Nissen group where there was vomiting of variable degree and frequency reported in 8 patients (P 0.025). Bloating was significantly less frequent in the Thal group (zero patients) when compared to the Nissen group (P 0.008). In this group 40% of patients (6 cases) suffered from bloating to a variable degree throughout their postoperative course. The number of patients that had to use medications to overcome postoperative vomiting or dysphagia (as prokinetic drugs or PPIs) was significantly lower in the Thal group (2 patients) when compared to the Nissen group (8 Patients) (P 0.025). There were no recurrent cases in the Thal group versus one recurrence in the Nissen group, but this lead to no statistical significance (P 0.500). In addition, comparing weight gain 2 weeks and two months post operatively showed no statistical significance among groups (P 0.419, P 0.417).



* Considered significant when P≤0.05.

Fig.1: Duration of Dysphagia in studied groups (days), Data expressed as no.

Discussion

Since the introduction of laparoscopic fundoplication in children in the early nineties[9], Nissen fundoplication has been the most commonly adopted procedure in many institutions all over the world. This can be attributed to its effectiveness and satisfactory long term results. However, the postoperative dysphagia and bloating frequently encountered raised the necessity for a more physiologic design of fundoplication. This is particularly important in children, as their ability to cope with distressing symptoms is limited. The development of Thal; as well as Toupet, partial wrap fundoplication gave surgeons an appealing alternative with seemingly similar effectiveness and much less postoperative side effects[10].

The hypothesis of the current study was whether the Thal anterior valve technique is of equal effectiveness as the Nissen fundoplication in addition to less dysphagia and bloating. The study included 30 patients that was assigned randomly to one of two groups: Group 1: Nissen Fundoplication Group 2: Thal Fundoplication.

In the current study, Operative time was found significantly long-

er in the Thal procedure (3 hours ± 52 minutes) than in Nissen (2 hours and 30 minutes ±48 minutes). By reviewing literature, only one comparative study considered operative duration as an aspect for comparison between the two techniques, yet, no significant difference was found in contrast to our study results. In that study, partial fundoplication showed a median of 142 (range, 78-710) minutes and complete fundoplication taking 120 (range, 60-205) minutes[5]. However, in the large retrospective comparative study by Esposito et al, the operative time ranged between one hour and 5 hours (median 70 minutes) without linking that wide variability to the type of wrap utilized. On the other hand, similar prospective studies on adult patients demonstrated no difference in operative time between the two techniques with generally shorter duration than in the current study[11].

In the current study, intra operative complication rate was 13.3% which was equal in the two study groups. The encountered complications ware in the form iatrogenic pleural perforation, bleeding during dividing the short gastric vessels, bleeding from hepatic abrasions and iatrogenic gastric perforation. Generally, the reported rate of these complications is between 0.5% and 11%. Bleeding, esophageal and gastric, vagus nerve injury, bowel injury, and pneumothorax have all been reported as intraoperative complications of laparoscopic antireflux surgery[12].

Moving to the outpatient follow up observations, dysphagia is the most common complaint which has been demonstrated in patients of either groups of the current study. It has been encountered in nearly 60% of patients with no difference in its incidence among either of the Thal or Nissen wrap cases. Meanwhile, the duration of dysphagia showed a wide variation in-between patients. Remarkably, post-Thal fundoplication patients reported earlier improvement of dysphagia, whilst post-Nissen patients suffering from dysphagia reported a significantly longer duration of this complaint. Kubiak et al[8] reported a significantly higher incidence of dysphagia after complete wrap of

11.8%; in a prospective study including 175 patients, compared to 2.4% after Thal fundoplication. Other studies reported no relation of dysphagia to the type of wrap [5,13]. Dysphagia after Thal procedure was reported in another study with an incidence of 2%[14]. Mauritz et al[15] studied 1280 patients in a meta-analysis where they reported postoperative dysphagia ranging from zero to 33% of patients. However, this rarely lasted after the first few months following surgery. In addition, they noted that post-operative dysphagia was more commonly reported after complete fundoplication than after partial fundoplication. Most of studies of adult patients generally show a lower incidence of post-operative dysphagia following a partial wrap when compared to a complete wrap[16]. Another prospective clinical trial even showed nil dysphagia after partial wrap versus 6% incidence after complete wrap[17]. Moreover, one prospective randomized double-blind trial between laparoscopic Nissen fundoplication and anterior partial fundoplication demonstrated earlier resolution of dysphagia after anterior wrap at 3 month of follow up[11].

In the current study, the high incidence of post-operative dysphagia can be explained by being overrated. This may be attributed to focusing on the presence of even any mild degree of difficulty in swallowing while leaving the child without strict limitation to various types of food. This is supported by observing that only one sixth of those patients (10%) had symptoms sever enough to require endoscopic evaluation. Moreover, all of them improved on follow up with no need for dilatation. Early dysphagia is mostly caused by postoperative edema, thus it is mostly transitory and resolves spontaneously with time. In contrast, in some cases with persistent symptoms, an over-approximation of the crura or a wrap that is too tight can be the cause[18]. Generally, pediatric patients undergoing laparoscopic antireflux surgery are expected to have not only a rapid recovery and return to normal activity, but also relief from gastroesophageal reflux symptoms equal to or better than that obtained with medical therapy. Thus, it is frustrating for the

surgeon and the patient alike when dysphagia occurs, or continues, postoperatively. Thankfully, with improvement in performing fundoplication with the rising learning curve, dysphagia rates fall significantly[19].

In the present study, postprandial epigastric fullness and discomfort; also described as bloating or gas bloat syndrome, was not encountered during the follow up of patients after Thal fundoplication. In comparison, 40% of Nissen fundoplication patients suffered from bloating to a variable degree throughout their postoperative course. This finding is in accordance to Watson et al [11], and Chrysos et al[20]. Generally, gas bloating, seen in 4% to 10% of cases, appears to be more common in the neurologically impaired[21]. Large body of literature documents a relatively higher incidence after complete wrap. Mathei et al[22] reported a 14% incidence of bloating in their series after complete wrap. Another earlier review reported a 35% incidence, but most of patients report fading out of symptoms over time Jones 23 [21]. Conversely, a recent meta-analysis including a total of 1280 children, no bloating was reported in any prospective study [15]. True gas-bloating syndrome is usually seen in neurologically impaired patients who are aerophagic. More commonly, patients who seem bloated are constipated and have a distended, air-filled colon rather than air in their stomach. These gas-bloating symptoms can be ameliorated by treating their constipation with fiber and appropriate laxative administration[24]. In addition, bloating and epigastric fullness are somewhat subjective symptoms and notoriously difficult to measure objectively. Inability to belch is perhaps a little more objective and is probably linked to the antireflux properties of an operation. Accordingly, inability to belch, is reported be lower after partial than complete fundoplication. The incidence of the increased flatus is usually found in correlation to bloating. However, this symptom often improves on follow up and as time progresses, it is anticipated that it will be less frequent. Furthermore, most adult patients reporting such symptom are rarely troubled by it[25].

In the current study, there was only one case of recurrence which was in the Nissen group (6.7%) with a revision rate of 3.3% of total patients. The cause of recurrence was wrap transmigration. This can be attributed to the presence of short esophagus in that particular case as she suffered from long standing esophagitis and esophageal stricture preoperatively. This case later had a laparoscopic redo where the hiatus was found to be wide and need repair. This finding is in contrast to other studies showing a significantly higher revision rate after partial wrap than in complete wrap (15.9% versus 5.9%)[8]. However, this study included a significant share of neurologically impaired children. On the other hand, other large studies showed no difference between either types of wrap (2.5%)[13]. In a study comparing the two techniques but on post-TEF repair patients, the need for reoperation was slightly more after Thal fundoplication (19%) than after Nissen (13%). However, this difference didn't reflect any statistical significance. Similarly, Coster et al[17] reported no difference between the two procedures in adult patients. It has been reported that recurrence show more likelihood in neurologically impaired children[26]. Conversely, this is not a constant finding in similar studies, most probably because of failure of controlling of potential confounding variables [27]. The most common cause of failure of laparoscopic fundoplication is transmigration of the wrap through the esophageal hiatus. There is some evidence that this complication is more common with the laparoscopic operation, perhaps due to decreased gastric adhesions with laparoscopy[28]. Wrap breakdown is another reported mechanism of failure. Decreased recurrence rates with the use of mesh have been shown in adults. Sutures between the esophagus and crura have been effectively utilized to minimize the failure rate[29].

Conclusion

The results of this study suggests that Thal fundoplication offers an effective alternative to Nissen fundoplication with apparently shorter duration of dysphagia and so earlier return to the normal eating pattern. Finally,

further studies with larger sample size, longer follow up and more objective post-operative evaluation will reveal more conclusive results.

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NISSEN VERSUS THAL LAPAROSCOPIC FUNDOPLICATION FOR TREATMENT OF GASTROESOPHAGEAL REFLUX DISEASE IN CHILDREN

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REIMPLANTATION OF AVULSED BRACHIAL PLEXUS ROOTS USING FIBRIN GLUE WITH TRANSPLANTATION OF STEM CELLS IN RATS

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Abstract

Introduction: *Brachial plexus root avulsion which can occur in both traumatic and obstetrical brachial plexus injuries remains a challenging problem to surgeons all over the world. So searching for a new method for repair supported by stem cells transplantation may be helpful for these patients.*

Materials & Methods: *This study was conducted in Mansora Research Center (MRC), Mansoura Faculty of Medicine. This study was carried out on 30 adult spraque Dawely rats divided into three groups; first group will be offered surgical reimplantation using micro-sutures, second group will be subjected for surgical reimplantation using fibrin glue and third group will be offered surgical reimplantation using fibrin glue combined with transplantations of highly purified neural stem cells.*

Results: *There is no significant difference in the clinical improvement in the three groups but pathological examination showed much more improvement in the third group which shows more rapid improvement in rats. Also, fibrin glue has the same effect as microsurgical sutures.*

Conclusion: *Reimplantation of avulsed roots of brachial plexus is a good method of brachial plexus repair specially if assisted by stem cells transplantation.*

Key words: *root avulsion, stem cells, fibrin glue, brachial plexus.*

Introduction

Brachial plexus root avulsion which can occur in both traumatic and obstetrical brachial plexus injuries remains a challenging problem to surgeons all over the world. Up till now a majority of neurotization procedures have been developed to reconstruct the function of injured plexus but due to the limited number of motor fibers of donor nerves, functional recovery is extremely restricted by the above procedures. To explore more effective fiber-offer, researchers have managed to reimplant the avulsed nerve roots into spinal cord and fortunately some encouraging but modest results are achieved upon rats, cats, monkeys and even human beings (Terzis and Kostopoulos, 2007).

Even in the hands of expert surgeons, the vast majority of patients with brachial plexus injuries remain handicapped, especially with regard to hand functioning. Further research is needed on innovative reconstructive procedures, especially for root avulsion treatments. Use of these new techniques, along with better understanding of central-peripheral function integration, may provide improved results and greater purposeful function for these patients in the future (Siqueira and Martins, 2011).

Neural stem and progenitor cells, particularly those derived from the human nervous system, have become highly appealing cells for the neuroscientist, both as cellular tools in drug discovery and developmental studies, and also as potential therapeutic elements for neurodegenerative conditions. These cells hold the promise of being able to replenish a cell-deficient environment, and also to supply bioactive molecules beneficial for the diseased recipient. In addition, they may also serve the purpose of expressing foreign genes, in the so-called ex-vivo gene transfer strategies. (Horner and Gage, 2000).

Based on that brief review of literature about sever brachial plexus injuries especially avulsion injuries and the modest result of the conventional microsurgical techniques and also the promising results of stem cells applications in various neurological disorders we decided to apply stem cells in

treatment of brachial plexus injuries hoping to improve the outcome of these patients

Materials and Methods

This study was conducted in Mansora Research Center (MRC), Mansoura Faculty of Medicine.

This study was carried out on 30 adult spraque Dawely rats each weighing 280-420 gram at the time of experiment and they were given standard diet, water adlibitum. They were subdivided into three groups.

1st group: will be offered surgical reimplantation using microsutures.

The 2nd group: will be subjected for surgical reimplantation using fibrin glue

The 3rd group: will be offered surgical reimplantation using fibrin glue combined with transplantations of highly purified neural stem cells.

The brachial plexuses of the rats will be approached posteriorly under general anesthesia using intraperitonial thiopental and atropine injection. After rats were deeply anesthetized fixed to card board through their limbs after elevation of the chest on small cylindrical pads to allow rat to respirate easily. Shaving of the hair on the back of the neck and scrubbing using betadine were done. Eye ointment was used to protect rats from exposure keratitis.

After skin incision we do subcutaneous dissection elevating two skin flaps. The cervical fascia is then encountered and incised to expose the paraspinal cervical muscles and the spinous processes the most prominent one is the spinous process of C2 then dissection through subperiosteal muscle stripping was done to expose spinous processes, laminae and facets of the cervical vertebrae of C3-C6with the C3 identified by an obvious nutrition foramina in its lamina.

To expose the roots of brachial plexus we should dissect laterally to be able to do laminectomy and partial facetectomy. The spinal cord and C5-C7 nerve roots will be exposed by elimination of C4-C6 vertebral laminas After

identification of C5-C6 nerve roots, they were avulsed gently from spinal cord by a little retractor. The avulsed C5 nerve root stumps were cut down and left alone. The C6 nerve root stump were trimmed sharply, and then reimplanted into spinal cord with the point being 1 mm posterior to the original site of anterior root and 0.5 mm of depth into the cord. The strategy of fixation of the root into the cord was different according to the group as described before. The next step is good haemostasis removal of the retractors then muscle closure in layers using 4\0 vicryl and anatomical closure without drain. After that the rat is transferred to a separate cage under a heater till full recovery

The motor power of the recovered rats were evaluated two days postoperative according to limping scoring system then these rats were monthly evaluated.

After six months we performed scarification of the rats is anesthetized and the old wound is reopened and explored. The cervical spinal cords and repaired nerve roots were dissected and obtained for histopathological examination. Sections from the spinal cord and emerging nerve roots were fixed in formalin 10% pathologically processed and paraffin sections were prepared and stained by H&E.

Results

Table (1) summarize the possible Intraoperative and postoperative complications and its incidence in our rats. Five of our rats suffered sever intraoperative bleeding in most cases through the nutrient foramen of C3 which was controlled by coagulation, fibrillar and bone wax. Exposure keratitis was found in two of our early cases which was then avoided by using eye ointment to protect their eyes during our long ptocedure. During performing laminectomy the spinal cord was injured in three of our rats two injuries were sever and the third was mild injury. Three of our rats showed wound infection. Two rats died one of them died intraoperative and the other died two weeks postoperative it had sever spinal cord injury.

First group:

This group contains ten rats sex females and four males. We

faced sever bleeding and spinal cord injury in 1st case in this group. Exposure keratitis occurred in one case. One rat arrested intraoperative and all trials of resuscitation failed.

Second group:

This group contains ten rats two females and eight males. We faced sever bleeding in one rat and spinal cord injury in another one in this group. Exposure keratitis occurred in one case.

Third group:

This group contains ten rats one females and nine males. We faced sever bleeding in two rat and spinal cord injury in one in this group.

Clinical improvement:

Table (2) shows the clinical improvement in the three groups which was judged according to improved limping and the ability of rats to use these limbs during eating. Our follow up shows no significant difference between the three groups. But what was significant is the rapid improvement occurred in the third group which occurred in 3-5 weeks in comparison two the other groups which took 6-8 weeks to improve.

Pathology Result:

Sections from the spinal cord and emerging nerve roots were fixed in formalin 10% pathologically processed and paraffin sections were prepared and stained by H&E.

The spinal cords were examined as well as the emerging nerve roots revealing intact cords, identified repaired roots connected to the cord with regenerated neurons and healthy sprouting rootlets containing myelinated fibers of several diameters and intraneural blood vessels evenly distributed.

Also new vascularizations were identified during pathological in two special sites at the surface of the spinal cord at the site of the repaired roots and inside the regenerating roots.

The other very interesting finding is the regeneration of some laminas which were identified during resurgery of some rats. These new laminas were formed of thin transparent bone. By pathological examination it was found to be newly formed metaplastic bone.

Table (1): Shows possible intraoperative and postoperative complications.

Complications	Sever bleeding	Exposure keratitis	Spinal cord injury	Wound infection	Mortality
No	5	2	4	3	2
%	16.6%	6.6%	13.3%	10%	6.6

Table (2): Clinical improvement.

	Group 1		Group 2		Group 3	
	Improved	*Not Improved*	*Improved*	*Not Improved*	*Improved*	*Not Improved*
No.	6	3	6	3	7	3
%	66.6%	33.4%	66.6%	33.4%	70%	30%

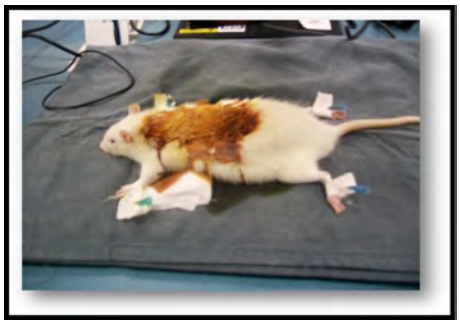

Fig. 1: Rat positioning.

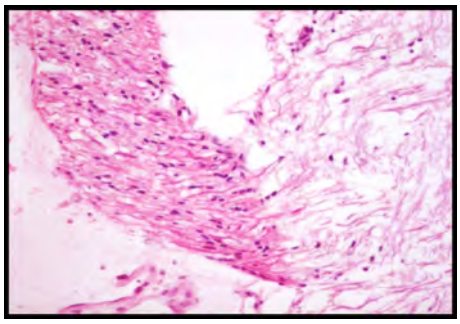

Fig. 2: Repaired healthy root with intact connections to the spinal cord.

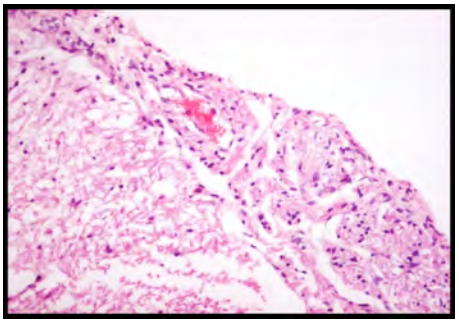

Fig. 3: New vascularizations at the surface of the cord at the site of sprouting roots.

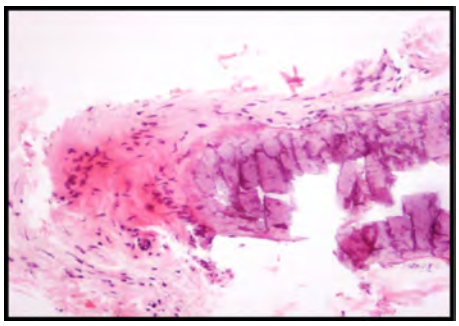

Fig. 4: Sheets of metaplastic bone.

Discussion

Reimplantation of the avulsed nerve roots into spinal cord has been attempted to treat brachial plexus root avulsion experimentally since 1970s. Fortunately some encouraging but modest results have been achieved upon animals such as rats, cats, and even monkeys. Following root avulsion, motor neurons respond with a changing polarity towards production of axons, sometimes even from the dendritic tree. Also avulsion usually leaves tufts of the most proximal parts of roots attached to the spinal cord surface. All these may help injured motor neurons regrow their axons into the implanted ventral roots on the surface of the spinal cord (Bertelli and Ghizoni, 2003).

It is proven to be difficult to obtain ideal functional recovery only by reimplanting the avulsed nerve roots back into spinal cord, because the microenvironment in injured spinal cord may become non-permissive and neurons necrosis may occur after brachial plexus root avulsion. Additionally, the mature neurons are destined to be terminal type without capacity of dividing and regenerating, lost neurons can not be replaced by survived ones (Li et al., 2007).

Transplantation of bone marrow stromal cells (BMSCs) or Schwann cells (SCs) can facilitate axonal regeneration in peripheral nerve injuries. The aim of this study was to compare the effects of transplantation of BMSCs and SCs on functional recovery after injury to the sciatic nerve in the rat. Electrophysiological and functional assessments showed a significant difference between the experimental groups compared with the control group. Electrophysiological measures were significantly better in the SCs transplantation group compared with the BMSCs treatment group. Functional assessments showed no statistically significant difference between the BMSCs and SCs groups. (Zarbakhsh et al., 2012).

Chen Lei et al. in their work proved that reimplantation of avulsed nerve roots combined with transplantation of neural stem cells modified by neurotrophin-3 gene is a relatively effective way to treat brachial plexus root avulsion

experimentally is most of the animals (83.3%) achieved partial biceps flexion function (Chen et al., 2008).

This result is comparable to our third group in which we used fibrin glue and stem cells for repair in this group 70% of rats were improved the difference may be due to the genetic modification of the used stem cells.

During re-exploration of a rat of the third grop we found a thin layer of transparent newly formed bone at the site of the previously removed lamina it was removed and sent for pathology which revealed newly formed metaplastic bone.

By comparing the results of the three groups we found no significant difference in the clinical improvement on the other hand pathological examination showed remarkable changes in the third group at the level of the number and size of the regenerated nerve bundles the number of nerve cells and the newly formed blood vessels. This can be explained by the fact that the biceps function is not a fine movement that need highly sophisticated nerve supply or that we need in the future to increase the number, purification or to use some suitable growth factors to achieve clinical improvement.

Now it is clear that in avulsion injuries the problem is the deficiency of the donor motor nerves so re-implantation of the avulsed roots can help to solve this problem. Fibrosis and necrosis at the site of trauma make regeneration and re-growth of the re-implanted roots very difficult and this explain the poor results of re-implantation. The combined use of fibrin glue and stem cells may help us in improving the results of this procedure.

Conclusion

Sever brachial plexus injuries are still unsolved surgical problem facing surgeons all over the world. Root avulsion which can occur in both obstetrical and traumatic injuries is a challenging injury due to deficiency of motor donors. Stem cells hold the promise of being able to replenish a cell-deficient environment, and also to supply bioactive molecules benefi-

cial for the diseased recipient. Reimplantation of avulsed brachial plexus roots combined with transplantation of stem cells may be available method for treatment of sever brachial plexus injuries.

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REIMPLANTATION OF AVULSED BRACHIAL PLEXUS ROOTS USING FIBRIN GLUE WITH TRANSPLANTATION OF STEM CELLS IN RATS

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EXPRESSION OF LIVIN IN CHILDHOOD ACUTE LEUKEMIAS: ANALYSIS OF RISK FACTORS

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Abstract

Background/Aim of the Study: *Livin, a member of the inhibitor of apoptosis proteins (IAP) has been considered to be a poor prognostic marker in malignancies. However, a little is known about the clinical relevance of Livin expression in childhood acute leukemia. This work was to assess the expression of Livin on leukemic blasts of de novo childhood acute leukemia and its relevance to clinical and hematological findings, and treatment outcome.*

Patients and Methods: *The expression of Livin was analyzed in 100 patients with newly diagnosed acute childhood leukemia (80acute lymphoblastic leukemia and 20 acute myeloblastic leukemia) using quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR).*

Results: *The results of the study revealed that the expression levels of Livin were higher in ALL patients with favorable prognostic factors. Furthermore, Livin expression was associated with a favorable early response to chemotherapy (leukemic blast <25% day 7 bone marrow response) [P<0.001]. Patients with +ve Livin expression were associated with significantly higher CR rate (P = 0.001) than those with -veLivin expression. While in AML patients Livin was neither significantly higher in patients with favorable prognostic factors nor better treatment outcome [P=0.054]*

Conclusions: *This study suggests that Livin expression could be a novel favorable prognostic marker in childhood acute lymphoblastic leukemia and thus, it could be incorporated into the patient stratification and treatment protocols, however not for acute myeloblastic leukemia.*

Keywords: *Acute lymphoblastic leukemia, Acute myeloblastic leukemia, Livin, RT-PCR.*

Introduction

Acute leukemia is an aggressive disease with generally poor prognosis. Acute lymphoblastic leukemia (ALL) is the most common type of pediatric malignancy, occupying 25% of childhood cancers and approximately 75% of childhood leukemia (Rana et al., 2009). 79% of these cases are acute lymphoblastic leukemia (ALL), followed by acute myeloblastic leukemia (AML) (Smith et al., 1999 & U.S. Cancer Statistics Working Group, 2003).

The outcome of childhood acute leukemia shows dramatic improvement over the last decades. ALL achieves an overall survival of over 80%. This success was achieved, in part, through the implementation of risk-stratified therapy. Patients presenting with features that are associated with a higher risk of relapse receive more intensive treatment, while those with features linked to a more favorable outcome are treated with more modest, less toxic therapy (Silverman et al., 2010).

The prognosis of children and adolescents with AML has improved significantly over the past decades with survival up to 65% of pediatric AML patients. This has been achieved not only by the more effective use of anti-leukemic agents, but also by improvements in supportive care and by better risk-group stratification (Kaspers and Zwaan, 2007).

As a result of the accumulation of knowledge on the molecular biology of malignancies, new diagnostic modalities are beginning to be incorporated into diagnostic and therapeutic strategies (Golub, 2001; Ramaswamy and Golub, 2002 & Ebert and Golub, 2004). One of these modalities is the quantitative reverse transcriptase polymerase chain reaction (RT-PCR) that allows the determination of messenger RNAexpression levels and, therefore, allows researchers to examine the expression patterns of a large number of genes at the RNA level (Choi et al., 2007).

A family of intracellular antiapoptotic proteins that has become increasingly prominent in the field of cancer is the inhibitor of apoptosis protein (IAP) family. IAP family members are able to inhibit apoptosis induced by a variety of stimuli mainly by binding and inhibiting

specific caspases, primarily 3, 7, and 9 (Nachmias et al., 2004).

Livin is one of the IAP family members. Cleavage of Livin results in truncated protein (tLivin) this form is pro-apoptotic upon apoptotic stimuli such as etoposide and NK cell cytotoxicity. As Livin (ML-IAP) plays adual role in tumorogenesis (antiapoptotic in the full length and on the other hand proapoptotic in the cleaved or truncated form) it may serve as potential target for cancer therapy. Cleavage of Livin, mediated by natural killer cell activity leads to apoptosis of the tumor cells and inhibition of tumor development. So, it can be implicated in Immune-mediated tumor destruction which can induce regression of tumors (Abd-Elrahman, 2009).

The clinical relevance of livin expression is still controversial in different types of malignancies. For example, livin expression was associated with poor clinical outcomes in neuroblastoma, whereas the livin expression level was not related to the survival of patients with nasopharyngeal cancer (Kim et al., 2005).

The aim of this study was to assess the expression of livin in peripheral blood and bone marrow samples from de novo childhood acute leukemia (ALL) and acute myeloid leukemia and comparing it with clinical and hematological findings, response to therapy and patients outcome.

Patients and Methods

From Novembre 2011 to August 2012, a total of 100 patients with newly diagnosed acute leukemia were recruited from pediatric clinical hematology unit, Mansoura University Hospitals, Egypt. The diagnosis was made based on the morphologic findings from Wright-Giemsa-stained smears of bone marrow aspirates and immunophenotype analyses of leukemic cells. Informed consent was obtained from every patient for laboratory studies according to the guidelines of Committee of Medical Ethics of Mansoura University Hospitals.

Blood sampling:

Peripheral blood (PB) or bone marrow samples were withdrawn from the patients before they received any treatment. The samples were divided into two aliquots. The first aliquot of blood was collected on vacutainer tube containing eth-

ylene diamine tetra-acetic acid (EDTA) for determination of hemoglobin, platelets and WBCs count, immunophenotyping, cytogenetics and livin expression. The second aliquot was collected on plain vacutainer tube for serum preparation for LDH determination.

Methods:

Patients were subjected to the following:

I. Full history taking with emphasis on presence of leukemia associated symptoms (fever, pallor, bleeding tendency, bone aches).

II. Thorough clinical examination with stress on the presence and extent of leukemia involvement including organomegaly (liver and spleen), lymphadenopathy and CNS infiltration.

III. Imaging studies: Chest radiography, testicular & renal ultrasonography, echocardiogram and ECG for patients with congenital or acquired heart disease.

IV. Laboratory investigations including:

1. Complete blood count: by (Sysmex N21) automated cell counter with examination of Leishman stained peripheral blood smears.

2. Bone marrow aspiration with examination of Leishman stained films.

3. Cytochemical examination: of peripheral blood and/ or bone marrow smears.

4. CSF examination for blast cells.

5. Evaluation of liver and kidney function using (Dimension E. S. chemical autoanalyzer).

6. Cytogenetic analysis using G banding technique: PB and BM samples on Lithium heparin were studied by G–Banding analysis.

7. Immunophenotyping was carried out by flow cytometry technique using diagnostic kits supplied by Beckman Coulter, Fullerton, CA, USA [15]. For establishment of the diagnosis, The following labeled monoclonal antibodies were used: triple color labeling specific markers for the three lineage groups of cells: cCD3,cCD79a and Myeloperoxidase, B-cell associated markers (CD19 and CD20), T-cell associated markers (CD3, CD5, CD7), Myeloid associated markers (CD13, CD33), monocytic marker (CD14), early markers (CD34, HLA-DR, CD10, TDT) and isotypic control markers IgG1 & IgG2a. The cut-off point to consider a sample positive for a certain marker was its expression by 20% of gated cells.

8-Serum lactate dehydrogenase was determined kinetically using commercially available kit supplied by Egy-Chem diagnostics, Egypt.

RNA isolation and real-time quantitative RT-PCR:

Mononuclear cells (MNCs) were isolated from 3 mL peripheral blood at diagnosis by Ficoll density gradient centrifugation. Total RNA was extracted from MNCs using RNAeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. One microgram total RNA was transcribed into cDNA using random hexamers and multiscribe reverse transcriptase enzyme (High Capacity cDNA Kit; Applied Biosystems, Foster City, CA, USA) under standard conditions and stored at -20 ˚C until use. Each sample was analyzed in duplicate; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and livin were co-amplified in the same tube. GAPDH probe (FAM- 5 CAAGCTTCCGTTCTCA-GCC-3-TMRA) with GAPDH forward (5-GAAGGTGAAGGTCGGAGTC3) and reverse (5-GAAG-AT GGTGATGGGAT-TTC-3) primers, and the predeveloped Assays-on-demand Gene Expression Set for the livin gene (Hs00223384 m1; GenBank accession no. NM 139317; Applied Biosystems). The reaction mixture was made in a final volume of 50 µl containing 5 µl cDNA, 400 µMof each primer and 25 µl Taq PCR master mix (2.5 units Taq DNA polymerase, 1x PCR buffer, and 200 µM of each dNTP) (Taq PCR master kit, Qiagen).

Reactions were performed using Real-time PCR 7000 sequence detection system (Applied Biosystems). Amplification was carried out at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 PCR cycles at 95°C for 15 seconds, and 60°C for 1minute. Livin mRNA expression was normalized to the simultaneously analyzed GAPDH. The comparative cycle threshold (CT) method was used to determine the relative expression levels of livin, and the cycle number difference (ΔCT=CT livin - CT GAPDH) was calculated for each sample. Relative livin expression values are expressed as 2 (ΔCT)

RESULTS

The relationship between Livin expression and other prognostic factors among ALL patients:

Livin mRNA was expressed in 53 (66.25%) of (80) ALL patients. Table (1) correlates between median level of Livin expression and other prognostic factors among ALL Livin positive group. The median Livin expression was higher in an age range [from 1 to <10 year] than those <1 or ≥10 years (P=0.017), also significantly higher in standard risk group than high risk one (P=0.019) and in pre- B ALL patients than those with T ALL (P=0.049). While no significant difference with respect to the sex, leukocyte count at diagnosis and cytogenetics (P=0.8, 0.09 and 0.15 respectively).

Furthermore, Livin expression was associated with a favorable early response to induction chemotherapy. The Livin expression was higher in patients with favorable response (leukemic blast <25%) on day 7 bone marrow than in patients with an unfavorable response (leukemic blast ≥25%) (The median was 130.99 versus 4.60 respectively, P<0.001).

Table (2) compares between prognostic factors among patients who expressed Livin (53) 0f 80 patients and those who didn't express (27). There was no significant difference as regards to the sex of negative and positive Livin patients 12male and 15 female versus 27 male and 26 female (P=0.582). The number of patients aged from 1year to <10 years was 18 and 9 patients aged less than 1 year or at least 10 years old among negative Livin group versus 47 and 6 among positive Livin one respectively (P<0.001). 10 patients with leukocyte count less than 50k/uL at diagnosis while 17 patients with leukocyte count at least 50k/uL or more at diagnosis among negative group compared to 45 and 8 among positive Livin group respectively (P<0.001).

There was significant difference between negative and positive Livin expression groups with respect to the immunophenotyping; 15 patients diagnosed as T ALL while 12 diagnosed as pre B ALL compared to 17 and 39 respectively (P=0.043). Also significant difference as regards to the cytogenetic analysis was observed: t(12,21) was expressed in 15 Livin negative patients versus 43 Livin positive patients, while t(9,22) was positive in 6 versus 2, 11q23 rearrangement was positive in 4 versus

4and other or no translocations were found in 2 vesus 4 respectively (P=0.034).

CSF involvement was reported in 2 of Livin positive patients while no reported cases in Livin negative group and mediastinal masses were detected in 2 of both groups , however this is insignificant (P=0.307 and P=0.481 respectively). Early response to chemotherapy using BM examination at day 7 shows significant difference among both groups (leukemic blasts <25% in 42 patients expressing Livin while only in 4 patients who didn't express Livin versus 11 patients and 23 with leukemic blasts ≥ 25% respectively) (P<.001).

The relationship between Livin expression and other prognostic factors among AML patients:

Livin mRNA was expressed in 11(55%) of 20 AML patients. Correlation between prognostic factors and median Livin expression levels among AML Livin positive patients was demonstrated in table (3). The median Livin expression level was significantly higher among Patients from 1 to <10 years (P=0.042), males (P=0.032), patients with -ve HLA-DR&CD34 (P=0.032) and t(8,21) (P=.042) compared to age group <1 or ≥10 years, females, HLA-DR or CD34 +ve patients and patients with absent or having translocations other than t(8,21). While no significant difference as regards to CSF involvement (P=0.474) and leukocyte count (P=0.7).

No statistical significance between positive and negative Livin expression levels among AML patients with respect to the age (P=0.653), sex (P=0.822), leukocyte count (P=0.194), CSF involvement (P=0.822), immunophenotyping (P=0.181) or cytogenetic analysis (P=0.964) table (4).

The effect of Livin expression on ALL patients' outcome:

Significant difference was reported as regards to treatment outcome between positive and negative Livin expression groups table (5). 37 of 53(69.8%) Livin positive ALL patients while 11of 27(40%) Livin negative ones achieved complete remission, 4 (7.5%) versus 14(51.9%) were relapsed respectively. 2 (7.5%) of 27 Livin negative patients were refractory while no refractory cases

among positive group.12 patients died during the induction course of chemotherapy (1st month) before being evaluated among Livin positive group versus no patients among the negative group (P<0.001) while no significant difference in the total death between the two groups 12 versus 8 respectively (p=0.495).

The effect of Livin expression on AML patients' outcome:

Table (6) compares treatment outcome between positive and negative Livin expression AML patients. 5 of 11Livin Positive group while only 1 of 9 negative one achieved complete remission, 2 versus 5 were relapsed respectively and 2 were refractory among Livin negative patients. 4 patients died during the induction course of chemotherapy before being evaluated among the positive group and 1 among the other group with borderline significant results (P=0.054). Total number of death cases were 4 among positive group and 8 among the negative one (P=0.017).

Table (1): Correlation between ALL patients' characteristics and Livin expression levels according to their prognostic significance.

Patient's characteristic	No	Livin expression level	*P* value
Age			
<1 or ≥10 years	6	8.17 [4.6 – 8.6]	0.017
1 to <10 years	47	127.67 [0.84 – 519.10]	
Sex			
Male	27	127.67 [0.84 – 350.40]	0.824
female	26	77.10 [0.84 – 519.10]	
WBC count:			
<50k/uL	45	128.13 [0.84 – 519.10]	0.091
≥50k/uL	8	10.65 [8.17 – 110.60]	
Risk group:			
Standard risk	43	128.13 [0.84 – 519.10]	0.019
High risk	10	8.60 [4.6 – 110.6]	
Immunophenotyping:			
Pre –B cell	36	130.99 [0.84 – 519.10]	0.049
T cell	17	12.70 [4.30 – 320.40]	
Cytogenetics:			
t(12,21)	43	110.6 [0.84 – 519.10]	
t(9,22)	2		0.156
t(11q23)	4	19.21 [8.17 – 30.24]	
others	4	210.85 [187.4 – 234.3]	
Day 7 BM response to treatment:			
<25%	42	130.99 [0.84 – 519.10]	< 0.001
≥25%	11	4.60 [0.84 – 12.70]	

(Mann-Whitney *U* test)

Table (2): ALL Patients, characteristics: Comparison between positive and negative Livin expression groups.

Characteristic	Acute lymphoblastic leukemia with -ve *Livin* expression n= 27	Acute lymphoblastic leukemia with +ve *Livin* expression n= 53	*P* value
Sex			0.582
Male	12 (44.4%)	27 (50.9%)	
Female	15 (55.6%)	26 (49.1%)	
Age			< 0.001
1-<10 y	18 (66.7%)	6 (11.3%)	
<1 or ≥10 years	9 (33.3%)	47 (88.7%)	
WBC count:			< 0.001
<50.000/mm^3	10 (37%)	45 (84.9%)	
≥50.000/mm^3	17 (73%)	8 (15.1%)	
Immunophenotyping			0.043
T ALL	15 (56.6%)	17 (32.1%)	
Precursor B ALL	12 (44.4%)	39 (67.9%)	
Cytogenetics			0.034
t(12,21)	15 (55.6%)	43 (81.1%)	
t (9,22)	6 (22.2%)	2 (3.8%)	
11q23 rearrangement	4 (14.8%)	4 (7.5%)	
Others	2 (7.4%)	4 (7.5%)	
CSF involvement			0.307
Yes	0 (0%)	2 (3.8%)	
No	27 (100%)	51 (96.2%)	
Mediastinal involvement			0.481
Yes	2 (7.4%)	2 (3.8%)	
No	25 (92.6%)	51 (96.2%)	
Day 7 BM response to treatment			< 0.001
Leukemic blast < 25%	4 (14.8%)	42 (79.2%)	
Leukemic blast≥25%	23 (85.2%)	11 (20.8%)	

(Pearson chi- square test)

Table (3): Correlation between AML patients' characteristics and Livin expression levels according to their prognostic significance.

Patient's characteristic	Number of patients	Livin expression level	*P* value
Age			
<1 or ≥10 years	6	17.6 [16.03 – 28.05]	0.042
1 to <10 years	5	75.3 [16.98 – 135.8]	
Sex			
Male	9	28.05 [16.98 – 135.8]	0.032
female	2	16.03	
CSF involvement			
No	9	28.05 [16.98 – 135.8]	0.474
Yes	2	17.60	
WBC count:			
<50.000/mm^3	7	28.05 [16.98 – 135.8]	0.702
≥50.000/mm^3	4	46.45 [17.60 – 75.30]	
Immunophenotyping:			
HLA-DR+ve	0		
CD34+ve	2	16.03	0.032
-ve HLA-DR&CD34	9	28.05 [16.98 – 135.8]	
Cytogenetics:			
t(8,21)	5	75.30 [16.98 – 135.8]	0.042
Others	6	17.6 [16.03 – 28.05]	

(Mann-Whitney *U* test)

Table (4): AML Patients, characteristics: Comparison between positive and negative Livin expression groups.

Characteristic	Acute myeloid leukemia with -ve *Livin* expression n= 9	Acute myeloid leukemia with +ve *Livin* expression n= 11	P
Age			
<1 or ≥10 years	4 (44.4%)	6 (54.5%)	**0.653**
1 to <10 years	5 (55.6%)	5 (45.5%)	
Sex			
Male	7 (77.8%)	9 (81.8%)	0.822
female	2 (22.2%)	2 (18.2%)	
CSF involvement			
No	7 (77.8%)	9 (81.8%)	0.822
Yes	2 (22.2%)	2 (18.2%)	
WBC count:			
<50k/uL	8 (88.9%)	7 (63.6%)	0.194
≥50 k/uL	1 (11.1%)	4 (36.4%)	
Immunophenotyping:			
HLA-DR+ve	1 (11.1%)	0 (0%)	0.181
CD34+ve	4 (44.4%)	2 (18.2%)	
-ve HLA-DR&CD34	4 (44.4%)	9 (81.8%)	
Cytogenetics:			
t(8,21)	4 (44.4%)	5 (45.5%)	0.964
Others	5 (55.6%)	6 (54.5%)	

(Pearson chi- square test)

Table (5): Treatment outcome among ALL patients: Comparison between +ve and -ve Livin expression groups.

Treatment outcome	Acute lymphoblastic leukemia with -ve *Livin* expression n= 27		Acute lymphoblastic leukemia with +ve *Livin* expression n= 53		X^2	p
	n.	%	n.	%		
Complete remission	11	40.7	37	69.8	28.164	<0.001
Relapse	14	51.9	4	7.5		
Refractoriness	2	7.4	0	0		
Death before evaluation	0	0	12	22.6		
Total death	8	29.6	12	22.6	0.466	0.495

(Pearson chi- square test

Table (6): Treatment outcome among AML patients: Comparison between +ve and -ve Livin expression groups.

Treatment outcome	Acute myeloid leukemia with -ve *Livin* expression n= 9		Acute myeloid leukemia with +ve *Livin* expression n= 11		X^2	p
	n.	%	n.	%		
Complete remission	1	11.1	5	45.5	7.629	0.054
Relapse	5	55.6	2	18.2		
Refractoriness	2	22.2	0	0		
Death before evaluation	1	11.1	4	36.4		
Total death	8	88.9	4	36.4	5.690	0.017

(Pearson chi- square test)

Discussion

In the current study we found that the expression levels of livin were higher in patients with favorable prognostic factors. This is in agreement with Choi et al. (2007) who showed that livin expression has independent favorable prognostic significance in childhood acute lymphoblastic leukemia. They recorded associations of this protein with several recognized favorable features of ALL patients: female sex, age 1 to 10 years, leukocyte count less than $50x10^9$/L, and the presence of t(12;21). It was associated with an increased apoptotic response to prednisolone treatment, a rapid early treatment response in vivo, and more importantly, a very favorable treatment outcome. Livin expression retained independent prognostic significance in a multivariate analysis.

Shurtleff et al. (1995) found

that livin expression rate was significantly higher in patients with favorable prognostic factors. It is particularly interesting to note that the Livin expression rate was very high in t(12;21) and was very low in t(9;22)/11q23 rearrangement, because these translocations are known to be strongly associated with the best and worst outcomes, respectively.

Most studies on the clinical relevance of livin expression, albeit limited in number, have shown that livin is an antiapoptotic regulator; therefore, Livin expression has been considered to be a poor prognostic factor in malignancies. Therefore, the observations from the current study were quite unexpected and suggest that the role of Livin in the apoptosis system in leukemogenesis or in the maintenance of leukemic cells might be different from what has been previously recognized. The possible explanation of the favorable prognosis of livin expression is that the cleaved form of livin may serve as a proapoptotic regulator.

The study of Kasof and Gomes (2001) showed that silencing of the livin β-isoform, but not the α-isoform, by RNA interference blocked the growth of HeLa cells in clonogenic survival assays, and sensitized the cells to various proapoptotic stimuli. A reasonable corollary of this idea is that leukemic lymphoblasts preferentially express the α-isoform of Livin, rendering them more susceptible to apoptosis.

Several investigators reported that some of the IAP family proteins could be changed to isoforms that have proapoptotic properties. Deveraux et al. (1999) demonstrated that the caspase-mediated cleavage of livin converts it from an antiapoptotic to proapoptotic factor. So, we could demonstrate the presence of the cleaved form of livin in the leukemic cells with livin expression. It seems plausible that this cleaved form might have proapoptotic activities.

Tamm et al. (2000) found that overexpression of livin protein could play an important role in carcinogenesis and progression of newly diagnosed children with acute leukemia. It provides a new strategy of gene therapy for in children.

Recently, Yan et al. (2011) suggested that the expression of livin α and livin β may be associated with the genesis and development of childhood acute leukemia .It seems that the expression of livin α and livin β may be used as a molecular marker of Childhood acute leukemia.

Yang et al. (2010) compared EFS and OS between childhoods ALL patients who did and did not express livin. They did not record any statistical significant difference between these two groups of patients.

On the other hand, El-Mesallamy et al. (2011) showed that increased livin expression in adult AML and ALL was associated with the presence of unfavorable prognostic factors at diagnosis. In addition, high livin expression was associated with a lower EFS and OS in both groups. This suggests that high livin expression is associated with a slower apoptotic response of leukemic cells to apoptotic stimuli provided by chemotherapeutic agents and eventually associated with a lower EFS and OS. It is particularly interesting to note that the livin expression level was higher in HLA-DR –ve and CD34-ve patients as they are accompanied worse outcome among AMLpatients.

Flotho et al. (2007) found that high expression of livin gene may be used as a marker of poor prognosis in acute lymphocytic leukemia. Expression of livin gene has associations with risk group and response to chemotherapy.

The cause of this controversy between our results and the results of the previous investigators may be attributed to the age difference between our patients and their which might affect the maturity of the apoptotic and anti apoptotic protein systems. Children, not fully matured, have a variety of different biologic features distinguishing them from adults. The regulation of apoptosis, the function of apoptotic regulators, or both in children appear to be different from that in adults. For example, Wuchter et al. (2004) reported that the expression patterns of apoptosis-related molecules were different between children and adults with de novo

acute myeloid leukemia. Another example is that the overexpression of Bcl-2, a well-known antiapoptotic protein, was significantly associated with a better prognosis in childhood ALL (Coustan et al., 1996), whereas it was not associated with distinct clinical or biologic characteristics in adult ALL (Campos et al., 1996). These findings suggest that livin expression might have a different biologic significance in childhood ALL compared with adult ALL and may also explain the better outcomes in the former than in the latter. Another explanation may be the predominance of the cleaved form of livin (truncated livin or t-livin) in children which is produced by caspase mediated cleavage of livin and is thought to act as a powerful proapoptotic factor.

Another interesting finding of our study is that the expression level of livin in ALL is significantly higher than in AML. This may be attributed to the difference in the genetic aberrations leading to leukemogenesis in the 2 types of leukaemia. In ALL, these genetic aberrations contribute to the leukemic transformation of hematopoietic stem cells by changing cellular functions. They alter key regulatory processes by maintaining or enhancing an unlimited capacity for self-renewal, subverting the controls of normal proliferation, blocking differentiation, and promoting resistance to apoptosis mostly of overexpression of IAPs (Hanahan et al., 2000). While in AML, genetic aberrations either activate signal transduction pathways resulting in enhanced proliferation and/or survival of leukemic progenitor cells or affect transcription factors or components of the transcriptional coactivation complex, resulting in impaired differentiation and/or aberrant acquisition of self renewal properties by hematopoietic progenitor (Dohner et al., 2008).

Livin expression in the present study was strongly associated with better DFS, and OS rates in patients with childhood ALL. The better early bone marrow response to induction chemotherapy suggest that livin expression is associated with a faster apoptotic response of leukemic cells to apoptotic stimuli provided by chemotherapeutic agents and

eventually associated with a better relapse-free survival (Kaspers et al., 1997).

In summary, the study suggested that high livin expression is associated not only with the presence of favorable prognosis but also with a significantly high DFS and OS in childhood acute leukemia patients. Therefore, livin expression can be a potential independent prognostic factor, and new investigational approaches, as part of well-controlled trials, would be needed to develop a modified risk stratification system based on the status of this new molecular biologic factor.

Conflict of interest all authors have no conflict of interest to report.

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EXPRESSION OF LIVIN IN CHILDHOOD ACUTE LEUKEMIAS: ANALYSIS OF RISK FACTORS

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PROGNOSTIC SIGNIFICANCE OF BCL2 EXPRESSION IN INVASIVE BREAST CARCINOMA, CORRELATION WITH NOTTINGHAM GRADING SYSTEM

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Abstract

Background: *Breast cancer is a heterogeneous disease. The existing prognostic factors are limited in accuracy, leading to unnecessary treatment of numerous cases. BCL2 has been proposed as a prognostic marker, but this effect is considered to relate to oestrogen receptor (ER) status. This study aimed to evaluate the clinical validity of BCL2 as an independent prognostic marker.*

Methods: *302 females with invasive breast cancer were analysed. Individual patient data included age, tumour size, grade, lymph node status, tumor recurrence and mortality rate. BCL2, ER, PR and HER2 levels were evaluated in all tumours. Bcl-2 expression was assessed in relation to age, grade, nodal status, tumor size and hormonal receptors. A Cox model incorporating the time-dependent effects of each variable was used to explore the prognostic significance of BCL2.*

Results: *In univariate analysis, ER, PR and BCL2 positivity were associated with improved survival and better clinical outcome. Tumor size, lymph node status, tumor grade and HER2 positivity were associated with inferior survival. In multivariate analysis, BCL2 positivity and tumor size retained independent prognostic significance.*

Conclusions: *BCL2 is an independent indicator of favourable prognosis. This study may establish the rationale for introduction of BCL2 immunohistochemistry to improve prognostic stratification.*

Introduction

Breast cancer is a heterogenous disease whose behavior is determined by the molecular characteristics of the tumour[1]. One of the greatest challenges in breast cancer management is to accurately predict outcome for each patient so that we can determine who will benefit from adjuvant therapy. To do this, we rely heavily on traditional pathologic variables such as lymph node status, tumor size, and tumor grade[2]. In many centers, these variables are combined into the Nottingham Prognostic Index (NPI) that is more predictive of outcome than any one individual feature. However, despite the broad applicability of clinicopathologic indices, such as the NPI, they cannot accurately predict outcome for all patients and we are still unable, for example, to separate the 30% of node-negative patients who will relapse from the 70% who will not; as a result, many patients receive unnecessary adjuvant treatment[3].

One of the limitations of Nottingham Grading System (NGS), is that 30-60% of breast cancers are still classified as grade 2 (G2), which is a category with ambiguous clinical significance due to its intermediate risk of recurrence[4]. Among the three components of NGS, ie mitoses, tubule formation, and pleomorphism, mitosis was found to be the principal prognostic component of NGS and remained an independent predictor of outcome[4].

Since Bcl2 protein is a prosurvival factor which blocks apoptosis, its overexpression in human tumors is expected to be associated with a more aggressive phenotype as well as resistance to treatments inducing cell death[5]. This is actually true for some human tumor types, including acute myeloid leukemia, aggressive non-Hodgkin's lymphoma, prostate carcinomas, neuroblastoma, and testicular carcinomas, where high Bcl2 expression was found to be associated with a worse clinical outcome, radio- and chemoresistance. However, in other human tumors including non-small-cell lung carcinoma, esophageal squamous cell carcinoma, cervical carcinoma, melanoma, and breast carcinoma, Bcl2 expression was

positively associated with favorable prognostic features and better survival[1].

Bcl2 immunostaining showed an independent prognostic capacity together with cell proliferation rate. It was proved to be a more accurate predictor of survival than ER status[6]. Many studies were done to establish the rationale for introduction of BCL2 immunohistochemistry to improve prognostic stratification. Further work is now needed to ascertain the exact way to apply BCL2 testing for risk stratification and to standardise BCL2 immunohistochemistry for this application[7].

Materials and Methods

This study included 302 selected cases of invasive breast carcinoma that have been obtained from the Mansoura University, Faculty of Medicine, Oncology Center, Egypt between December/ 2009 to december/2013. Tumors were resected by modified radical mastectomy operation. All patients receive postoperative therapy, hormonal, chemo or radiotherapy. Clinical and postoperative follow up data were obtained from oncology center database. Follow up period is variable among patients for about 12-48 months.

Paraffin wax embedded sections prepared from tumors were stained with haematoxylin and eosin. Tumors were diagnosed according to the new WHO classification 2012. (270) cases were diagnosed invasive ductal carcinoma (NOS). (25) cases diagnosed as invasive lobular carcinoma. (5) cases diagnosed as mixed ductal mucinous carcinoma. Tumors were graded according to Nottingham grading system regarding tubular differentiation, nuclear pleomorphism and mitotic count.

Tissue microarray Construction:

Hematoxylin and eosin (H&E) tissue sections of 10% formalin-fixed paraffin-embedded tissue blocks were used as a guide to select the regions for sampling. Tissue microarray (TMA) was assembled manually[8]. First, a hole in the recipient TMA block was made using a mechanical pencil tip. A cylindrical 0.8 mm core sample from the donor block was obtained and deposited onto TMA block at a

distance of 1mm between each core. Three cores were punched from each donor block to minimize the number of cases which cannot be evaluated due to tissue loss[9].

Immunohistochemistr:

After deparaffinization and rehydration, 4-µm thick sections on coated slides were heat-pretreated in a citrate buffer (pH 7.3 at 92C) and immunostained using monoclonal primary antibodies against the following antigens: estrogen receptors (ER) (Clone 1D5 DAKO Corporation at dilution rate of 1:50); progesterone receptors (PR) (Clone PR 636 DAKO Corporation at 1:50); Her2/neu (CB11; Novocastra Laboratories, Newcastle, U.K. at 1: 50); and Bcl-2 (mouse monoclonal antibody clone 124; Dako at 1:100). The avidin-biotin technique was applied using diaminobenzidin (DAB) and hematoxylin for counterstaining. Appropriate negative controls, consisting of histological sections of each case processed without the addition of primary antibody, were prepared for each antigen, The internal positive control were normal breast duct epithelia and intratumoral lymphocytes.

Evaluation of immunohistochemistry:

Immunohistochemical results were scored. A cutoff value was applied to each marker to indicate positive or negative staining. According to ASCO/CAP guidelines for ER and PR expression, tumors were considered positive if at least 1% of the tumor cells showed unequivocal nuclear staining[10]. For Her2/neu, membranous staining was scored for as follows: 0, no staining or faint incomplete staining in <10% cells; 1, faint incomplete staining in >10% cells; 2, weak to moderate complete staining in >10% of cells; 3, strong complete staining in >10% cells. Only score 3 was considered as positive[11]. Cytoplasmic and membranous staining were scored for Bcl-2. Both the intensity of staining and the percentage of positive cells were recorded and a cutoff value of 10% positive cells was used. Cases were regarded positive when they showed either moderate or strong staining for these markers[5].

Statistical analysis:

Data were tabulated, coded then analyzed using the computer

program SPSS (Statistical package for social science) version 17.0 to obtain.

Descriptive data:

Descriptive statistics were calculated for the anthropometric measurements and laboratory data in the form of:

1. Mean ± Standard deviation (SD).
2. Frequency (Number-percent).

Analytical statistics:

In the statistical comparison between the different groups, the significance of difference was tested using one of the following tests:

1- Student's t-test: Used to compare between mean of two groups of numerical (parametric) data.

2- Inter-group comparison of categorical data was performed by using chi square test (X^2-value), Wilcoxon signed rank test was used for two values within the same group.

Spearman's correlation coefficient ® test was used correlating different parameters.

Univaraite and multivariate survival analyses were performed with the Cox proportional hazards model. Survival curves were constructed according to the Kaplan-Meier method. Finally, a log-rank test was performed to evaluate the statistical significance of differences in survival. A P value <0.05 was considered statistically significant. And a P value <0.0001 was considered highly significant in all analyses.

Results of Bcl-2 immunohistochemical expression were correlated with clinical and histopathological prognostic factors in breast cancer including: patient's age, tumor histological subtype, grade, nodal status and tumor size. Statistical analyses were also used to evaluate correlations between expression of BCL2, HER2, oestrogen and progesterone receptors.

Results

The mean age of cases was 54.6 years (range, 28-88; SD, 11.9). As regard menopausal state, 116 cases are premenopausal (38.4%) and 186 cases are postmenopausal (61.1%). As regard tumor size 6% of cases are T1, 57.6% are T2, 36.4% are T3. As regard lymph node status, 24.8% are N0, 24.8% are N1, 29.1% are N2 and 21.2% are N3. As regard tumor stage 3%

of cases are stage 1, 18.9% are stage 2a, 20.2% are stage 2b, 0.3% are stage 2c, 34.4% are stage 3a, 1.3% are stage 3b and 21.2% of cases are stage 3c. About 73.5% of cases (200) are alive and 26.4% of cases (72) are dead. 34.5 % of cases (94) had local or distant recurrence. 9.9% of cases (30) were lost follow up.

Histopathology: This study included 302 cases. 89.4% of cases in this study (270 cases) were diagnosed as invasive duct carcinoma, not otherwise specified (NOS). 8.3% of cases (25 cases) were diagnosed as invasive lobular carcinoma. 1.6% of cases (5 cases) were diagnosed as mixed ductal mucinous carcinoma. 85 cases were graded as grade 1 (28%). 135 cases were graded as grade 2 (45%). 82 cases were graded as grade 3 (27%).

Immunohistochemistry results:

54% of cases are PR positive and 46% are PR negative. 59.9% of cases are ER positive and 40.1% are ER negative. 18.9% are Her2neu positive and 81.1% are negative. 41% of cases are Bcl2 negative and 59% are Bcl2 positive.

Correlation of BCL2 expression with clinicopathological characteristics.

Mean age in Bcl2 positive cases was 54.15 compared to mean age in Bcl2 negative cases was 55.27. No statistically significant correlation was found between age and Bcl2 expression. Bcl2 expression was detected in all cases with tumor size <5cm but was negative in all cases with large tumor size >5cm. This difference was statistically significant with P value<0.001. As regard lymph node status: Bcl2 expression was detected in the majority of lymph node negative cases with reduced expression in lymph node positive cases. This difference was statistically significant with P value<0.001. The positive rates of BCL-2 in histologic grade I, II, III respectively were 64.7%, 64.4%, 43.9%. These positive rates tend to assume the declining trend. The difference have stastistical significance (P<0.005) (Table 1)

Correlation of BCL2 expression with ER, PR and Her2 neu expression.

Bcl2 expression was positively

related to ER, PR expression with p value<0.001. 73% of Bcl2 positive cases were ER positive. 65% of Bcl2 positive cases were PR positive

Bcl2 was negatively correlated with Her2neu expression with p value 0.004 as 88% of Bcl2 positive cases were Her 2 negative (Table 2).

Relationship between Bcl2 and mortality risk and tumor recurrence: 95.8% of dead cases were Bcl2 negative and 4.2% were Bcl2 positive. 73.4% of Bcl2 negative cases have developed local or distant recurrence.compared with Bcl2 positive cases. Only 1.7% of positive cases developed local or distant recurrence. The difference was statistically significant with P value <0.001.

Survival analysis:

Tumor size, Tumor grade and lymph node status were positively correlated with mortality risk and tumor recurrence (P value 0.0001). Among the three parameters assessed in tumor grade only mitotic count show positive correlation with mortality risk and tumor recurrence (P value <0.001). ER and PR show negative correlation with mortality risk and recurrence (p value 0.003 and 0.0001 respectively). Her2 neu show positive correlation with mortality risk and recurrence (P value 0.004 and 0.003 respectively) BCL2 expression was negatively correlated with mortality risk and recurrence with p value 0.0001.

• In the univariate analyses for disease free survival carried out on the entire series of patients, all variables tested achieved statistical significance (Table 3) When variables were included in a multivariate model, Bcl2 expression and tumor size only achieved statistical significance as independent predictors for disease free survival.

• In the univariate analyses for overall survival carried out on the entire series of patients, all variables tested achieved statistical significance (Table 4). When variables were included in a multivariate model, Bcl2 expression only achieved statistical significance as independent predictors for overall survival.

Table (1): Relationship between Bcl2 expression with tumor size, lymph node and tumor grade.

		Bcl2				X^2	P value
		N		P			
		No	%	No	%		
tumor size	<2	6	4.8%	12	6.7%	253.9	<0.001
	2-5	8	6.5%	166	93.3%		
	>5	110	88.7%	0	0.0%		
LN	n	6	4.8%	69	38.8%	45.06	<0.001
	p	118	95.2%	109	61.2%		
tumor grade	Grade 1	30	35.3%	55	64.7%	10.5	<0.005
	Grade 2	48	35.6%	87	64.4%		
	Grade 3	46	56.1%	36	43.9%		

Table (2): Relationship between Bcl2 expression with ER, PR and Her2neu expression.

		Bcl2				X^2	P value
		N		P			
		No	%	No	%		
ER	N	77	62.1%	62	34.8%	21.9	<0.001
	P	47	37.9%	116	65.2%		
PR	N	73	58.9%	48	27.0%	30.9	<0.001
	P	51	41.1%	130	73.0%		
Her2	N	87	70.2%	158	88.8%	16.5	0.004
	P	37	29.8%	20	11.2%		

Table (3): Cox Proportional Hazard Models Showing Predictors of Disease free survival

	Univariate			Multivariate		
	P value	95%CI	HR	P value	95%CI	HR
ER	0.001	1.3-3.11	2.05	0.58	0.66-2.06	1.17
PR	0.000	1.5-3.4	2.3	0.68	0.51-1.5	0.89
Her-2	0.009	1.2-2.8	1.8	0.17	0.4-1.2	0.7
Grade	0.000	2.67-5.3	3.7	0.06	0.33-1.02	0.58
Tumor size	0.000	7.4-22.9	13.02	0.01	1.3-6.4	2.8
Lymph nodes	0.000	3.4-25.1	9.2	0.7	0.15-3.7	0.8
Bcl2	0.000	18.9-189.6	59.9	0.000	16.1-251.4	63.7

Table (4): Cox Proportional Hazard Models Showing Predictors of Overall survival.

	Univariate			Multivariate		
	P value	95%CI	HR	P value	95%CI	HR
ER	0.007	1.2-3.1	1.9	0.7	0.48-1.7	0.90
PR	0.001	1.4-3.7	2.3	0.8	0.5 -1.7	0.92
Her-2	0.005	1.2-3.4	2.06	0.7	0.49-1.6	0.88
Grade	0.000	4.9-13.6	8.1	0.057	0.97-8.2	2.8
Tumor size	0.000	7.2-25.9	13.7	0.9	0.3-3.1	0.96
Lymph nodes	0.000	2.7-20.1	7.3	0.08	0.03-1.2	0.19
Bcl2	0.000	15.11-153.1	48.1	0.000	7.02-117.4	28.7

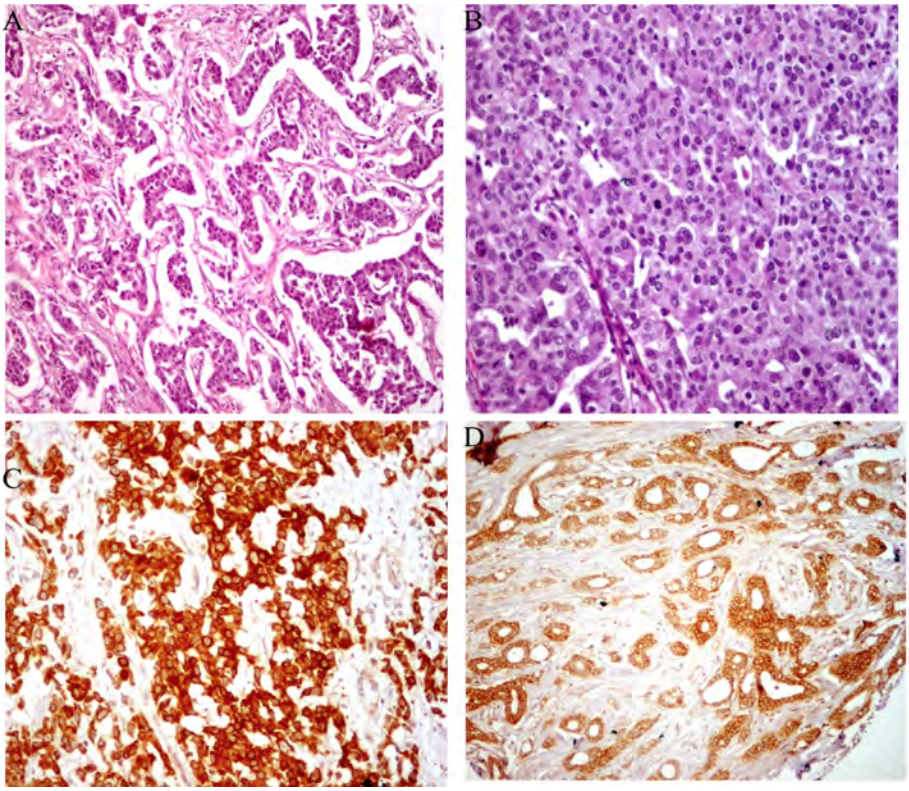

Fig. 1: A- Grade 2 infilterating duct carcinoma Hx&E x 200. **B-** Grade 3 infilterating duct carcinoma Hx & Ex 200. **C-** strong positive cytoplasmic staining for Bcl2 in nearly all tumor cells x 200 case of Grade 2 infilterating duct carcinoma. **D-** strong positive cytoplasmic staining for Bcl2 in nearly all tumor cells x 200 in a case of Grade 1 infilterating duct carcinoma.

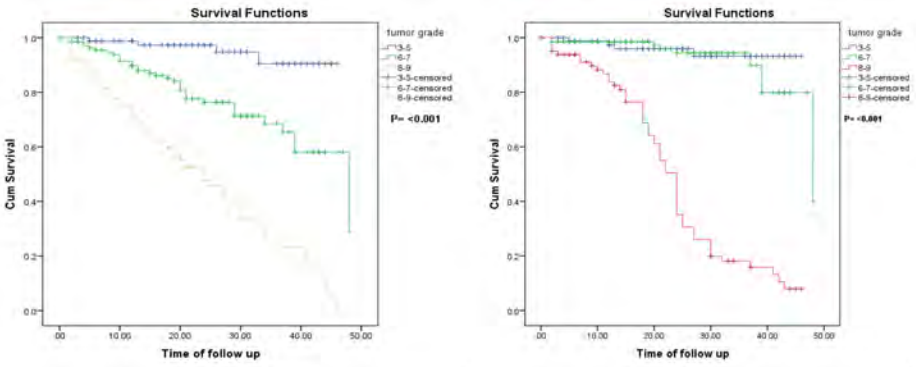

Fig. 2: Prognostic significance of tumor grade, Kaplan meire curve of cumulative survival right (disease free survival), left (overall survival).

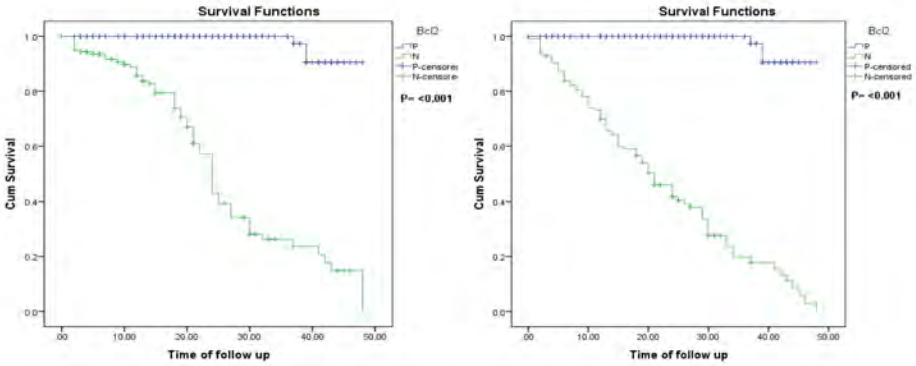

Fig. 3: Prognostic significance of Bcl2 expression, Kaplan meire curve of cumulative survival right (disease free survival) and left (overall survival).

Discussion

Breast cancer is a heterogeneous disease which is characterized by the proliferation and abnormal differentiation of malignant cells. These cells carry aberrations that deregulate hundreds or even thousands of genes[12,13]. The heterogeneous nature of breast cancer has resulted in an overwhelming interest in the search for prognostic markers. These markers help to identify patients who might benefit from the available therapeutic modalities[2].

The prognosis varies, not only with the state of disease, but also with its biological behavior[14].

Several prognostic markers have been identified in breast cancer.

Among these biologic variables, apoptosis markers, in particular Bcl-2 expression, is related to cellular response to genotoxic damage in experimental tumors[15].

This work was done to spot light up on the prognostic yield of BCL2 and mitotic count in invasive breast carcinoma and their correlation with histopathologic grading. Immunohistochemical analysis of BCL2 protein expression is a simple, well-validated, inexpensive and widely available test[7].

The present study represents a prospective and retrospective analysis in 302 women with invasive breast cancer which were classified and graded according to the last revised WHO classification.

In the present study 270 cases were diagnosed as invasive ductal carcinoma (NOS)(89.4%). 25 cases were invasive lobular carcinoma (8.3%). 5 cases were mixed ductal mucinous carcinomas (1.6%). The mean age of cases was 54.6 years (range, 29-88; SD, 11.9). This coincide with some authors who reported a range 28-89[6].

Abdel-Fatah et al. reported that 49.3% of cases were grade 3, 32.2% of cases were grade 2 and 18.5% of cases were grade 1[4]. This is quite different from results in the present study, the majority of cases were grade 2 (44.7%), grade1 (28.1%) and grade 3 (27.2%) This may be explained by differences in the number of studied cases.

In the present work, the pattern of Bcl-2 immunostaining was cytoplasmic and membranous, a compatible feature with its localization in the outer membrane of the mitochondria. About 58.9% of tumors are positive and 41.1 % are negative. This is different from the reported frequency of Bcl-2 expression in the literature ranged from 69-73% in previous studies [7,16]. Another frequencies which is close to the current study about 61.25% was reported[17] and 55% was also reported[18]. This discrepancy may be explained by marked heterogeneity of the tumor, types of cases studied or variation between the methodologies regarding staining score.

The positive rates of BCL-2 in

histologic grade I, II, III respectively were 64.7%, 64.4%, 43.9%. The positive rates of BCL-2 in breast cancer histologic grades I, II, III tend to assume the declining trend, the difference have stastistical significance (P<0.005). These values were close to some reported data[18], however These findings may be little different from those mentioned by other studies[17,19]. This may be explained by differences in the number of studied cases and genetic heterogeneity of tumors.

Bcl-2 expression show significant statistically relationship with age and menopausal state[17]. In this study no significant relation was found. Other studies also noted absence of this relation[16]. This may be explained by differences in the number of studied cases.

Bcl-2 expression was found to be associated with favorable prognostic factors. Bcl2 positivity is statistically greater in cases with small tumor size <5 cm with p value <0.001. Abd El-Mageed et al., also reported this association[20]. Also the majority of lymph node negative cases were Bcl2 positive compared to lymph node positive cases, the majority were Bcl2 negative. The difference was statistically significant with P value <0.001. These findings were similar to those mentioned in literature[5,19].

Bcl-2 expression was found to be statistically greater in the ER and PR positive breast tumors compared to ER and PR negative tumors with p value <0.001. These findings are also confirmed by many studies[21,2]. Bcl2 was inversely correlated with Her2neu expression, and lymph node status with p value <0.05%. This goes hand in hand previous reported results[4]. Bcl2 expression was inversely related with mortality risk and tumor recurrence. This relation was statistically significant with P value 0.0001. This was confirmed in literature[22].

A statistically significant positive correlation was found between, tumor size and lymph node status with mortality risk with p values <0.001. A statistically significant positive correlation was found between tumor size, lymph node status and tumor recurrence with

P value (0.001, 0.007 and 0.0001) respectively. These findings are also confirmed in literature[5].

As regard tumor grade, our findings concluded that tumor grade show positive correlation with tumor recurrence and mortality risk. This is quite similar to finding reported by some papers [23]. Among different parameters assessed in NGS, only mitotic count was positively correlated with tumor recurrence. Abdel Fattah et al., also reported these results[4]. A statistically significant correlation was found between lower mitotic count and improved overall survival and better clinical outcome with P value <0.001. This relation was found also by some studies[24,25]. A statistically significant correlation was found between Bcl2 expression and prolonged overall survival and improved clinical outcome with P value about 0.006 and 0.0001 respectively. These findings are coincident with most findings in literature[7,26] and also against some other studies[2].

In univariate analysis using Cox Proportional Hazard Models involve all tested variables. ER, PR, Her2, tumor size, lymph node, tumor grade and Bcl2 a were confirmed to be Predictors of both OS and DFS. Syed et al., confirmed these findings[22]. However in multivariate analysis, only Bcl2 and tumor size retain their value as predictors of DFS and OS.

The biological mechanisms of BCL2 as a prognostic factor for breast cancer remain largely unclear. The BCL2 (B-cell CLL/ lymphoma 2) gene is located in 18q21. It encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells, such as lymphocytes[5].

Bcl-2 has two opposing activities, one (anti-apoptotic) that promotes tumorigenesis, and another (anti-proliferative), which is anti-tumorigenic. Although BCL2 is well-known as an anti-apoptotic oncogene in lymphoma. The paradoxical function of the tumor suppressor gene has been reported in many solid tumors, including breast cancer. BCL2 may be both oncogenic and tumor suppressive in specific cell types or under spe-

cific conditions. It is postulated that the tumor suppressive effect is more prominent in breast cancer[5].

In contrary to the above favourable prognostic data, others suggest that Bcl-2 expression correlates with aggressive, prometastatic and chemotherapy resisting behavior in breast cancer[27].

Conclusion

Bcl2 is considered a favourable prognostic marker in invasive breast carcinoma. It helps in better stratification of patients into low and high risk groups. This helps to determine which category will benefit best from therapy and which will need more aggressive therapy.

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IMMUNOHISTOCHEMICAL EVALUATION OF P27 (KIP1) AND CYCLIN D1 IN PLEOMORPHIC ADENOMAS, WARTHIN'S TUMORS AND MUCOEPIDERMOID CARCINOMAS OF SALIVARY GLANDS

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Abstract

Background: *A family member of Cyclin Dependent Kinase (CDK) inhibitors named $P27^{kip1}$ has key position in cell cycle regulation, leading to arrest of cell proliferation. It enables a repair process of DNA damage through inhibition of different CDKs and therefore control the cell cycle by balancing the activity of CDKs. Changes in $P27^{kip1}$expression which can be shown by (immunohistochemistry) can alter the normal progression of the cell cycle and its reduced expression has been shown in some tumors. Cyclin D1 also indirectly promotes cell proliferation by sequestering p21 and $P27^{kip1}$, leading to the activation of CDK2. The present study aims at investigating the expression of $P27^{kip1}$ and cyclin D1 proteins in selected benign lesions (pleomorphic adenoma, warthin's tumor) and mucoepidermoid carcinoma and their possible correlation with clinicopathologic features.*

Patients and Methods: *Clinicopathological features of 50 patients (30 MEC-10 PA, 10 warthin's tumors), including age, sex and tumor size were obtained from medical records in the period 2009-2013, from files of pathology department, faculty of medicine- Benha University and Egyptian national cancer institute (NCI). Cases of MEC graded into 18 low grade, 12 intermediate and high grade. Stages (16 cases of stage I,II and 14 cases of stage III, and IV). Immunohistochemical staining with $P27^{kip1}$ and cyclin D1 was performed for each specimen.*

Results: *This study revealed: Highly statistical significant difference in P27kip1 expression between PA, warthin's tumor & MEC. Inverse statistical significant correlation between P27kip1 expression and tu-*

mor stage. High inverse statistical significant correlation between $P27^{kip1}$ expression and tumor grade. Inverse correlation between $P27^{kip1}$ expression and age, tumor size and nodal status but with no statistical significance. High positive statistical significant correlation between cyclin D1 expression and tumor size, nodal status, stage, tumor grade, and p27 expression. Insignificant difference in cyclin D1 expression between PA, warthin's tumor and MEC. Significant difference in cyclin D1 expression between males and females

Conclusion: *In summary, our results provide evidence that reduced expression of $P27^{kip1}$ may be involved in initiation, clinical progression and poor prognosis of MEC patients. Also, our findings indicate that cyclin D1 overexpression may play a pivotal role in the biological behavior of MEC and may provide a strong prognostic implication.*

Introduction

Salivary gland tumors (SGT) are relatively uncommon, comprising no more than 1% of all tumors and 3% of all head and neck neoplasias[1]. Their morphologic diversity and relative rarity frequently pose a challenge in their diagnosis and treatment[2]. As the clinical course and final outcome of many patients with salivary gland tumors cannot be reliably predicted on the basis of histomorphologic features, it is highly desirable to find new prognostic markers aimed at better characterizing tumor aggressiveness[3].

Mucoepidermoid carcinoma (MEC) is one of the most frequent salivary gland malignant tumors (SGMT). It is more common in women and peaks in the fifth decade. However, it is also the most common SGMT to arise in children and adolescents under 20 years of age. Among SGMTs, mucoepidermoid carcinoma is unique in that it demonstrates a broad spectrum of aggressiveness, which can be predicted by microscopic grading. High-grade mucoepidermoid carcinoma is a highly aggressive, while its low-grade counterpart usually demonstrates a favorable outcome. However, metastasis also occurs in low-grade mucoepidermoid carcinomas and can be lethal[4-5].

Mucoepidermoid carcinoma is composed of varying proportions

of mucous, epidermoid, columnar, intermediate, and clear cells. It is thought to arise from the salivary excretory duct. However, some authors have shown that there are essentially two basic types of cells, luminal and intermediate, and that the intermediate cell exhibits characteristics of modified salivary gland myoepithelial cells. Uncertainties regarding mucoepidermoid carcinoma histogenesis is contributed by the lack of known precursor lesions. Information on molecular events causing or accompanying malignant transformation is also very limited. Several studies have reported the occurrence of 6q deletions, a common change in salivary gland carcinomas, which suggests inactivation of tumor suppressor genes[6].

Despite recent progress in molecular medicine, there is still a paucity of data on the involvement of cell-cycle regulatory proteins in the pathogenesis of head and neck tumors, particularly of salivary glands[1-7].

Progression from the G1 to the S phase of the cell cycle is regulated by the formation of cyclin/cyclin-dependent kinase complexes, but their kinase activity is inhibited by a number of specific proteins belonging to the INK4 and CIP/KIP families (p21, p27, p57). $P27^{kip1}$ is a member of the universal CDKI family[8]. It can block this progression by binding cyclin E-cdk2 and cyclin A-cdk2, and defective regulation of this major checkpoint may contribute to resistance against growth inhibitors, the deregulation cell proliferation, and oncogenic changes[9].

$P27^{kip1}$ gene is located on chromosome 12p13 at the 12p12-12p13.1 junction, and is normally expressed in the nuclei of quiescent cells, whereas its activity is lost in actively proliferating cells since it responds to different signals and its level changes reciprocally as cells progress through G1, being high in quiescent cells and decreasing during the G0/S phase interval[10-11].

The intracellular levels of $P27^{kip1}$ increase in response to contact-dependent growth inhibition and a large number of extracellular antimitogenic signals and growth factors, such as TGF-b, cyclic AMP,

lovastatin, rapamycin and tamoxifen[12]. It has been attributed with various functions. As the loss of $P27^{kip1}$ expression may lead to tumor development and progression, it has a potential function as a tumor suppressor gene and may play a role in regulating drug resistance in solid tumors[13].

D-type cyclins are proteins involved in cell cycle regulation that are essential for G1 phase progression, which act during late G1 phase by complexing with cyclin-dependent kinases (CDK)[14]. The regulatory function of cyclins, CDK (CDK4 and CDK6) complex are due to phoshorylation of the proteins involved in cell cycle control, such as pRb, and there is evidence that both cyclins and pRb belong to the same regulatory pathway that releases the repression of E2F-dependent transcription and allows the expression of the genes required for progression to the S-phase of the cell cycle[15].

Cyclin D1 also indirectly promotes cell proliferation by sequestering p21 and p27, thus leading to the activation of CDK2. This protein degradation is mediated by phosphorylation-triggered, ubiquitin-dependent proteolysis. The overexpression of cyclin D1 contracts the G1 phase, decreases cell size, and reduces the serum requirements for growth and the transition from the G1 to the S phase[16]. DNA amplification is the most frequent abnormality affecting the CCND1 gene and, in the majority of the cases, correlates with the overexpression of cyclin D1 protein, a frequent event in a large number of primary human neoplasms and cell lines[17].

Aim of The Work

This study ends at evaluation of the role of Cyclin D1 and $P27^{kip1}$ in both benign lesions (pleomorphic adenoma, warthin's tumor) and mucoepidermoid carcinoma and their possible correlation with clinicopathologic data and comparison between Cyclin D1, $P27^{kip1}$ immunohistochemical expression in studied cases of salivary gland neoplasms.

Materials and Methods

Clinical investigations and tissue samples:

The present study was based on 50 cases of salivary gland neo-

plasms including 30 non-consecutive retrospective selected mucoepidermoid carcinoma and 10 cases of pleomorphic adenoma, and 10 cases of Warthin tumors. Cases were collected in the period 2007-2013. They were selected from files of pathology department of faculty of medicine- Benha University and Egyptian national cancer institute (NCI). Cases were selected according the availability of clinical and follow-up data.

Each specimen was assessed for tumor extension by inked specimen margin as well as extension into peri salivary tissues. Only patients with primary mucoepidermoid carcinoma who had not undergone previous treatment were included in the study. Two experienced pathologists blindly and independently confirmed the histological diagnosis of each salivary lesions and agreed on the grading. Histologic subtypes were based on the classification of the Armed Forces Institute of Pathology (AFIP) grading system[18], and comprised 18 low grade, 12 intermediate and high grade tumors. Stages of the cases were 16 of stage I, II and 14 of stage III, and IV).

Apparently normal salivary gland tissue neighboring to tumor area in the examined specimens was used as a normal control. Formalin-fixed, paraffin-embedded salivary gland tissues were used. Three sections of 4 micron thickness were obtained from each case. One section was H&E stained for diagnosis. Other sections were mounted on positively-charged slides, immunohistochemically stained with mouse monoclonal antibodies against cyclin D1 (clone BF683; Santa-Cruz Biotechnology), diluted 1:200), p27 1B4 (clone 57, Zymed laboratories, USA diluted at 1:100) monoclonal antibody, using the Ultra Vision Detection System (Anti-polyvalent, HRP/DAB, ready-to-use, Lab Vision corporation).

Immunohistochemical staining: Paraffin-embedded tissue sections, 3-4 micron thick were mounted on positively-charged slides and heated at 60℃ for 30 minutes then deparaffinized and rehydrated through a series of xylene and alcohol before staining. After antigen retrieval with micro-

wave treatment in 10mM citrate buffer (Neo-Markers, Cat. # AP-9003), pH 6.0, endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 minutes. Sections were washed 3 times with cold 0.01 M phosphate buffered saline (PBS). After blocking with 10% normal rabbit serum, sections were incubated with polyclonal antibody against $P27^{kip1}$, Cyclin D1. Slides were incubated for 90 minutes with theses antibodies at room temperature. The prepared DAB-substrate-chromogen solution was applied and incubated for 5-15 minutes until color intensity has been reached. Lastly, sections were counterstained with Mayer's hematoxylin. Mantle cell lymphoma and tonsillar specimens were used as positive controls for cyclin D1, and $P27^{kip1}$ staining, respectively. Negative control consisted of substituting normal mouse serum for the primary antibody and reaction without primary antibody[12].

$P27^{kip1}$ and cyclin D1 scoring of immunohistochemistry analysis:

Sections stained for $p27^{kip1}$ and cyclin D1 were assessed and the percentage of immunostained cells was determined. The percentages of $p27^{kip1}$ and cyclin D1 immunopositive tumor cells were counted in 5 consecutive microscopic fields (magnification 100x) per tumor sample in area that showed the highest density of these cells. In each field, 100 tumor cell nuclei were evaluated and the mean for every 5 fields were calculated. A distinct granular brown stain was scored as positive. A cutoff value of <5% immunopositive cells was considered negative, and >5% immunopositive cells was considered positive[19].

For immunolocalization of p27 and cyclin D1 protein, percentages of immunopositive cells in 100 cells were counted in 5 different microscopic fields (magnification 100x) and the mean of were evaluated according to Viglietto, et al; $P27^{kip1}$ and cyclin D1 were scored 'nuclear' when more than 50% of $p27^{kip1}$ and cyclin D1-expressing cells presented nuclear staining, and 'cytoplasmic' when there was nucleo-cytoplasmic or exclusively cytoplasmic staining in at least 35% of $p27^{kip1}$ and cyclin D1-expressing cells[20].

For $p27^{kip1}$ expression evaluation, the positive samples were scored according to the frequency of immunopositive cells as 5%, 25%, 26% to 50%, 51% to 75%, and >75%. Samples from patients with <50% p27-positive tumor cells were considered low expressors, whereas those with >50% p27-positive tumor cells were considered high expressors[19].

For cyclin D1 expression evaluation, the positive samples were scored according to the intensity expression. Immunostaining was further graded as 1 when there was mild staining, 2 for moderate, and 3 for intense staining[21].

Statistical analysis: Statistical analysis was performed using the SPSS (16.0 for windows) software package according to Pearson's correlation coefficient. Correlation between several variables was computed using Fisher's exact test. P value less than 0.05 (<0.05) was considered significant and <0.01 was highly significant.

Results

A- immunohistochemical results of $P27^{kip1}$ staining (Table 1):

$P27^{kip1}$ immunoreactivity was observed in almost all adjacent apparently normal tissues, and in the majority of fibroblasts and lymphocytes. These cells served as an internal positive control.

All examined 10 cases of pleomorphic adenoma (100%), and 10 cases of Warthin's tumors (100%) showed high $P27^{kip1}$ expression, while among the 30 cases of mucoepidermoid carcinoma, 6 cases (20%) showed high expression, and the other 24 cases (80%) showed low expression, and this difference was statistically highly significant (P<0.01).

In relation to the tumor grade, all the 18 cases (100%) of low grade mucoepidermoid cases showed low expression of $P27^{kip1}$, however among the 12 cases of intermediate, high grades, 6 (50%) showed high expression, and 6 (50%) showed low expression, and this relation was statistically highly significant (P<0.01).

Among 16 cases of Stage I, II, one case (6.3%) showed high $P27^{kip1}$ expression and 15 (93.7%)

showed low expression. While, in the 14 cases of stage III, IV, 5 (35.7%) showed high expression, and 9 (64.3%) showed low expression, and this inverse relation was statistically significant (P<0.05).

Concerning to lymph node status (lymph node metastasis), 3 out of the 22 lymph node negative cases (13.6%) showed high $P27^{kip1}$ expression, and 19 (86.4%) showed low expression. On the other hand, among 8 lymph node positive cases, 3 (37.5%) showed high expression, and 5 (62.5%) showed low expression. This relationship was statistically insignificant (P>0.05).

A- immunohistochemical results of cyclin D1 staining (Table 2):

No specific cyclin D1 immunoreactivity was observed in the adjacent apparently normal tissue. Among the examined 10 cases of pleomorphic adenoma, 4 (40%) showed negative/mild Cyclin D1 expression and 6 (60%) showed moderate expression, among 10 cases of Warthin tumors, 3 (30%) showed negative/mild expression and 7 (70%) showed moderate expression. While, among the 30 cases of mucoepidermoid carcinoma, 15 (50%) showed moderate and intense expression, and 15 (50%) showed negative/mild expression, a statistically insignificant difference between benign and malignant lesions (P>0.05).

In relation to the tumor grade, among the 18 cases of low grade mucoepidermoid cases, 3 (16.7%) showed moderate Cyclin D1 expression, and 15 (83.3%) showed negative/mild expression. However, among cases of intermediate, high grade cases, 6 (50%) showed moderate expression, and 6 (50%) showed intense expression and this relation was statistically highly significant (P<0.01).

Among 16 cases of Stage I, II, one case (6.25%) showed intense Cyclin D1 expression and 2 (12.5%) showed moderate expression, and 13 (81.25%) showed negative/mild expression. Among the 14 cases of stage III, IV, 5 (35.7%) showed intense expression, 7 (50%) showed moderate expression, and 2 (14.3%) showed negative/mild expression, and this inverse relation was statistically

highly significant (P<0.01).

Concerning lymph node status, 2 out of the 22 lymph node negative cases (13.6%) showed intense Cyclin D1 expression, and 5 (22.7%) showed moderate expression. Among 8 lymph node positive cases, 4 (50%) showed intense expression, and 4 (50%) showed moderate expression. This relation was statistically highly significant (P<0.01).

Table (1): correlation between $P27^{kip1}$ immunoexpression and clinicopathological data in examined patients.

	No.	P27 expression (No. & %)		P value
		High	Low	
***Diagnosis:**				
- PA[1]	10	10 (100)	-	
- Warthin's tumor	10	10 (100)	-	
***-* MEC[2]**	30	6 (20)	24 (80)	**<0.01****
***Age**				
- ≤60	13	2 (15.4)	11 (84.6)	
- >60	17	4 (23.5)	13 (76.5)	>0.05
***Sex**				
- Male	12	2 (16.7)	10 (83.3)	
- Female	18	4 (22.2)	14 (77.8)	>0.05
***Tumor size**				
- ≤2cm	13	1 (7.7)	12 (92.3)	
- >2cm	17	5 (29.4)	12 (70.6)	>0.05
***Nodal status**				
- Negative	22	3 (13.6)	19 (86.4)	
- Positive	8	3 (37.5)	5 (62.5)	>0.05
***Stage**				
- I, II	16	1 (6.25)	15 (93.75)	
- III, IV	14	5 (35.7)	9 (64.3)	**<0.05***
***Histologic grade**				
- Low	18	0	18 (100)	
- Intermediate, High	12	6 (50)	6 (50)	**<0.01****

[1]PA= Pleomorphic adenoma, [2]MEC= Mucoepidermoid carcinoma

N.B.: The table showed highly statistical significant difference in p27 expression between PA, Warthin's tumor & MEC. High statistically significant inverse correlation between p27 expression and histologic grade. Inverse statistically significant correlation between p27 expression and tumor stage. Inverse correlation between p27 expression and age, tumor size and nodal status but with no statistical significance.

Table (2): correlation between Cyclin immunoexpression and clinicopathological data in examined patients.

	No.	Cyclin D1 expression (No. & %)			P value
		Negative/mild	Moderate	Intense	
***Diagnosis:**					
- PA	10	4 (40)	6 (60)	0	
- Warthin's tumor	10	3 (30)	7 (70)	0	
***MEC**	30	15 (50)	9 (29)	6 (21)	>0.05
***Age**					
- ≦60	13	10 (76.9)	1 (7.7)	2(15.4)	
- >60	17	5 (29.4)	8 (47.1)	4 (23.5)	>0.05
***Sex**					
- Male	12	10 (83.3)	0	2 (16.7)	
- Female	18	5 (27.8)	9 (50)	4 (22.2)	**<0.05***
***Tumor size**					
- ≦2cm	13	11 (84.6)	1 (7.7)	1 (7.7)	
- >2cm	17	4 (23.5)	8 (47.1)	5 (29.4)	**<0.01****
***Nodal status**					
- Negative	22	15 (68.2)	5 (22.7)	2 (9.1)	
- Positive	8	0	4 (50)	4 (50)	**<0.01****
***Stage**					
- I, II	16	13 (81.25)	2 (12.5)	1 (6.25)	
- III, IV	14	2 (14.3)	7 (50)	5 (35.7)	**<0.01****
***Histologic grade**					
- Low	18	15 (83.3)	3 (16.7)	0	
- Intermediate, High	12	0	6 (50)	6 (50)	**<0.01****
* **P27 expression**					
- Low	24	15 (62.5)	8 (33.3)	1 (4.2)	
- High	26	7 (16.9)	14 (53.9)	5 (19.2)	**<0.01****

N.B.: The table showed high statistical significant positive correlation between cyclin D1 expression and tumor size, nodal status, stage and histologic grade. There was significant difference in cyclin D1 expression between males and females. High statistically significant relation was found between cyclin D1 and p27 expression. There was positive relation between cyclin D1 expression and patient age but with no statistical significant difference. There was insignificant difference in cyclin D1 expression between PA, warthin's tumor and MEC.

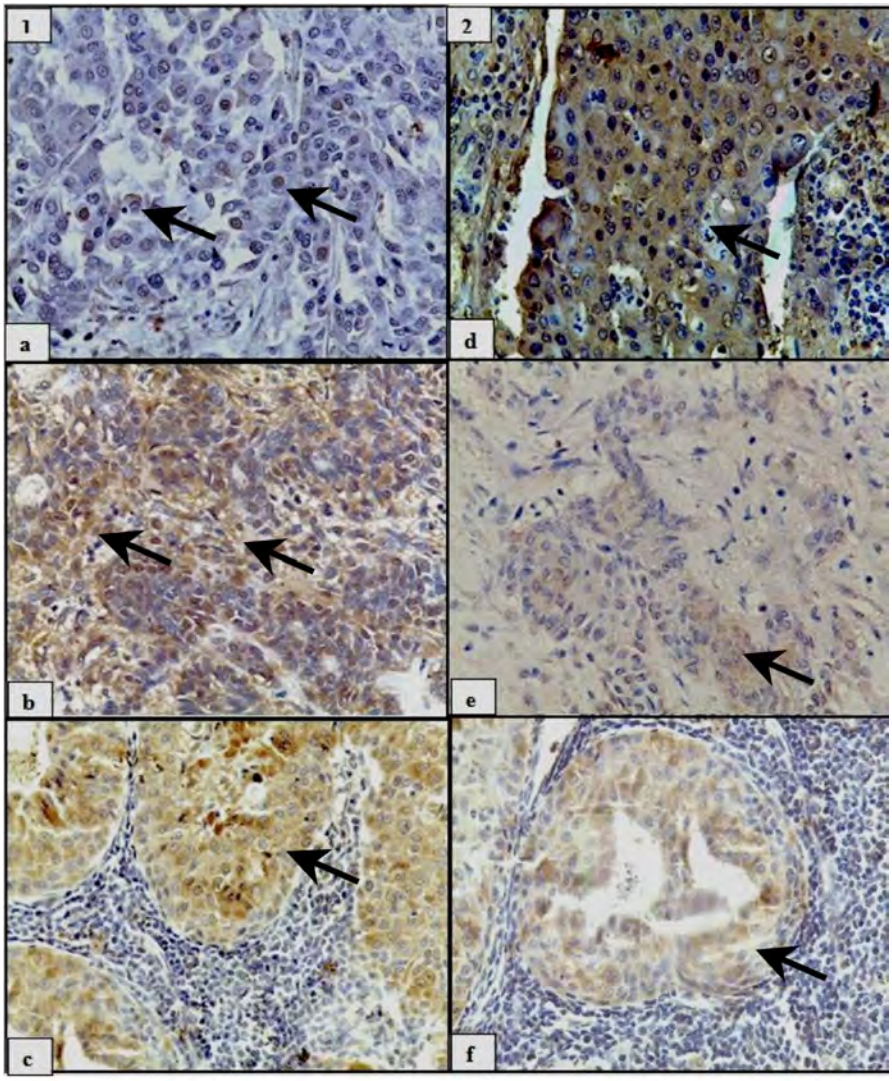

Fig.: 1) P27 immunostain. a) High grade MEC showing low expression. **b)** PA, Most tumor cells showed obvious staining (high expression). **c)** Warthin's tumor showing immunopositive neoplastic epithelial cells (basal and columnar cells) forming papillary growth (high expression). The C.T. stroma is negative. **2) Cyclin D1 immunostain. d)** High grade MEC showing epidermoid cells expressing intense Cyclin D1 (3+) immunoreactivity. **e)** PA showing weak (1+) Cyclin D1 staining in epithelial and myoepithelial tumor cells. **f)** Warthin's tumor showing intermediate Cyclin D1 (2+) staining in epithelial tumor cells (basal and columnar cells). All figures **(Streptavidin-Biotin DAP, X400). DAP, 3'diaminobenzidine tetrahydrochloride.**

Discussion

Salivary gland tumors are relatively rare and comprise a group of morphologically and biologically heterogeneous lesions[15]. The significance of CIP/KIP protein expression in malignant neoplasms of the salivary glands is still unclear. The few reports in literature are contradictory[22].

Tumorigenesis frequently involves disruption to proteins in the pathway of the pRb. Currently, it is known that tumor cells typically sustain damage to genes that regulate G1-S progression. The retinoblastoma gene product; Rb protein serves as a checkpoint that restricts entry into the S phase by binding to transcription factor E2F and blocks the transcription of the S phase genes. Normally, this inhibition by Rb is relieved at the appropriate time by phosphorylation of the Rb protein, a process initially triggered by cyclin D–Cdk4/Cdk6 complexes[14]. The activities of cyclin D–Cdk4/Cdk6 complexes are constrained by inhibitors such as $P27^{kip1}$. Thus, the loss of $P27^{kip1}$ or Rb and cyclin D overexpression have similar effects on G1 progression and represent a common pathway to tumorigenesis. Abnormalities in the Rb pathway can lead to uncontrolled cell proliferation and tumorigenesis in various types of human tumors[23].

Here we report cyclin D1 expression in non-neoplastic and neoplastic tissues obtained from salivary glands; immunohistochemical staining of examined salivary gland lesions showed insignificant difference in cyclin D1 expression between (PA, Warthin's tumors) and MEC. Sixty percent of pleomorphic adenoma showed moderate expression of cyclin D1, and (40%) showed negative/mild expression, also among the cases of Warthin tumors, (70%) showed moderate expression of Cyclin D1, and (30%) showed negative/mild expression. Among Examined cases of mucoepidermoid carcinoma, (50%) showed positive cyclin D1 expression, and (50%) showed negative/mild expression, and this relation was statically insignificant ($P>0.05$). These results were in agreement to results reported by Jour, et al who found that Cyclin D1 is expressed similarly in malignant and benign salivary

gland tumors (p=0.146)[15]. Also Patel, et al found that Cyclin D1 was more likely to be expressed in the malignant components of CXPA than in the benign components of PA (50% versus 31%), but the trend was not statistically significant[17]. On the contrary, Greer, et al found that Cyclin D1 overexpression was present in 90% (35/39) of salivary gland adenoid cystic carcinoma examined[24].

As regard the grade of mucoepidermoid carcinoma, among the cases of low grade, only (16.7%) showed moderate and intense expression of Cyclin D1, while (100%) of intermediate and high grade showed moderate and intense expression, and this relation was statistically highly significant (P<0.01). These results matched with those reported by Zhao-peng, et al who demonstrated that the Cyclin D1 expressions had significant differences in different histological grades of MEC[25]. Also Ning, et al found that There was a significant difference in expression of Cyclin D1 between the high grade and the low grade of MEC [26]. On the contrary, Pignataro, et al reported that No significant correlation was found between cyclin D1 and histological grade of laryngeal carcinoma[27].

Concerning to lymph node status, 31.8% of lymph node negative cases showed weak/mild Cyclin D1 expression, while moderate/intense expression was seen in 100% of lymph node positive cases, this relationship was statistically significant (P<0.05). These results were parallel with those of Pignataro, et al who reported that Cyclin D1 overexpression was significantly higher in cases with lymph node metastases than in those without (p = 0.014)[27].

Regarding the stage of mucoepidermoid carcinoma, Stage I, II cases showed moderate/intense Cyclin D1 expression in (18.8%), stage III, IV showed moderate/intense expression in (85.7%). A statistically highly significant relation between expression of cyclin D1 and stage of mucoepidermoid carcinoma was found (P<0.01). Parallel to such results, Pignataro, et al found that Cyclin D1 overexpression was significantly higher in T_3-T_4 than in T_1-T_2 tumors (p=0.016) in cases of laryngeal

carcinoma[27].

The proliferation and progression of cancer cells may be caused by abnormalities of various positive and negative cell cycle regulators. CIP/KIP family proteins entitled p21(WAF1/CIP1) and p27 (KIP1) have key positions in cell cycle regulation leading to an arrest of cell proliferation. They are supposed to enable a repair process of DNA damage[28]. $P27^{kip1}$ tumor suppressor protein regulates progression from the G1 to S phase of the cell cycle by inhibiting cyclin-D or -E dependent kinase activity[12-29].

Reduced expression of $P27^{kip1}$ was found in various cancers and was correlated with their malignancy and poor prognosis[28]. The role of these cell cycle regulators in tumors of salivary gland origin is still unclear[22].

In the current study, we demonstrated reduced immunohistochemical expression of $P27^{kip1}$ in 80% of mucoepidermoid carcinoma, whereas all benign cases (pleomorphic adenomas and warthin's tumors) showed high expression, a statistically high significant relation. These findings suggest that the reduction of $P27^{kip1}$ protein may be involved in the development of mucoepidermoid carcinoma. This was supported by Akrish, et al who demonstrated that all cases of PA strongly expressed $P27^{kip1}$ while 85.7% of mucoepidermoid carcinomas showed reduced $P27^{kip1}$ expression[30]. Shahsavari, et al also found that $P27^{kip1}$ expression was significantly lower in adenoid cystic carcinoma than pleomorphic adenoma[31].

In this work, all specimens of warthin's tumor showed high $P27^{kip1}$ expression. Both layers of the neoplastic epithelium (basal and columnar cells) revealed positive immunostaining, indicating the low proliferation rate of these cells. The surrounding stroma was negatively stained. Basal epithelial cells showed a faint immunppositive reaction indicated their higher rate of proliferation[32]. Myoepithelial cells were negatively stained. This was similar to results of[10].

Also, the most noteworthy find-

ing of the current study is that the reduced immunoexpression of $P27^{kip1}$ protein in MEC was closely associated with histologic grade as we found that 100% of low grade MEC cases showed reduced $P27^{kip1}$ expression compared with 50% only of high grade cases. This was parallel with Akrish, et al who found that 100 % of low grade MEC showed reduced $P27^{kip1}$ expression in relation to 33% of high grade cases[30]. Many other studies demonstrated that $P27^{kip1}$ had significant differences in different histological grades[12-33-6]. Russo, et al also reported a positive correlation between $P27^{kip1}$ positivity of expression and tumor differentiation[1]. Incontrast, Talaat reported no correlation between Tumor grade and $p27^{kip1}$ expression[7].

This study also demonstrated inverse statistical significant correlation between $P27^{kip1}$ expression and tumor stage as 93.75% of stage I,II showed reduced $P27^{kip1}$ expression, and 64.3% of stage III, IV showed reduced expression. This was supported by several studies which reported significant correlation between low $P27^{kip1}$ expression and advanced clinical stages[33-6-13].

On the other hand, we found that age, sex, tumor size and nodal status were inversely correlated with $P27^{kip1}$ expression but with no statistical significance. Similarly, Ben-Izhak, et al found Significant correlation rates between age and reduced $p27^{kip1}$ expression, indicating $P27^{kip1}$ reduction as part of a general phenomenon-age related mutagenesis. In contrast, they demonstrated significant $P27^{kip1}$ reduction resulted in significantly larger tumor size (T value) and higher spread of neck metastasis[13]. Also, Wanzhong, et al reported that $P27^{kip1}$ had significant differences with lymph node metastasis[6]. Other study found no correlation with age, sex, tumor size, nodal metastasis, clinical stage[12].

In this study, we also reported high statistically significant correlation was found between cyclin D1 and p27 expression. This was parallel with several studies in different tumors such as breast[34-35], in renal cell carcinoma[36], and eosophageal carcinoma[37].

The correlation between high $p27^{kip1}$ expression and its opposing partner cyclin D1ycdk4 led us to hypothesize that the true determining factor for cell proliferation is the balance between the two opposing regulators of cell proliferation, $p27^{kip1}$ and cyclin D1/cdk4.

In Conclusion

- Reduced immunohistochemical expression of the $P27^{kip1}$ protein in this study suggests that $P27^{kip1}$ is enrolled in the carcinogenesis, clinical progression and poor prognosis of the mucoepidermoid carcinoma with significant values as regard tumor stage which can be used during therapy and patient's follow up.

- We demonstrated a close correlation between cyclin D1 expression and clinical relevance and identified cyclin D1 as new marker for tumor cell proliferation. If our results are confirmed in a larger study, overexpression of cyclin D1 may play a pivotal role for the biological behavior of MEC and may provide the strongest prognostic implication.

- Decreased $P27^{kip1}$ expression and cyclin D1 overexpression, alone and in combination, predict poor prognosis in patients with mucoepidermoid carcinoma of salivary gland.

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IMMUNOHISTOCHEMICAL EVALUATION OF P27 (KIP1) AND CYCLIN D1 IN PLEOMORPHIC ADENOMAS, WARTHIN'S TUMORS AND MUCOEPIDERMOID CARCINOMAS OF SALIVARY GLANDS

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NOVEL INSIGHTS IN DIAGNOSIS OF CUSHING SYNDROME: ROLE OF HAIR AND SALIVARY CORTISOL

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Abstract

Objectives: *Measurement of cortisol in human hair as a biomarker of long-term systemic exposure, to establish a proper noninvasive technique suitable for diagnostic screening for subclinical Cushing syndrome and create retrospective timelines of cortisol exposure that correspond with symptomatic periods in patients suspected of cyclic Cushing 's syndrome. Overnight salivary cortisol is a promising diagnostic tools for diagnosis of Cushing syndrome. This study was conducted to establish the reference range of overnight salivary cortisol in African-Egyptian and to evaluate its usefulness in the diagnosis of CS*

Subjects and Methods: *40 Patients diagnosed by conventional investigations in addition to radiological diagnosis using CT abdomen and chest, in addition to dynamic MRI pituitary gland were involved in this study and are subdivided into 3 groups, iatrogenic cushing, endogenous hypercortisolemia, undifferentiated group with Cushinoid features, in addition to control group (n=32). Overnight salivary sample, hair in addition to 24 H UFC, ACTH, 1mg overnight dexamethasone suppression test and overnight serum cortisol were taken from the patients and the results of the salivary and hair samples were compared with the other investigations in the same patients and with salivary, hair cortisol taken from the control group.*

Results: *Overnight salivary cortisol concentration was significantly higher in patients with Cushing's syndrome in comparison with patients who have cushingoid features and control group while in case of using hair cortisol, there was no significant difference between Cush-*

ing's syndrome in comparison with patients who have cushingoid features and control group, however male patients with Cushing's syndrome had significantly higher results of hair cortisol than female patients with Cushing's syndrome, patients with cushingoid features and control group.

Conclusion: *Overnight salivary cortisol is an accurate diagnostic tool for diagnosis of Cushing syndrome and can be used as a first line diagnostic test in the diagnosis of CS in African- Egyptian population while hair cortisol needs a lot of prerequisites before being sampled especially hair length.*

Introduction

Cushing's Syndrome (CS) consists of signs and symptoms caused by prolonged exposure to elevated glucocorticoid levels. The severity of the symptoms depends on the degree and duration of glucocorticoid excess[1]. This can be caused by taking glucocorticoid drugs, or by tumors that produce cortisol or adrenocorticotropic hormone (ACTH) or corticotrophin releasing hormone (CRH)[2].

In iatrogenic CS, these factors are usually well documented. In endogenous CS, the duration of hypercortisolism can usually only be estimated based on subjective patient reports detailing the time course of symptom development and progression[3].

The diagnosis of endogenous CS is among the most difficult in clinical endocrinology. The phenotype is very common in the population, and the frequency of the disorder is relatively uncommon. Therefore, accurate, convenient, and relatively inexpensive screening tests are essential if the large number of patients with suspected CS can be evaluated[4,5].

The positive diagnosis of CS requires that chronic hypercortisolism is unequivocally demonstrated biologically, using 24-h urinary free cortisol (UFC), late-evening plasma or salivary cortisol, midnight 1-mg or the classic 48-h-low-dose dexamethasone suppression test (LDDST), etc., all with essentially the same diagnosis potencies. The search for the responsible tumour then relies on the assessment of the corticotroph

function, and imaging[6].

It is important to suspect the existence of CS in order to start adequate clinical investigations[7,8]. The best approach today is the midnight (salivary) cortisol measurement[9] or the overnight LDDST[10] followed by a 24-h UFC (3-4 times) and/or a cortisol daily profile[8].

Hair may provide a long-term "memory" that documents chronic substance abuse and can provide a positive result in cases where blood or urine tests may have been falsely negative[11]. Sauvé et al. documented the use of hair cortisol as a biomarker of cortisol exposure in non-obese subjects[12].

Aim of The Work

The aim of this study was to establish the reference range of overnight salivary cortisol in African-Egyptian and to evaluate its usefulness in the diagnosis of CS and measurement of cortisol in human hair as a biomarker of long-term systemic exposure, to establish a proper noninvasive technique suitable for diagnostic screening for subclinical CS and create retrospective timelines of cortisol exposure that correspond with symptomatic periods in patients suspected of cyclic CS.

Subjects and Methods

The study population consisted of 40 subjects (32 women, age 31±12 mean±SD), divided into three groups attending the Department of Endocrinology in Mansoura specialized medical hospital, the 1st group is diagnosed being endogenous hypercortisolemia (Cushing syndrome (n=6), Cushing disease (n=9) and cyclic Cushing (n=2)) diagnosed basically on clinical presentation, hormonal evaluation (overnight serum cortisol, LDDST and 24 h UFC in addition to ACTH), and radio-diagnostic techniques, the 2nd group is iatrogenic CS (n=8) diagnosed basically on clinical presentation while the patient is on treatment with corticosteroid, the 3rd group are undifferentiated group either has Cushingoid features (n=12) with negative laboratory tests (suspected CS) or those who attend the urology and nephrology centre for incidentally discovered adrenal adenoma (n=3) with incidentally discovered with elevated 24 hour UFC or overnight serum cortisol,

in addition to control group (n=32) who is divided into two subgroups obese (n=12) and non obese (n=20). None of the patients was taking corticosteroids or oral Contraceptives except the iatrogenic group. The study was approved by the local Ethical Committee. The 4 groups of patients will undergo, history taking as regard to age, marital status, having children or not and negative history of corticosteroid intake, clinical examination as regard presence or absence of proximal myopathy, moon face, malar flush, truncal obesity, buffalow hump, hollow buttock , stria rubra, stria alba , irregular menses, intertrigo, acne, hair fall, diabetes and hypertention, Investigations in the form of 24hours UFC, midnight salivary cortisol at 11 pm, Midnight serum cortisol at 11 pm, 1-mg dexamethasone suppression test, ACTH, 8-mg dexamethasone suppression test for those with high ACTH level and Hair cortisol, in-addition to Radiodiagnostic techniques including CT abdomen for all patients , MRI brain and CT chest for those with high ACTH levels. Patients excluded from this study include those with kidney, liver disease, oral diseases or injury. Individuals who had previously applied cosmetic coloring, shampoo, creams or oils and Grey colored hair and bald person, also those who used glucocorticoid during the experimental period (except cases of iatrogenic cushing), or use antibiotics drugs within 2 days before the sampling day. 4 patients with Cushing's syndrome underwent appropriate surgeries (3 unilateral adrenalectomy and 1 pituitary adenoma resection) and the pathologic findings confirmed the diagnosis and 3 received Gamma knife radiosurgery.

Sample acquisition:

All participants were asked to stop taking some medicines (such as birth control pills, aspirin, morphine, methadone, lithium, monoamine oxidase inhibitors, and diuretics) for 24 to 48 hours before the samples are drawn. All participants collected saliva and blood sample at 23:00 h for overnight salivary and serum cortisol, 24 h UFC from 9 am to 9 am next day and hair cortisol. CS, Cushing disease, incidentally discovered adrenal adenoma and cyclic Cushing do in addition, 9 am blood sample

for serum cortisol after 1 mg dexamethasone suppression test and ACTH.

Serum samples were collected by venous puncture from all study participants. Patients were admitted in the hospital and samples at midnight (11 pm) were obtained. 5ml of blood was collected into plastic EDTA tubes, Samples were immediately separated in a cooling centrifuge at 4°C (3500 rpm, 7 min). An aliquot of plasma was kept at -20°C until processing.

Because salivary cortisol secretion is characterized by high reactivity, marked circadian changes and considerable day-to-day variability within an individual(13), salivary cortisol concentration was strongly affected by a sampling time and environmental disturbances occurring at times. Salivary cortisol 20-30 minutes after awakening the patient at 11 pm (overnight) was adopted here because it could receive less environmental disturbances relative to other time points. Salivary cortisol was collected in special tubes consisting of a small tube containing a cotton wool contained into a bigger tube. Patients cleaned their mouth, are not allowed to do vigorous physical activity eat, drink or smoke for 1 h and rinsed their mouth with cold water and wait at least 10 minutes after rinsing to avoid sample dilution before obtaining the sample. The patient chewed the cotton wool for 2-3 minutes then the cotton placed back to the small tube, centrifuged for 10 minutes at 3000 Rpm for falling of the saliva from cotton wool into the bigger tube and stored at -20°C until processing. Serum (overnight serum cortisol and ACTH) and salivary cortisol were obtained at the 1st day of admission at the same time.

Hair samples with longer than 1 cm in the posterior vertex region were cut as close as possible to the scalp so that hair follicles are not included. The collected hair strands were cut with clean iron scissors and stored in dry tubes at -20°C for cortisol analysis. It is important that the hair follicle itself is not included in the analysis, as a previous study found that hair follicles are capable of producing cortisol in response to corticotropin-releasing hormone stim-

ulation[14] and thus may skew the results. Additionally, prior studies have shown that this area of the scalp has the lowest coefficient of variation (15.6%), as compared to hair sampled from other areas (30.5%)[12].

Hair cortisol was prepared for analysis according to Gow et al., 2010[11] as follow: hair was washed with isopropranolol, and allowed to dry. The appropriate section of hair were cut and weighed then 50 mg of hair were finely minced with surgical scissors, then incubated with 1.5 ml methanol for 16 h at 50°C. The methanol is dried under a gentle steam of nitrogen at 50°C then reconstituted with 250 ml of phosphate buffer saline. The samples were vortexed then analyzed for hair cortisol.

The patient is asked to start collecting urine with empty bladder from 9 am in the 1st day till 9 am in the next day and the volume is documented and an aliquot was kept at -20°C till analysis, 24 UFC were calculated as: (Urinary cortisol (ng/ml) x Urine volume in litre).

In the 2nd day after collecting the 24 urinary cortisol, the patient is given 2 tablets of dexamethasone 0.5 mg at 11 pm, 5 ml of blood was collected into plastic EDTA tubes, on the 3rd day at 8 am samples were immediately separated in a cooling centrifuge at 4°C (3500 rpm, 7 min). An aliquot of plasma was kept at -20°C until processing.

Only patients who had high ACTH levels (15 pg/ml) undergo high dose dexamethasone suppression test, the patient was given 16 tablets of dexamethasone 0.5 mg together with omeprazole 40 mg at 11 pm, 5 ml of blood was collected into plastic EDTA tubes, on the next day at 8 am, samples were immediately separated in a cooling centrifuge at 4°C (3500 rpm, 7 min). An aliquot of plasma was kept at -20°C until processing.

Triphasic CT abdomen was done for all patients included in the study (n=32) except iatrogenic and control groups, dynamic MRI on the pituitary gland was done only for the patients who had high ACTH levels, a series of MRI images are taken quickly over several minutes

after the gadolinium has been injected and if the result was normal, CT chest was done for them.

All participants provided written informed consent before inclusion. The health Science Research Ethics Board of Mansoura University approved the study.

Cortisol measurement:

Plasma and urine were assayed for cortisol by competitive immunoenzymatic method and ACTH by enzyme chemilumenscence immunoassay using Immulite 1000 (Seimens USA) while hair and salivary cortisol were assayed by competitive immunoenzymatic salivary cortisol kits supplied by DiaMetra (Italy).

Statistical analysis:

Statistical analysis was performed using SPSS software, version 20 (SPSS 20.0; SPSS, Inc., Chicago, IL). Values are presented as median and range. Comparison was performed using Student's t-test or Mann- Whitney U-test as indicated. Bivariate correlation analyses between variables were tested by Pearson correlation test. P values less than 0.05 were considered significant. The diagnostic performance of midnight salivary cortisol and hair cortisol measurements were evaluated using receiver operator characteristic analysis (ROC), and these data were compared to those obtained from ROC analysis of the overnight serum cortisol.

Results

I. Comparing the results in patients who proved to have Cushing's syndrome, Cushing's disease and cyclic Cushing (excluding one case from cyclic Cushing (group A) Vs patients in whom Cushing's syndrome was excluded by detailed hormonal testing (cushingoid and adrenal incidentaloma group (group B)

a. Midnight salivary, hair cortisol, midnight plasma cortisol, and LDDST:

As shown in Table 4, 5, 6 the overnight salivary cortisol concentration was significantly higher (group A) compared to (group B), control obese and control non obese group. As expected, patients with Cushing's syndrome had significantly higher overnight serum cortisol compared to those measured in the 3 groups separately. However there was no significant

difference between hair cortisol levels in group A, when compared to the 3 groups separately.

II. Comparing the results of hair cortisol in male patients (group A) Vs female patients (group A) and Vs control group:

Hair cortisol levels were significantly higher in males (group A) median (range) (15.65 (2.2-72.1) ng/ml Vs females (group A) 3.6 (1.7-6) ng/ml, P value= 0.05, and between males (group A) median (range) 15.65 (2.2-72.1) ng/ml Vs control group 3.8 (2.3-12.1), p value= 0.047.

III. Analytical performance of salivary cortisol measurements in the diagnosis of Cushing's syndrome:

To study the analytical performance of salivary cortisol measurements in the diagnosis of CS, the cut-off values and the corresponding analytical sensitivity and specificity were calculated according to the ROC curves. Bedtime measurements showed that salivary cortisol levels have high sensitivity and specificity. Thus, salivary levels could be used as a fist line screening test for CS in the general population because of the high sensitivity and specificity that reduces the number of false positives. These findings are in agreement with other studies that have found slightly higher sensitivities, also employing healthy people as controls[15]. There are other studies that employ healthy people and obese patients as control group[16]. Other studies have also concluded that salivary levels at midnight are an adequate screening test, but they argue a lack of validation of diagnostic criteria[17]. Thus, we present reference values, obtained from patients with Cushing's disease, CS and healthy people in order to obtain representative values of the general population. Therefore, 4.2 ng/dL in saliva at 11 pm appears to be an appropriate cut off point to distinguish patients with CS with 93.75% sensitivity and 94.7% specificity (0.984 area under roc curve 95% Confidence Interval 0.95-1) (fig. 1) and perform subsequent confirmation.

VII. Correlation between salivary and plasma cortisol measurements:

Cortisol concentrations in ser-

um significantly correlated with cortisol concentrations in saliva in the whole studied population (r=0.775, p=0.000) (Fig. 2).

Table (1):

Hormone measurement	group A n=16	group B n=15	P value
11pm sr. cortisol (ng/ml) median (range)	337(145-625)	78 (7.9-337)	P=0.000
11pm salivary. cortisol (ng/ml) median (range)	11.1 (3.5-83.8)	3.5 (0.9-9.7)	P=0.000
Hair cortisol(ng/ml) median (range)	4 (1.7-72)	3.3 (1-8.6)	P=0.129
24 h UFC (ug/24h) median (range)	853 (169-1750)	378 (28-706)	P=0.000
Sr. cortisol after LDDST (ng/ml) median (range)	212 (22-507)	3.4 (0.6-80)	P=0.000

Table (2):

Hormone measurement	Study group A n=16	control non-obese n=20	P value
Age in ys mean ± SD	29±10	26±7	P = 0.169
Sex (females)	10 (62.5%)	12 (60%)	P = 0.87
BMI mean ± SD	37±7	28±2	P = 0.000
11pm sr. cortisol (ng/ml) median (range)	337(145-625)	9.75 (2.5-88.6)	P = 0.000
11pm salivary. cortisol (ng/ml) median (range)	11.1 (3.5-83.8)	2.35 (0.8-4.5)	P = 0.000
Hair cortisol (ng/ml) median (range)	4 (1.7-72)	4 (2.3-103)	P=0.987
24 h UFC ug/24h median (range)	853 (169-1750)	140 (64-295)	P = 0.000

Table (3):

Hormone measurement	Study group A n=16	control obese n=12	P value
Age in ys mean ± SD	29±10	34±9	P = 0.239
Sex (females)	10 (62.5%)	10 (83.3%)	P = 0.4
BMI mean ± SD	37±7	36±4	P = 0.455
11pm sr. cortisol (ng/ml) median (range)	337(145-625)	6.5 (4.1-136.9)	P = 0.000
11pm salivary. cortisol (ng/ml) median (range)	11.1 (3.5-83.8)	1.3 (0.3-3.1)	P = 0.000
Hair cortisol median (ng/ml) (range)	4 (1.7-72)	4.9 (3.1-117.6)	P=0.732
24 h UFC ug/24h median (range)	853 (169-1750)	113.5 (26-200)	P = 0.000

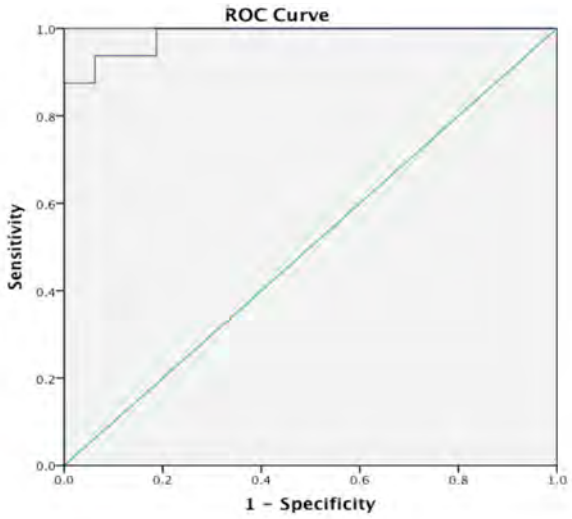

Fig. 1: ROC analysis of midnight salivary cortisol.

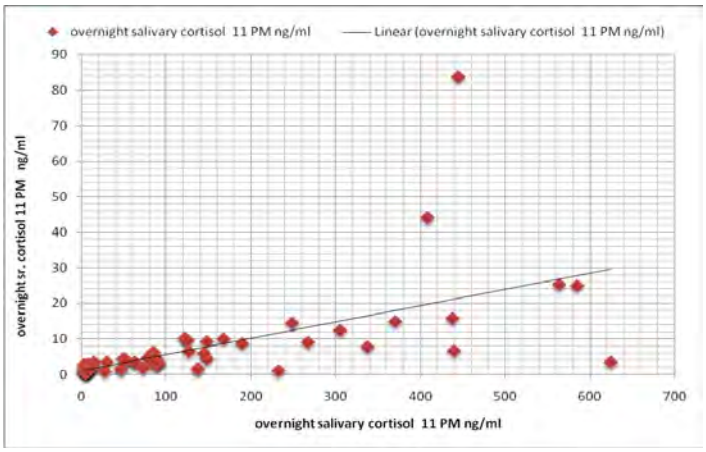

Fig. 2: Cortisol levels measured in samples of serum correlate (r=0.775, p=0.000) with those found in saliva obtained at the same time point from 32 healthy people, from 17 patients with Cushing's syndrome, from 8 patients with iatrogenic Cushing, from 3 with adrenal incidentaloma and from 12 patients with Cushingoid features.

Discussion

Serum and salivary cortisol levels in Cushing's syndrome:

Measurement of serum cortisol is the common practice for the initial screening of CS. Interestingly, in the present study, serum and salivary cortisol levels significantly correlated in 72 individuals. This finding is in agreement with previous reports and shows, as other groups have also demonstrated, that the association of serum and salivary cortisol appears to be independent of the status of health of the patient[18,19,20]. As expected, serum cortisol levels were different in patients with CS compared with controls and these differences are reproduced in saliva. Both serum and saliva cortisol levels are lower in healthy subjects compared with Patients with CS suggesting that saliva measurements are appropriate in handling this disease. Patients with CS present higher serum levels of cortisol at midnight than controls without differences between patients with CS. One important and relevant finding of the present study for the clinical practice is that salivary cortisol levels at 23:00 h allows distinguishing patients with CS from patients with cushingoid features or healthy control subjects. Based on our data, healthy controls present suppressed values of cortisol at midnight.

Advantages of salivary measurements:

Thus, as salivary measurements present similar or better analytical characteristics in the detection of patients with CS, our data also support that it is possible to replace the serum determination with salivary cortisol measurements as a screening test, because they present several advantages. First, in addition to eliminating venipuncture, saliva can be easily obtained at home, avoiding admission in the hospital. Salivary cortisol measurements could minimize this bias improving test performance. Furthermore, most currently screening tests for CS have the disadvantage of a high false positive rate. In our study we have shown that in patients with CS, bedtime salivary cortisol presents high sensitivity which could be due to the fact that salivary levels of cortisol reflect the biologically active

free form. Moreover, salivary cortisol measurements allowed distinguishing between patients with CS and those with cushingoid features. Thus, overall the rate of false positive results is reduced avoiding unnecessary tests[21]. It is fortunate that cortisol is stable in saliva at room temperature for at least a week, and samples can be mailed to the laboratory by standard postal service[22,23]. In summary, in this study we have shown bedtime salivary cortisol levels, due to their accuracy and convenience are a valuable alternative to help in the diagnosis of patients with CS.

Cortisol analysis from hair samples:

While the analysis of cortisol in hair is an exciting and informative tool to assess chronic systemic cortisol exposure, one must be aware of its potential limitations. Many different factors could influence the results of hair testing. These may include hair growth rates, gender, age, hair colour, environmental exposures and others. Hair colour is another important consideration that is not well understood. As mentioned earlier, Sauvé et al. found chemically treated hair (dyed hair) to have significantly lower hair cortisol concentrations than those with untreated hair[12]. Washing with shampoo and water gave rise to cortisol loss in monkey's hair and hair cortisol loss increased with washing frequency[24]. The wash-out effect was suggested to possibly cause inter-segment loss in hair cortisol along hair shaft from the scalp-near segment to distal segment[25,11,26]. Compared with the hair segments far from the scalp, the 1-cm hair segment closest to the scalp might show less variability and keep more cortisol because it received less influences of washing and other environmental factors. The inter-individual differences in these external factors might give rise to some variations in hair cortisol content among subjects[12].

In our study there was no significant differences between hair cortisol levels in the cases already diagnosed being CS compared with the control non obese group, P=0.987, control obese group, P=0.732 and when compared with cases with cushingoid features

P=0.129, however there was a statistically significant difference between hair cortisol levels in male versus female cases diagnosed being CS, P value= 0.05, and there was also a statistically significant difference between hair cortisol levels in male CS versus the whole control group, p value= 0.047.

Our results of hair cortisol were not conclusive and this may be attributed to many factors, firstly, Cutting too short hair in males versus females, unknown duration of exposure to high serum cortisol levels including cases with cyclic Cushing, may be there is undiagnosed cases with cyclic Cushing who are diagnosed during the cycle of over-activity with long periods of hypo-reactivity, heterogeneity of the studied group with the different aetiologies and finally repeated wash of the hair.

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NOVEL INSIGHTS IN DIAGNOSIS OF CUSHING SYNDROME: ROLE OF HAIR AND SALIVARY CORTISOL

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B CELL SUBPOPULATIONS ANALYSIS IN PATIENTS WITH PRIMARY HUMORAL IMMUNODEFICIENCY

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Abstract

Background: *Primary antibody deficiencies are the most common primary immunodeficiencies and are characterized by a defect in the production of normal amounts of antigen-specific antibodies. The pathogenesis of some primary humoral immunodeficiency diseases, includes defects in maturation of B lymphocytes at different stages of development.*

Objective: *The aim of the present study is to analyze peripheral B cell subpopulations in patients with, common variable immunodeficiency (CVID), unclassified hypogammaglobulinemia UCH, selective (Ig) A deficiency (sIgAD) and selective (Ig) M deficiency (sIgMD).*

Methods: *The study population comprised 25 patients with hypogammaglobulinemia (CVID, 10; UCH, 5; sIgAD, 5; sIgMD, 5) admitted to Mansoura University Children`s Hospital between January 2011 and April 2012, as well as 50 healthy controls, B cell subpopulations (Naïve, non switched & switched memory cells) were analyzed using flowcytometry.*

Results: *Switched (CD19+CD27+IgD-) memory B cell values were found to be significantly lower in patients with CVID than in healthy controls. Non-switched (CD19+CD27+IgD+) memory B cell values were found to be significantly lower in patients with selective IgM deficiency than in healthy controls. No significant differences in B cell subsets were found in patients with selective IgA deficiency and UCH.*

Conclusions: *The present study observed that class-switched memory B cell values were lower in patients with CVID only, however there is a significant decrease in non switched memory cells in cases of selective IgM deficiency these observations suggestsa final maturation defect could play a role in the pathogenesis of CVID and selective IgM deficiency.*

Introduction

Immunity-the state of protection from infectious disease -has both a less specific and more specific component. The less specific component, innate immunity, provides the first line of defense against infection. In contrast to the broad reactivity of the innate immune system, which is uniform in all members of a species, the specific component, adaptive immunity, does not come into play until there is an antigenic challenge to the organism. Adaptive immunity responds to the challenge with a high degree of specificity as well as the remarkable property of "memory"[1].

The immune system consists of many different organs and tissues that are found throughout the body. These organs can be classified functionally into two main groups. The primary lymphoid organs (thymus & bone marrow) organs provide appropriate microenvironments for the development and maturation of lymphocytes and the secondary lymphoid organs (lymph nodes, spleen and mucosa associated lymphoid tissues) trap antigen from defined tissues or vascular spaces and are sites where mature lymphocytes can interact effectively with that antigen[2].

Primary immunodeficiency diseases (PID) are a heterogeneous group of disorders defined by defects in genes involved in host defense. During the past 25 years or so expansive increases in our knowledge of basic immunology and human genetics have led to recognition of several distinct immunodeficiency disorders and their underlying genetic causes. More than 95 clinically distinct PID are now recognized and this number continues to increase rapidly[3].

Accurate diagnosis and classification of PID is necessary to decide on appropriate clinical management, to enable informed genetic counseling and to permit the systematic collection of data on PID through registries that will facilitate further study of these rare diseases.PID have been classified into five broad groups encompassing: (a) combined immunodeficiencies; (b) primarily antibody deficiencies; (c) phagocyte disorders;

(d) other welldefined immunodeficiency syndromes; and (e) complement deficiencies[4].

Hypogammaglobulinemia is defined as a decrease of 2SD in the levels of at least 1 of the immunoglobulin (Ig) isotypes compared to mean values for age. IgG levels reach their lowest values at 3-6 months of age (normal-physiologic hypogammaglobulinemia). Hypogammaglobulinemia persisting beyond 6 months of age is referred to as transient hypogammaglobulinemia of infancy (THI)[5].

THI is characterized by prolonged hypogammaglobulinemia that resolves spontaneously by 2-3 years of age, although improvement may not be seen until 5-6 years in some patients[6]. Because of this conflict in terminology, patients with persistent hypogammaglobulinemia after 3 years of age are considered to have "unclassified hypogammaglobulinemia" (UCH) [7]. Although the pathogenesis of THI remains unclear, the disease is thought to be due to a maturation defect of Ig synthesis[8].

Common variable immunodeficiency (CVID) is an idiopathic antibody deficiency with an estimated prevalence of 1:25,000. It is defined by serum IgG levels below 2 SD of normal controls in the presence of decreased IgA and/orIgM levels, recurrent infections, impaired response to immunization, exclusion of defined causes of hypogammaglobulinemia,and an age above 2 years. Less than10% of the CVID patients have a positive family history and a genetic defect has only been identified in less than 10% of the patients who have been reported to the ESID (European society of immunodeficiencies) primary immunodeficiency database with the clinicalphenotype of CVID. Reported defects involve B cella ctivation (CD19 and CD81 deficiency), costimulation (ICOS deficiency) and B cell survival (BAFF-R deficiency)[9].

Selective IgA deficiency (sIgAD) is the most prevalent primary humoral immunodeficiency, the various pathogenic mechanisms postulated for sIgAD include IgA-specific suppressor T-cell activity, inadequate helper T-cell function, an intrinsic B-cell defect, and decreased expression of CD49 on

monocytes[10]. sIgAD is considered to be genetically associated with common variable immunodeficiency (CVID), since CVID can develop from sIgAD[11].

Selective IgM deficiency (sIgMD) is a rare dysgammaglobulinemia characterized by a decreased serum IgM level <2SD or <20 g/L with normal levels of the other Ig isotypes. For the diagnosis of primary sIgMD, the other immunodeficiencies and IgMD secondary to other diseases must be ruled out[12]. The pathogenesis of sIgMD is also unclear, although intrinsic IgM-secreting B-cell defects, lack of the helper function of $CD4^+$ cells, and excessive activity of $CD8^+$ suppressor cells have been hypothesized as the mechanisms[13].

The objective of this study is to analyze B-cell subpopulations in CVID, UCH, IgAD, and sIgMD patients.

Subjects and Methods

This study was performed on 25 children (10 CVID, 5 UCH, 5 sIgAD and 5 sIgMD), attending Mansoura University Children Hospital with mean age ± SD (4.25y`±0.64), also 50 apparently healthy children of matched age & sex with mean age ± SD (3.87y`±0.58) were selected to act as a control group.

Written consents were taken from children`s mothers before doing the laboratory investigations. Patients & controls were routinely investigated at Mansoura university children`s hospital for CBC (with total and differential leukocytic count), complete liver function tests, renal function tests, ESR & CRP.

Also the immune competence of the studied groups was investigated in the following order:

1- Measurement of serum IgG, IgM& IgA: by using commercial kits (Orion DiagnosticaTurbox assay, Finland).

2- Assessment of specific antibody response: by measurement of serum level of anti-tetanus toxoid antibodies using commercial kits (IBL international GMBH, Germany) before and after vaccination with tetanus toxoid.

3- Assessment of B cells surface markers: by using commercial kits (CD_{19}FITCBecton & Dick-

inson Bioscience, Ireland) / (CD_{27}PEBecton & Dickinson Bioscience, Ireland) /(Surface IgD-PerCPMiltenyiBiotec GmbH, Germany).

Immunophenotyping of B-cell subpopulations: After gating on lymphocytes according to forward (FSC) /side scatter (SSC), B cells are characterized by CD19 staining. By staining for CD27 and IgD, naive $CD19^+IgD^+CD27^-$ cells, $CD19^+IgD^+$ $CD27^+$nonswitched memory B cell and $CD19^+IgD^-CD27^+$ switched memory B cells were distinguished.

Statistical analysis and separation between control and the tested groups was done using independent samples T test through theStatistical Package for Social Sciences (SPSS 20) a p. value ≤0.05 was considered to be significant.

Results

Patients in this study were divided into 4 groups (group I CVID, group II UCH, group III sIgAD and group IV sIgMD) and their results were compared to those of healthy controls.

- There is a statistically highly significant decrease in serum IgG level and serum IgA level in patients (5.20 g/L ± 1.23 g/L) and (0.54 g/L ± 0.26 g/L) respectively, compared to that of healthy controls (16.65 g/L ± 3.13 g/L) and (1.21 g/L ± 0.14 g/L) respectively, P value 0.002 and 0.001 respectively, however there is no significant difference in serum IgM level in patients (1.69 g/L ± 1.28 g/L) compared to that of healthy control group (2.62 g/L ± 1.02 g/L), P value >0.05.

- All cases of CVID show subnormal specific antibody response to tetanus toxoid vaccination.

- There is no statistically significant difference in $CD19^+$ B lymphocytes (19.32%±4.95) in patients compared to that of healthy control group (19.52%±4.13), P value >0.05.

- There is no statistically significant difference in $CD19^+IgD^+CD27^-$ cells (naïve B lymphocytes) and $CD19^+IgD^+CD27^+$ (non switched memory B cells) (85.37%±3.91), and (6.67%±2.07) respectively compared to that of healthy control group

(84.28%±4.39) and (6.92%±1.83) respectively, P value > 0.05, however there is a statistically highly significant decrease in $CD19^+IgD^-CD27^+$ (switched memory B cells) in patients (0.86% ± 0.06) compared to that of healthy control group (5.24%±1.74) P value 0.003.

- There is a statistically highly significant decrease in serum IgG level in patients (6.89 g/L ± 0.47 g/L) compared to that of healthy controls (16.65 g/L ± 3.13 g/L), P value 0.001, however there is non significant difference in serum IgM and serum IgA levels in patients (1.59 g/L ± 0.61 g/L) and (1.21 g/L ± 0.14 g/L) respectively compared to that of healthy control group (2.62 g/L ± 1.02 g/L) and (2.47 g/L ± 1.21 g/L) respectively, P value >0.05.

- All cases of UCH show normal specific antibody response to tetanus toxoid vaccination.

- There is no statistically significant difference in $CD19^+$ cells (B lymphocytes) (16.97%±3.62) of patients compared to that of healthy control group (19.52%±4.13), P value >0.05.

- There is no statistically significant difference in $CD19^+IgD^+CD27^-$ cells (naïve B lymphocytes), $CD19^+IgD^+CD27^+$ (non switched memory B cells) and $CD19^+IgD^-CD27^+$ (switched memory B cells) (80.91%±4.32), (8.26%±2.61) and (5.12%±1.75) respectively compared to that of healthy control group (84.28%±4.39), (6.92%±1.83) and (5.24%±1.74) respectively, P value >0.05.

- There is no significant difference between serum IgG and serum IgM inpatients (13.27 g/L ± 5.07 g/L) and (2.52g/L ± 1.07 g/L) respectively, and in healthy controls (16.65g/L ± 3.13 g/L) and (2.62 g/L ± 1.02 g/L) respectively, P value > 0.05, however there was a statistically highly significant decrease in serum IgA of patients of this group (0.21 g/L ± 0.08 g/L) compared to healthy control group (2.47 g/L ± 1.21 g/L), P value 0.008.

- All cases of sIgAD show normal specific antibody response to tetanus toxoid vaccination.

- There is no statistically significant difference in $CD19^+$ cells (B

lymphocytes) (20.47%±3.35) in patients compared to that of healthy control group (19.52%±4.13), P value >0.05.

- There is no statistically significant difference in $CD19^+IgD^+CD27^-$ cells (naïve B lymphocytes), $CD19^+IgD^+CD27^+$ (non switched memory B cells) and $CD19^+IgD^-CD27^+$ (switched memory B cells) (83.1%±4.74), (7.05%±2.17) and (5.43%±1.37) respectively compared to that of healthy control group (84.28%±4.39), (6.92%± 1.83) and (5.24%±1.74) respectively, P value >0.05.

- There are no significant differences between serum IgG and serum IgA levels in patients (11.95g/L ± 1.32 g/L) and (1.21g/L ± 0.23 g/L) respectively, and in healthy controls (16.65g/L ± 3.13 g/L) and (2.47 g/L ± 1.21g/L) respectively, P value > 0.05, however there is a statistically highly significant decrease in serum IgM of patients of this group (0.23 g/L ± 0.01g/L) compared to healthy control group (2.62g/L ± 1.02 g/L), P value 0.004.

- All cases of sIgM show normal specific antibody response to tetanus toxoid vaccination.

- There is no statistically significant difference in $CD19^+$ cells (B lymphocytes) (18.92%±3.67) in patients compared to that of healthy control group (19.52%±4.13), P value > 0.05.

- There is no statistically significant difference in $CD19^+IgD^+CD27^-$ cells (naïve B lymphocytes)and $CD19^+IgD^-CD27^+$ (switched memory B cells) (83.32%±7.52), and (5.88%±1.85) respectively compared to that of healthy control group (84.28%±4.39), and (5.24% ±1.74) respectively, P value >0.05, however there is a statistically highly significant decrease in $CD19^+IgD^+CD27^+$ (non switched memory B cells) in patients (2.65%±0.09) compared to that of healthy control group (6.92%± 1.83) P value 0.005.

Table (1): Classification of studied patients.

Group	No.	
1	10	Common variable immunodeficiency (CVID)
2	5	Unclassified hypogammaglobulenemia (UCH)
3	5	Selective IgA deficiency (sIgAD)
4	5	Selective IgM deficiency (sIgMD)

Table (2): Serum IgG, IgM and IgA levels in patients with common variable immunodeficiency (group I), compared to control.

Test		Group I	Control	P. value
Serum IgG g/L	Mean	5.21	16.65	0.002*
	SD ±	1.23	3.13	
Serum IgM g/L	Mean	1.69	2.62	0.587
	SD ±	1.28	1.02	
Serum IgA g/L	Mean	0.54	2.47	0.001*
	SD ±	0.26	1.21	

Table (3): B lymphocyte subpopulations in patients with common variable immunodeficiency (group I) compared to healthy controls.

Test		Group 1	Control	P. value
$CD19^+$ cells%	Mean	19.32	19.52	0.512
	SD ±	4.95	4.13	
Naïve B cells%	Mean	85.37	84.28	0.387
	SD ±	3.91	4.39	
Non switched memory B cells%	Mean	6.67	6.92	0.842
	SD ±	2.07	1.83	
Switched memory B cells%	Mean	0.86	5.24	0.003*
	SD ±	0.06	1.74	

Table (4): Serum IgG, IgM, and IgA levels in patients with unclassified hypogammaglobulinemia (group II), compared to control.

Test		Group II	Control	P. value
Serum IgG in g/L	Mean	6.89	16.65	0.001*
	SD ±	0.47	3.13	
Serum IgM in g/L	Mean	1.59	2.62	0.235
	SD ±	0.61	1.02	
Serum IgA in g/L	Mean	1.21	2.47	0.459
	SD ±	0.14	1.21	

Table (5): B lymphocyte subpopulations in patients with UCH (group II) compared to healthy controls.

Test		Group II	Control	P. value
$CD19^+$ cells%	Mean	16.97	19.52	0.271
	SD ±	3.62	4.13	
Naïve B cells%	Mean	80.91	84.28	0.519
	SD ±	4.32	4.39	
Non switched memory B cells%	Mean	8.26	6.92	0.372
	SD ±	2.61	1.83	
Switched memory B cells%	Mean	5.12	5.24	0.287
	SD ±	1.75	1.74	

Table (6): Serum IgG, IgM, and IgA levels in patients with selective IgA deficiency (group III) compared to healthy controls.

Test		Group III	Control	P. value
Serum IgG in g/L	Mean	13.27	16.65	0.181
	SD ±	5.07	3.13	
Serum IgM in g/L	Mean	2.52	2.62	0.504
	SD ±	1.07	1.02	
Serum IgA in g/L	Mean	0.21	2.47	0.008*
	SD ±	0.08	1.21	

Table (7): B lymphocyte subpopulations in patients with selective IgA deficiency (group III) compared to healthy controls.

Test		Group III	Control	P. value
$CD19^+$ cells%	Mean	20.47	19.52	0.786
	SD ±	3.35	4.13	
Naïve B cells%	Mean	83.10	84.28	0.878
	SD ±	4.74	4.39	
Non switched memory B cells%	Mean	7.05	6.92	0.953
	SD ±	2.17	1.83	
Switched memory B cells%	Mean	5.43	5.24	0.523
	SD ±	1.37	1.74	

Table (8): Serum IgG, IgM and IgA levels in patients with selective IgM deficiency (groupIV), compared to control.

Test		Group IV	Control	P. value
Serum IgG in g/L	Mean	11.95	16.65	0.186
	SD ±	1.32	3.13	
Serum IgM in g/L	Mean	0.23	2.62	0.004*
	SD ±	0.01	1.02	
Serum IgA in g/L	Mean	1.21	2.47	0.328
	SD ±	0.23	1.21	

Table (9): B lymphocyte subpopulations in patients with selective IgM deficiency (group IV), compared to control.

Test		Group IV	Control	P. value
$CD19^+$ cells%	Mean	19.82	19.52	0.562
	SD ±	3.67	4.13	
Naïve B cells%	Mean	83.32	84.28	0.891
	SD ±	7.52	4.39	
Non switched memory cells%	Mean	2.65	6.92	0.005
	SD ±	0.09	1.83	
Switched memory cells%	Mean	5.88	5.24	0.248
	SD ±	1.85	1.74	

Discussion

Primary immunodeficiency diseases (PIDs) are considered rare and phenotypically variable that is why many primary health care physicians are not familiar with these disorders, many patients arrive at immunology specialists too late for proper diagnosis and treatment; often there are complications secondary to manifestations of the disease, which are associated with a compromised quality of life, increased morbidity, and mortality[14].

Most registries from Western countries have reported a predominance of antibody production defects over other forms of PID[15]. Additionally, there are a few reports from Arabian countries and the Middle East. A group from Tunisia reported 152 patients with PID over a period of 8 years, with a mortality of 24%[16]. One center in eastern Saudi Arabia found that over a period of 6 years, 31 patients were diagnosed with humoral immunodeficiency[17]. Kuwait reported 76 patientswith PID between 2004 and 2006, of which 98% presentedin childhood with 77% consanguinity[18]. An Egyptian study reported 64 patients with a higher prevalence of combinedimmunodeficiency over other forms and 62% consanguinity and a mortality of 23%[19]. The Iranian Primary Immunodeficiency Registry reported 440 patients with PID seen overa period of 20 years. There was a predominance of antibody production and phagocytic defects over other PID as compared with other registries, and only 3 patients with complement deficiencies were identified[20]. In other Asian countries, like Taiwan, over a 20-year period, 37 pa-

tients were diagnosed with PID, antibody production defects and phagocytic function defects predominated over other forms of PID, and no patients were identified with complement deficiencies [21].

This study was conducted on 4 groups (group I, group II, group III and group IV) CVID, UCH, sIgAD and sIgMD respictively.Group I included 10 patients diagnosed as CVID showing significant decrease of serum IgG, serum IgM levels and switched memory B cells ($CD19^+CD27^+IgD^-$) compared to that of healthy control, tables (2) & (3), the same results were published by Aghamohammadi et al [21], Warnatz et al.[22], Sanz et al.[23] and Vodjgani et al.[24]. In an analysis of B-cell subsets in 56 CVID, Bukowska-Strakova et al [25] showed that the percentage of switched memory B cells decreased in those patients. The authors suggested that patients with profoundly decreased switched memory B-cell values should be monitored for development of CVID.

Normally, after antigen-independent development in the bone marrow, immature B cells leave the bone marrow and gather in the longer-lived mature, naive ($CD19^+CD27^-IgD^+$) B-cell pool. When these cells are stimulated by antigen in the presence of the appropriate co-stimulation they will engage in a germinal center (GC) reaction and develop into plasma cells or memory B cells. In most patients with CVID the development of both cells types is greatly disturbed while mature B cells are present in normal numbers, indicating defects in the late B-cell differentiation[26].

Warnatz et al.[22] used the percentage of switched memory B cells ($CD19^+CD27^+IgD^-$) of the PBL fraction as a reliable marker for a classification of CVID patients in their study where the authors reported 77% of CVID patients in that study with normal B-cell counts contained less than 0.35% of switched memory B cells, this group of patients was designated as CVID group I. The remaining 23% of the patients showed less significant reduction in switched memory cells (less than 0.9%) they were classified as

group II CVID. Group I was furtherly subdivided according to the percentage of $CD21^-$ B cells of total B cells ($CD19^+$cells) into 2 subgroups Ia with an increased proportion of $CD21^-$ B cells and Ibwith normal numbers of this cell population.

As regard grpup II (UCH) the current study showed 5 patients having statistically lower serum IgG level compared to healthy control group, normal specific antibody response to tetanus toxoid and normal percentages of B cell subsets, the same findings were reported by Cipe et al[27] whom diagnosed them as unclassified hypogammaglobulinemia.

In this study the group III includes 5 patients with selective IgA deficiency having serum IgA (0.21 g/L ± 0.085) which is significantly lower than that of healthy control (2.47 g/L ± 0.121), P. value 0.008 table (6). According to Crisanti et al[28], the prevalence of sIgAD in Europe varies between 1:163 and 1:875. While, the incidence is much lower in Asian populations according to Kanoh et al.[29].

All the 5 patients with sIgAD in our study showed normal antibody response to tetanus toxoid and normal percentages of B lymphocytes (20.47%±3.35) and B cell subsets: naïve cells (83.1%±4.74), non switched (7.05%±2.17) and switched memory cells (5.43%± 1.37), table (7), these results are in agreement with those reported by Cipe et al[27], and Litzman et al[30], in their study on 41 and 36 patients in Turkish and Polish population, respectively on the other hand Bukowska-Strakova et al(26), reported marked decrease in switched memory B cells ($CD19^+CD27^+IgD^-$), this difference may be due to variation in the mechanism of the disease because IgA deficiency represents a heterogeneous group of genetic abnormalities which includes mutations in transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI, TNFRSF13B) TACI. B-cell surface ligand for BAFF and APRIL, has a role in isotype switching in B cells, also a shared cytotoxic T lymphocyte-associated protein-4-inducible costimulator mutation is considered a risk locus for IgA deficiency, T helper cell dysfunction, and

suppressor T cells have all been reported in IgA deficiency. Abnormalities in the cytokine network such as lack of IL-4, IL-6, IL-7, IL-10, TGF-β, and most recently IL-21 have also been proposed to play a role in IgA deficiency[31].

In the current study 5 cases were diagnosed as sIgMD with no significant difference between serum IgG and serum IgA in patients (11.95 g/L ± 1.32 g/L) and (1.21 g/L ± 0.23 g/L) respectively, and in healthy controls (16.65 g/L ± 3.13 g/L) and (2.47 g/L ± 1.21 g/L) respectively, P value > 0.05, however there was a statistically highly significant decrease in serum IgM of patients of this group (0.23 g/L ± 0.01 g/L) compared to healthy control group (2.62 g/L ± 1.02 g/L), P value 0.004 table (8). These results were in agreement with that reported by Yel et al[31], on defining the immunological finding of 15 cases of sIgMD in the American population.

For the 5 cases of sIgMD in this study there was no statistically significant difference in $CD19^+$ cells (B lymphocytes) (18.92%± 3.67) of patients compared to that of healthy control group (19.52%± 4.13), P value >0.05.There was no statistically significant difference in $CD19^+IgD^+CD27^-$ cells (naïve B lymphocytes) and $CD19^+IgD^-CD27^+$ (switched memory B cells) (83.32%±7.52), and (5.88%±1.85) respectively compared to that of healthy control group (84.28%± 4.39), and (5.24%±1.74) respectively, P value >0.05, however there was a statistically highly significant decrease in $CD19^+IgD^+CD27^+$ (non switched memory B cells) in patients (2.65%±0.09) compared to that of healthy control group (6.92%±1.83) P value 0.005 the same as reported by Cipe et al[27]. In contrast Belgemen et al[32] on their study for a case of sIgMD represented by recurrent impetigo, they declared a marked decrease in switched memory B cells ($CD19^+CD27^+IgD$) unlike the results of our study. In contrast, Revy et al[33] reported a very high level of non switched memory B cells in a patient with autosomal recessive type 2 hyper IgM syndrome. In the light of these findings, we can speculate that low IgM levels are due to low percentages of non switched memory B cells.

Conclusions

The present study observed that class-switched memory B cell values were lower in patients with CVID only, however there is a significant decrease in non switched memory cells in cases of selective IgM deficiency these observations suggests a final maturation defect could play a role in the pathogenesis of CV|ID and selective IgM deficiency.

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B CELL SUBPOPULATIONS ANALYSIS IN PATIENTS WITH PRIMARY HUMORAL IMMUNODEFICIENCY

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ANTISOCIAL PERSONALITY DISORDER AMONG PRISONERS; FACTS AND MISLEADS

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Abstract

Introduction: *Antisocial personality disorder is one of cluster B personality disorder which is characterized by pervasive pattern of disregard for and violation of the rights of others, consistent irresponsibility, failure to conform to social norms and deceitfulness, occurring since age 15 years. It is not easy to diagnose antisocial personality disorder in the community. Among all societies there are many hateus crimes so deviated from the common human behavior norms that can make even psychiatrists astonished. On the other side there is a great myth in the community that any person was incarcerated once before is a villain, this research was done to highlight the magnitude of antisocial personality disorders among Egyptian prisoners and throw the light upon the causes of self-injurious behaviors among prisoners.*

Patients and Methods: *This is a cross- sectional study on adult prisoners. The target sample size is 1350 prisoners, in a ratio of 50 prisoners for each thousand. The interview with prisoners was applying standardized psychiatric assessment, the structured clinical interview for DSM_ IV axis 2 disorders (SCID II).*

Results: *The overall point Prevalence of antisocial personality disorder among studied prisoners is 12.3%.*

Conclusions: *Really a lot of Egyptian prisoners are incarcerated due to social, environmental and financial rationales; there is no excuse for any person to break the law and regulations, but bad bringing up of some families for their kids, not to grow them on values, manners and religion make them risky to acquire different forms of corruption which was disseminated in the Egyptian society all over the past 60 years.*

Introduction

Prevalence rate of antisocial personality disorder is between 0.2% and 3.3%. The highest prevalence of antisocial personality disorder (greater than 70%) is among substance abusers, forensic psychiatry settings and prisons. Prevalence is higher in samples affected by adverse socioeconomic and sociocultural factors such as poverty and migration. Antisocial personality disorder must be distinguished from criminal behavior undertaken for gain that is not accompanied by the personality features characteristic of this disorder[1]. About 9 million people are imprisoned worldwide, but the number with serious mental disorders (psychosis, major depression, and antisocial personality disorder) is unknown. Worldwide, several million prisoners probably have serious mental disorders, but how well prison services are addressing these problems is not known. The field of prison psychiatry research still growing, and there are many difficulties face the researcher at this field[2]. In Egypt, the situation is not different, although a few small scale studies were conducted to explore the real state of the mental health of the prisoners, the data about the situation of the mentally ill offenders in Egypt still not clear, especially with the increasing number of prisons, prisoners and with the multiplicity of penalties and crimes[3]. The percent of personality disorder are 42% & 65% in both men &women respectively; the percent of antisocial personality disorder are 21% & 47% in both women & men respectively[2]. The prevalence of personality disorder among prisoners was 37.1% for antisocial personality disorder[4]. Self injurious behavior among prisoners especially antisocial personality disorder persons is so disruptive because it may lead to actual death. Self-injurious behavior of prisoners include categories of non suicidal intentional self-injury, non suicidal habitual self injury and of suicide attempts, these typologies are related to the intent of some of these individuals to demonstrate the self-harm as an attempt to cope with the residual emotional effects of trauma such as undesired transfers and restrictive housing[5]. A hunger strike is an example of passive

self-injury which they are on the scale of disruptive behaviors[6]. Staff should know that these behaviors have multiple etiologies and require planned intervention rather than emotional reactions[7]. This research was done to highlight the magnitude of antisocial personality disorders among Egyptian prisoners.

Patients and Methods

This is a cross- sectional study on adult prisoners of both sexes with different crimes and different penalties, from age of 18 years old to 65 years old that have spent at least one year in the prison. Exclusion criteria: political prisoners and foreigners. This study was done in 16 prisons all over Egypt, during a period of 2 years from the 1st of March 2012 to end of February 2014. The target sample size is 1350 prisoners, in a ratio of 50 prisoners for each thousand. Sampling Technique was stratified proportional sample. The interview with prisoners was applying standardized psychiatric assessment, the structured clinical interview for DSM_ IV axis 2 disorders (SCID II). The Structured Clinical Interview for DSM-IV Axis II Disorders (SCID-II) Arabic version was translated by[8]. The SCID-II is a version of the SCID developed for the assessment of DSM-IV-TR Personality Disorders. Unlike the SCID-I, there is only a single version of the SCID-II (i.e., there are no separate research and clinician versions). There are three components to the SCID-II, the interview itself covers the eleven DSM-IV Personality Disorders (including Personality Disorder NOS) and the appendix categories Depressive Personality Disorder and Passive-Aggressive Personality Disorder[9]. A list of 16 prisons was provided by The Prison Sector of Ministry of Interior. Administrative approvals: a approval of the assistant of the Minister of the Interior for the prisons sector, approval of the section of the Public Relations Sector of the ministry of the interior and approval of each prison director for conducting the interviews with the inmates. The interviews were held at the hospital of the prison, library, buildings of the staff, to less extent in the buildings of the prisoners and lastly, inside the unit of execution by speaking with some prisoners who will be executed through the

window of the door of his highly secure room. The investigator conducted the interview with each prisoner privately after arrangement with the director of the prison, the police officers, police officer investigators, the doctors at the hospital of prisons, or the employers of the library. Oral consent was taken from each prisoner after discussing all the details regarding all the steps of the study, its futuristic results and its importance. Confidentiality was assured; prisoner has the right to withdraw from the interview at any time.

Results

The overall point Prevalence of personality disorder among studied prisoners is 12.3% for antisocial type. Overall point prevalence for paranoid, narcissistic, schizoid, schizotypal, borderline and depressive personality disorder is 0.1%. Prevalence of passive aggressive personality disorder is 0.4% and personality disorder not otherwise specified is 0.3%. The estimated highest prevalence was for antisocial type.

Table (1): Prevalence of different diagnoses of personality disorders among 1350 studied prisoners.

Diagnosis	Frequency	Percent
Antisocial Personality disorder.	166	12.3 %
Paranoid Personality disorder.	2	0.1 %
Schizotypal Personality disorder.	1	0.1 %
Schizoid Personality disorder.	1	0.1%
Borderline Personality disorder.	1	0.1 %
Narcissistic Personality disorder.	2	0.1 %
Personality disorder Nos.	4	0.3 %
Passive aggressive personality disorder.	5	0.4 %
Depressive Personality disorder.	1	0.1%
Total	183	13.6%

Discussion

In this study, the estimated overall point prevalence of antisocial personality disorders is 12.3%. International studies are not greatly different from the results of our study. The percent of personality disorder is 42% & 65% in both men & women respectively; the percent of antisocial personality disorder is 21% & 47% in both women & men respectively[2]. The prevalence of personality disorders among prisoners in the USA studies is for antisocial personality disorder 37.1%[4]. There is a great myth between lay persons that any person was incarcerated once before is avillain, but this is not the truth. Incarceration inside the prison is a stigma all over the world not in Egypt only. Really a lot of Egyptian prisoners are incarcerated due to social and financial rationales; there is no excuse for any person to break the law and regulations, but bad bringing up of some families for their kids, not to grow them on the ethics and religion make them risky to catch different forms of corruption which was disseminated in the Egyptian society all over the past 60 years. In this study, most of self injury behaviors were informed to the investigator by the staff, but at the same time were denied by prisoners because all of these behaviors are legally prohibited inside the prison, as a result, these behaviors were not estimated. Correctional staff stated that these behaviors happen as an emotional reaction, also as a psychopathic behavior, it is a prisoner claim for accusing officers of maltreatment. The situation is different regarding international studies, Study of 15 women who engaged in potentially lethal self-injury, institutional transfers and losses of a girlfriend are primary risk factors for suicide and self-harm[10]. Study of 143 self-injuring prisoners; found that those who self-injured without intent to die had precipitating factors related to negative emotions and the desire to reduce anger and frustration[11]. Disruptive behavior may be self-harm (Self-injurious behaviors may include cutting, scratching, burning, insertion, ingestion, asphyxiation, amputation, evisceration, and other creative damage to one's body) or inappropriate or aggressive sexual and emotional abuse (public

masturbation, lewd comments, coercive sex) or offences that make property damages (flooding or setting fires, failing to exit the cell). All of these strange behaviors happen in the Egyptian correctional settings, these behaviors were informed to the investigator by the staff, and no clear evidence exists to suggest that such behaviors occur in direct response to abuses of authority by officers as it was informed to the investigator by the staff. These behaviors may at times be viewed as tools of last resort for achieving a sense of control within restrictive environments[12].

Conclusions

Incarceration inside the prison is a stigma all over the world not in Egypt only. There is a great myth between lay persons that any person was incarcerated once before is avillain, but this is not the truth. Really a lot of Egyptian prisoners are incarcerated due to social, environmental and financial rationales; there is no excuse for any person to break the law and regulations, but bad bringing up of some families for their kids, not to grow them on values, manners and religion make them risky to acquire different forms of corruption which was disseminated in the Egyptian society all over the past 60 years. Prisoners in Egyptian prisons need insight development programs for problem adjustment issues, coping with a life and family issues and cycle of violence, also need programs for information and skill building such as communication skills, life span development, occupational therapy, parenting problem solving and all rehabilitation programs that are living up to international studies.

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ANTINUCLEAR AUTOANTIBODIES (ANA) AND C- REACTIVE PROTEIN (CRP) IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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Abstract

Background: *Despite its high and increasing prevalence and mortality, the pathogenesis of COPD remains unclear and there is no effective treatment available that halts the irreversible and progressive tissue destruction and small airway wall fibrosis which are characteristic of the disease.*

Aim of the work: *The present study was designed to investigate the ANA and CRP in different populations of COPD patients; it also investigated the relations of ANAs and CRP to some patient's characteristics (smoking behavior, gender, age, lung function, and physical characteristics.*

Patients and methods: *It carried out on 80 subjects, 40 of them had COPD; 20 stable COPD (group 1) and 20 during exacerbation of COPD (group2); another 20 non COPD smokers (group3) and finally 20 non COPD non- smokers (healthy controls) were included in the present study. All included individuals were subjected to the followings: Full medical history taking, thorough clinical examination, plain X-Ray chest, ECG, high Resolution Computed Tomography scanning of the chest (HRCT), spirometry, ANA & CRP measurement, body mass index was calculated. Finally, ANA and CRP were estimated according to methods described by (Jaskowski et al., 1996), and (Koenig et al., 1999) respectively.*

The results of the present study revealed that following: *There was statistically significant variance between studied groups as regard FEV1 and FEV1/FVC. CRP was positive in 26 cases (32.5% of all studied cases). There was significant difference between groups as regard positive CRP (90% of COPD exacerbation group had positive CRP, 25.0% of stable COPD, 10% of non COPD smokers and 5.0% of non - smoker non COPD groups had positive CRP). ANA was positive in 24 cases out of 80 cases (30.0%) and there was significant difference between studied groups (it was positive in 65.0%, 45.0%, 5.0% and 5.0% in COPD exacerbation, stable COPD, non COPD smokers and non-smokers non COPD groups respectively). There was significant association between CRP and ANA with ECG changes, but no association was found with BMI.*

Conclusion: *The present study showed increased levels of CRP and ANAs in the serum of COPD patients and correlated with the severity of the disease. These results confirmed the inflammatory nature of COPD and autoimmunity play a role. In addition, the results of the present study might suggest a role for immunosuppressive or immunomodulating anti-inflammatory drugs in treatment of COPD in the future.*

Introduction

Chronic obstructive pulmonary disease (COPD) is a widespread disease. Despite its high and increasing prevalence and mortality the pathogenesis of COPD remains unclear and there is no effective treatment available that halts the irreversible and progressive tissue destruction and small airway wall fibrosis which are characteristic of the disease[1].

The immune system is chronically activated in COPD. Markers of systemic inflammation such as C-reactive protein (CRP) and interleukin 6 (IL-6) are present instable COPD and their levels relate to disease severity[2].

Studies showing increased levels of antinuclear antibodies (ANAs)[3] indicate an antigen-specific self-reactive response in patients with COPD[1].

In addition, increased levels of

anti-elastin autoantibodies and Th1 responses against elastin have been found in emphysema[4]. This suggests the presence of antibody-mediated degradation of extracellular matrix which might contribute to inflammation. Other papers reported no evidence for systemic auto-antibodies directed against elastin peptides in chronic inflammatory lung disease[5].

Interleukin (IL)-6 is increased in the systemic circulation of COPD patients, particularly during exacerbations, and may account for the increase in circulating acute phase proteins such as C-reactive protein (CRP) found in COPD patients as it induces the release of acute phase proteins from the liver. CRP is an acute phase protein, which is increased in the plasma of COPD patients, particularly during acute infective exacerbations. In stable COPD, plasma concentrations are related to or cause mortality in mild to moderate patients, but not in severe and very severe patients[6].

Aim of The Work

This study is going to investigate the ANA and CRP in different populations of COPD patients; it will also investigate the relations of ANAs and CRP to some patient's characteristics (smoking behavior, gender, age, lung function, and physical characteristics.

Patients and Methods

The present study was conducted in the Departments of Chest Diseases at Al-Azhar University Damietta Hospital. It included 80 subjects who were attending the chest clinic or were admitted to inpatient wards where COPD patients had the clinical features or chest radiographs suggestive of chronic airflow obstruction and the spirometry revealed irreversible or partly-reversible airflow obstruction indicative of COPD according to the GOLD criteria.

The study subjects were divided into 4 groups each contains 20 subjects: Group I: contains 20 stable COPD patients of different grades of severity according to GOLD criteria, Group II: contains 20 COPD patients during exacerbation, Group III: contains 20 cigarette smokers non COPD, healthy individuals of matched

age and sex, Group IV: contains 20 apparently healthy, non-smokers individuals of matched age and sex as control group.

Inclusion Criteria: Patients who had a smoking history of more than 20 pack years; Patients had airflow limitation that was not fully reversible (post bronchodilator FEV_1 less than 80% of the predicted value in combination with FEV_1 /FVC % not more than 70%); patients who had an increase in FEV1 less than 200 mL and less than 12% of baseline value 30 minutes after the inhalation of 400 μg of β2-agonist salbutamol.

Exclusion Criteria: Patients had any evidence of coexisting bronchiectasis, interstitial lung disease, tuberculosis, cystic fibrosis, bronchial asthma, bronchogenic carcinoma, previous lung surgery, diabetes mellitus, liver cirrhosis or uremia.

All included individuals were subjected to the followings: Full medical history taking, thorough clinical examination, plain X-Ray chest, ECG, high Resolution Computed Tomography scanning of the chest (HRCT), spirometry, ANA & CRP measurement, body mass index calculation from the following equation: BMI = weight (kg)/(height (m))^2[7]. ANA was estimated according to method described by(8), while CRP was estimated according to method described by[9].

Statistical analysis of data: The obtained data of every patient were collected in a separate patient sheet then tabulated in a master table and fed to the computer on IBM SPSS version 20, statistical program for analysis of data. Appropriate statistical tests were used. For quantitative data, mean and standard deviation were calculated. For qualitative data, frequency and percentage distribution were calculated. Test for significance of qualitative data is Chi- square. Test for significance of quantitative data is one way analysis of variance (ANOVA) (F) test. A two-tailed P value of less than 0.05 was considered statistically significant.

Results

In the present work, there was no significant difference between

studied groups as regard age, COPD severity in groups I & II or for BMI. On the other hand, in the first three groups (smoking groups), smoking packs per year ranged from 21 to 52 pack with a mean of 31.39±6.79 pack and there was significant increase of packs in COPD exacerbation group and stable COPD group in comparison to non COPD smokers group (34.11±7.90, 32.09±7.20 vs 28.30±3.44 respectively). As regard pulmonary functions tests, there was statistically significant variance between studied groups as regard FEV1 and FEV1/FVC (healthy controls followed by non COPD smokers had the better functions, while COPD exacerbation and stable COPD groups had the worst respectively). Regarding arterial blood gases, there was statistically significant difference between studied groups as regard SaO_2, pH, PaO_2 and $PaCO_2$. In the present work, cases with COPD exacerbation and stable COPD had highest WBCs count, higher hemoglobin while non COPD smokers and non COPD nonsmokers groups had the lowest WBCs count and lower hemoglobin concentration with significant difference between groups (table 1).

In the present study, ECG was normal in 58 cases (72.5%); p-pulmonale presented in 12.5%, sinus tachycardia in 3.8%, right axis deviation in 6.2%, AF in 3.8% and left axis deviation in 1.2% of cases with significant difference between groups (all cases in non-smoker non COPD group had normal ECG, 95% of non COPD smokers had normal ECG, 20% in COPD exacerbation and 75% of stable COPD had normal ECG. In addition, All cases in first two groups showed hyperinflated chest and increased retrosternal space in PA and Lateral views X-rays respectively. In addition, all cases in stable COPD showed centrilobular emphysema on HRCT, while 19 cases showed the same changes in COPD exacerbation group and one case showed panacinar emphysema (data not tabulated).

Regarding CRP, it was positive in 26 cases (32.5% of all studied cases). There was significant difference between groups as regard positive CRP (90% of COPD exac-

erbation group had positive CRP, 25.0% of stable COPD, 10.0% of non COPD smokers and 5.0% of non-smoker non COPD groups had positive CRP). Regarding ANA distribution, it was positive in 24 cases out of 80 cases (30.0%) and there was significant difference between studied groups (it was positive in 65.0%, 45.0%, 5.0% and 5.0% in COPD exacerbation, stable COPD, non COPD smokers and non-smokers non COPD groups respectively). In addition, CRP values ranged from 3 to 48 with a mean of 11.73±13.22, while ANA ranged from 0.4 to 4.1 with a mean of 1.25±0.97 and there was statistically significant difference between studied groups as regard both CRP and ANA (The highest values were in group II, then group I, III and finally control group) (table 2).

In the present study, positive CRP cases, the most common grade was grade III (52.2%), then grade II (26.1%) and finally grade IV (21.7%) while majority of negative cases were grade II (58.8%) with no significant difference between positive and negative cases. In addition, positive ANA cases, the most common grade was grade II (45.5%), then grade III (31.8%) and finally grade IV (22.7%), while majority of negative cases were grade III (55.6%) with no significant difference between positive and negative cases. In addition, there was significant increase of ECG abnormalities in positive CRP cases in comparison to negative cases. The most prominent changes were P-pulmonale and right axis deviation. Furthermore, there was significant increase of ECG abnormalities in positive ANA cases in comparison to negative cases. The most prominent changes were P-pulmonale and right axis deviation (table 3).

In the present work, there was no significant relation between positive and negative CRP cases as regard BMI. On the other hand, positive cases for ANA had significantly lower BMI than negative cases. Also, there was significant decrease of PaO2 and SaO2 in positive CRP cases in comparison to negative cases. On the other hand, no relation was found between positive and negative ANA (table 4).

Table (1): General characteristics, pulmonary functions, arterial blood gases and laboratory investigations of studied cases.

		Group I	Group II	Group III	Controls	test	p
Age (mean±SD)		64.11±2.48	64.15±2.25	63.75±1.94	64.50±2.24	0.41	0.74(NS)
Smoking packs/year		32.09±7.20	34.11±7.90	28.30±3.44	-	3.69	0.031*
GOLD Grading (n,%)	II	10(50.0%)	6(30.0%)	-	-	1.67	0.43(NS)
	III	7(35.0%)	10(50.0%)	-	-		
	IV	3(15.0%)	4(20.0%)	-	-		
BMI		27.16±2.82	26.99±2.52	26.80±2.78	28.89±3.02	2.45	0.07(NS)
FEV1		52.60±16.98	52.32±18.32	90.40±3.06	95.75±2.67	69.16	<0.001*
FEV1/FVC		53.55±10.06	53.10±9.70	83.15±3.55	92.50±3.95	147.10	<0.001*
SaO_2		91.50±1.19	86.85±1.34	94.50±1.10	95.45±0.60	248.6	<0.001*
pH		7.40±0.016	7.34±0.015	7.41±0.015	7.42±0.012	95.5	<0.001*
PaO_2		73.60±2.70	56.90±1.37	74.10±1.65	91.65±1.30	181.9	<0.001*
PCO_2		43.0±2.07	50.60±2.62	40.85±2.68	34.25±2.40	150.3	<0.001*
WBCs x 10^3		8.83±4.54	17.92±6.52	6.32±2.02	6.80±2.29	32.30	<0.001*
HB		16.20±1.54	16.30±1.08	11.83±0.95	11.90±0.67	104.5	<0.001*

Table (2): CRP and ANA distribution and mean values in studied cases.

	Group I	Group II	Group III	Controls	test	p
Positive CRP (n,%)	5(25.0%)	18(90.0%)	2(10.0%)	1(5.0%)	42.16	<0.001*
Positive ANA (n, %)	9(45.0%)	13(65.0%)	1(5.0%)	1(5.0%)	25.71	<0.001*
CRP (mean±SD)	8.55±7.10	28.65±15.88	5.10±2.19	4.65±2.83	32.97	<0.001*
ANA (mean±SD)	1.11±0.31	2.44±1.26	0.73±0.23	0.73±0.26	28.97	<0.001*

Table (3): Relation between CRP, ANA and disease severity and ECG changes.

		Positive CRP		Positive ANA	
		No	%	No	%
Disease severity	II	6	26.1%	10	45.5%
	III	12	52.2%	7	31.8%
	IV	5	21.7%	5	22.7%
ECG	Normal	3	13.0%	8	36.4%
	P pulmonale	10	43.5%	5	22.7%
	Sinus tachycardia	3	13.0%	3	13.6%
	Right axis deviation	4	17.4%	4	18.2%
	AF	2	8.7%	1	4.5%
	Left axis deviation	1	4.3%	1	4.5%

Table (4): Relation between CR, ANA and disease severity.

	CRP		P value	ANA		P value
	Positive	Negative		Positive	Negative	
BMI	27.21±2.66	26.82±2.69	0.64(NS)	25.31±1.39	29.16±2.25	0.001*
PaO_2	60.34±6.79	71.88±6.36	<0.001*	64.0±9.08	66.77±8.23	0.32(NS)
SaO_2	88.04±2.63	90.70±1.86	<0.001*	88.54±2.53	89.94±2.68	0.10(NS)

Discussion

The present study was designed to investigate the ANA and CRP in different populations of COPD patients; it also investigated the relations of ANAs and CRP to some patient's characteristics (smoking behavior, gender, age, lung function, and physical characteristics.

In the present study, age ranged from 59 to 70 years with a mean of 64.12±2.21 years, and there was non-significant difference between studied groups as regard age distribution. These results are in agreement with those reported by(10) who reported that, the mean age of COPD patients was 64± 9 years. On the other hand,(11) included patients with mean age of 68±8.6 years that is slightly higher than those reported in the present study and this may be explained by different inclusion criteria, e.g., their cases were 90% males and 10% females, while all cases in the present study were males.

In the first three groups (smoking groups), smoking packs per year ranged from 21 to 52 pack with a mean of 31.39±6.79 pack and there was significant increase of packs in COPD exacerbation group and stable COPD group in comparison to non COPD smokers group (34.11±7.90, 32.09±7.20 vs 28.30±3.44 respectively). In agreement with these results, it was reported that, smoking is a major risk factor not only for development of COPD but also many other chronic diseases. It triggers a local inflammatory response in lungs and it also causes systemic inflammation that result in comorbidities like cardiovascular or metabolic disorders(12). It is not clear yet whether this inflammation spreads from lung to systemic circulation or multiple organ systems including lung are affected due to a systemic inflammatory response(6).

In contradiction to these results(13) found significant increase of packs per year in controls (40 packs/year) when compared to stable COPD patients (20 packs/year) and these results can be explained except by individual habitual variations.

Regarding BMI, it ranged from

22 to 35 kg/m2, with a mean of 27.43±2.88 and there was non-significant difference between studied groups. In addition, there was no significant relation between BMI, CRP or ANA. These results are comparable to those reported by(11) who reported that, the mean BMI of COPD subjects was 28.1±3.4 kg/m2 with non-significant difference between cases with positive and negative ANA antibodies, or between different grades of COPD. Also, they included patients with metabolic syndrome in their study and this may be another explanation.

In the present study, there was statistically significant variance between studied groups as regard FEV1 and FEV1/FVC (healthy controls followed by non COPD smokers had the better functions, while COPD exacerbation and stable COPD groups had the worst respectively). These results are in agreement with that reported by [14] who found progressive decrement of lung function with the increase of COPD severity. In addition,[15] reported that, mean FEV1 in COPD patients was 47.4±16.6 and FEV1/FVC was 54.7±11.1 and these results are in agreement with that of the present study.

In the present study, 16 cases out of 40 cases (40.0%) were GOLD stage II, 17 cases (42.5%) were GOLD stage III and 7 cases (17.5%) were GOLD stage IV, and there was non-significant increase of GOLD stages III and IV in COPD exacerbation group, when compared to stable COPD group (50%, 20% vs 35%m 15% respectively). These results are similar to those reported by[13] who found that GOLD stage II are the most prevalent grade in their patients and correlated with elevated CRP levels.

In the present study, ECG was normal in 58 cases (72.5%); p-pulmonale presented in 12.5%, sinus tachycardia in 3.8%, right axis deviation in 6.2%, AF in 3.8% and left axis deviation in 1.2% of cases with significant difference between groups (all cases in non somoker non COPD group had normal ECG, 95% of non COPD smokers had normal ECG, 20% in COPD exacerbation and 75% of stable COPD had normal ECG.[14]

reported that, their data demonstrate that COPD is associated with an abnormal cardiac autonomic modulation at rest. Indeed, heart rate variability (HRV) parameters were progressively reduced with the increase of COPD severity. Heart rate variability (HRV) expresses cyclic fluctuations of heart rate (HR) and reflects autonomic modulation by sympathetic and parasympathetic efferent nervous impulses of heart rhythm. In addition, HRV has been found to be reduced in subjects with COPD and related to the severity of the disease[16].

In the present study, all cases in first two groups (COPD groups); showed hyperinflated chest and increased retrosternal space in PA and Lateral views x rays respectively. In addition, all cases in stable COPD showed centrilobular emphysema on HRCT, while 19 cases showed the same changes in COPD exacerbation group and one case showed panacinar emphysema. These results are logic findings in cases with COPD as reported everywhere in the literature[14].

In the present study, CRP was positive in 26 cases (32.5% of all studied cases). There was significant difference between groups as regard positive CRP (90% of COPD exacerbation group had positive CRP, 25.0% of stable COPD, 10% of non COPD smokers and 5.0% of non-smoker non COPD groups had positive CRP). CRP values ranged from 3 to 48 with a mean of 11.73±13.22, while ANA ranged from 0.4 to 4.1 with a mean of 1.25±0.97 and there was statistically significant difference between studied groups as regard both CRP and ANA (The highest values were in group II, then group I, III and finally control group) (value were 28.65±15.88, 8.55±7.10, 5.10±2.19 and 4.65±2.83 for CRP and 2.44±1.26, 1.11±0.31, 0.73±0.23, 0.73±0.26 for ANA in groups II, I, III and IV respectively). These results are in agreement with previous studies showed that markers of systemic inflammation like high-sensitivity C-reactive protein (CRP), interleukin (IL)-6 were higher in blood of COPD patients than the ones without COPD[17,2].

(2) were emphasized the importance of high CRP levels in COPD

patients, confirming the systemic inflammation in the stable phase of the disease.

Studies of circulating CRP levels in COPD demonstrated that CRP was further elevated during exacerbations, and it was found to predict mortality[18].

In addition,[13] reported that, high sensitive-CRP level increased in 53.8% of COPD patients. Whereas high CRP levels were found only in 26.2% of control group (p= 0.005). High CRP levels seemed to be more prominent in GOLD stage II patients. Their findings are higher than that reported in the present study (25% in stable COPD patients compared to 5% in control group). This variation can be explained by different inclusion criteria. In addition, they determined the high sensitivity CRP.

C-reactive protein reflects the total systemic burden of inflammation in several disorders and has been shown to upregulate the production of proinflammatory cytokines[19].

The reasons for the inverse association between systemic inflammation and reduced pulmonary function are not fully understood, but several mechanisms may be involved, e.g., reduced lung function may be responsible for the observed systemic inflammation. Inflammatory lung or pulmonary epithelial cells have been shown to express CRP and IL-6 [20]. Interleukin-6 may reach the liver via the blood stream, stimulating the production of CRP and other inflammatory mediators by the liver, sequentially activating pulmonary inflammatory cells during transit through the pulmonary circulation[21,17].

An alternative mechanism-reverse causation-cannot be excluded: high levels of cytokines and acute-phase reactants in the peripheral circulation may be a cause rather than a consequence of poor lung function. There is increasing evidence that cytokines play a major role linked to the activation of inflammatory cells and their adhesion to the pulmonary capillary endothelium, leading to changes in endothelial function and increases in pulmonary vascular filtration[22]. Besides, it is known

that COPD may even influence venous circulation. Thus, persistence of systemic inflammation may result in damage to the airways, accelerating decline in FEV1 of COPD patients[23].

Regarding ANA distribution, it was positive in 24 cases out of 80 cases (30.0%) and there was significant difference between studied groups (it was positive in 65.0%, 45.0%, 5.0% and 5.0% in COPD exacerbation, stable COPD, non COPD smokers and non-smokers non COPD groups respectively). These results are in agreement with[11] who reported that, a quarter and a third of patients with clinically stable COPD have abnormal titers of circulating ANA (34%), a prevalence much higher than determined in healthy controls (3%); and also higher than that reported in previous studies in the general population [24]. In 1976,[25] reported that 28% of 50 patients with "severe chronic bronchitis," most likely what today would be called COPD, had increased titers of circulating ANA, a figure that was much higher than that determined simultaneously in age and sex-matched "non-bronchitic" controls (4%). This study, however, has gone mostly unnoticed to date. It is also possible, as in fact our results show, that not all patients with COPD have evidence of autoimmunity and that only a subset of them develop it, as previously suggested[26]. Furthermore,[1] quantified the fraction of patients that are positive for ANAs and other autoantibodies, and investigated which characteristic of the patient population is associated with higher ANAs. They found that 44% of the patients with COPD were ANA-positive, significantly more than the prevalence of 22% in controls. The presence of ANAs was not associated with smoking behavior. Among the patients with COPD, higher ANAs were more often found in patients with low BMI.

A significant group of patients with COPD has been reported to carry serum autoantibodies against elastin or antinuclear targets[3], whereas other reports have not shown an antibody response against elastin in patients with COPD[5,27] reported that 68% of 47 patients with COPD were ser-

um positive for ANAs.

It remains unclear how immune tolerance is broken in patients with COPD and autoimmunity against nuclear antigens arises.[1] suggested that, the lung B cells may play a role. They showed for the first time that lung B cells of patients with COPD produce antibodies that carry a high frequency of positively charged amino acids in the IgH-CDR3, a feature which is also observed with ANAs. They therefore hypothesize that the development of COPD is associated with infiltration of B cells in the lung that are directed at infectious material or residues of destroyed lung tissue. B cell follicular structures then arise in which germinal centres can develop, enabling B cell isotype switching and affinity maturation by somatic hypermutations.

With ongoing smoking, long-term inflammation and increasing lung damage in COPD, inflammatory cells are exposed to nuclear antigens derived from damaged epithelial cells and probably many other antigenic protein residues of damaged cells and structures. This exposure may last for years, even after smoking cessation, and can cause the unintended recognition of (nuclear) antigens by somatically hypermutated antibodies that were originally targeted to non-self-antigens[28].

(1) data thus suggest that B cells in the lungs of patients with COPD generate antibodies (or antibody receptors) which share a feature that is characteristic for ANAs-positively charged residues in the IgH CDR3 region, one of the regions in antibodies that is critical for antigen binding.

As smoking was found to be related to the prevalence of ANA in a large cohort study of the general (non-COPD) population and as COPD is recognized mostly as a smoking-related disease, one might expect that the autoantibody levels are related to smoking history in COPD as well[29].

In the present study, there was significant association between CRP and ANA with ECG changes, but no association was found with BMI. It had been reported that, raised CRP relates to health stat-

us, exercise capacity and body mass index (BMI)[30] as well as cardiovascular disease[31], indicating a clear association with both lung-related and systemic features. On the other hand,[32] reported that, twin studies show a highly significant hereditable component in base-line CRP values that is independent of age and BMI. These results are in agreement with the present study.

In short, results of the present study showed increased levels of CRP and ANAs in the serum of COPD patients and correlated with the severity of the disease. These results confirmed the inflammatory and autoimmune nature of COPD.

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REPRINT

BENHA MEDICAL JOURNAL

ANTINUCLEAR AUTOANTIBODIES (ANA) AND C- REACTIVE PROTEIN (CRP) IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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STUDY OF TOLL-LIKE RECEPTORS GENOTYPE POLYMORPHISM IN EGYPTIAN CHILDREN WITH CHRONIC VIRAL HEPATITIS C

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Abstract

Background: *Hepatitis C virus (HCV) is a public health problem and is one of the main causes of chronic liver disease worldwide, with high prevalence in Egypt. Toll-like receptors (TLRs) play an important role in the immune system. Genetic variations in TLRs alters susceptibility to infections. One common type of these genetic variations is the single nucleotide polymorphisms (SNPs). TLRs SNPs in patients with chronic hepatitis C have shown different findings and results.*

Objective: *To assess the frequency of TLR2 Arg753Gln and TLR4 Asp299Gly, Thr399Ile polymorphisms among Egyptian children with chronic hepatitis C, in comparison to healthy matched controls, and to study the association of these polymorphisms with HCV infection and response to treatment.*

Methods: *This is an observational case control study that was conducted in Mansoura University Children's Hospital (MUCH) in compliance with Department of Clinical Pathology and Insurance outpatient clinic, from February 2012 through May 2013. The study included 100 Egyptian children with chronic hepatitis C (63% males) with mean age of 13.01 years (±2.81 SD). In addition, 100 healthy Egyptian children with matched age and sex were enrolled as control. Both groups were subjected to clinical and laboratory assessments, with molecular diagnosis to detect TLR2 and TLR4 gene polymorphisms by using restriction*

fragment length polymorphism (RFLP) technique.

Results: *We found that, TLR2 Arg753Gln, TLR4 Asp299Gly were not polymorphic at all, and TLR4 Thr399Ile polymorphism was low in frequency (3 out of 100).*

Conclusion: *we concluded that, racial difference could be found in the frequency of TLR2 and TLR4 polymorphisms.*

Keywords: *Toll-Like Receptor, Single nucleotide polymorphism, Molecular diagnosis.*

Introduction

Hepatitis C virus is a hepatotropic non-cytopathic virus able to evade immune system as mechanism to persist in infected hosts [1]. It is a major cause of chronic liver disease leading to progressive hepatic fibrosis with long-term progression to cirrhosis[2]. The development of hepatic fibrosis and cirrhosis in patients with chronic hepatitis C infection results from inflammatory response. This process is associated with marked inter-patient variation, which is difficult to predict[3,4].

Toll-Like Receptors are a class of proteins that play a key role in the immune system. They recognize structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs, which activate immune cell responses[5].

Toll-Like Receptors participate in orchestrating the innate (and subsequently adaptive) immune responses against HCV[6]. Experimental studies revealed that Arg753Gln SNPs impairs TLR2 recognition of HCV Core and non-structural 3 proteins[7]. TLRs polymorphism was found to influence the fate of liver transplant for chronic hepatitis C. The association was assessed between SNPs in genes that encode TLR2 and TLR4 in a historical cohort of 92 liver transplant patients. Homozygous TLR2 Arg753GLn polymorphism was associated with liver allograft rejection and mortality[8]. Furthermore, TLR4 SNPs were studied in relation with liver cirrhosis in patients with chronic hepatitis C[9]. There is significant

variability in the prevalence of TLR SNPs in different populations, probably due to evolutionary and environmental pressures[10].

Subjects and Methods

This is an observational case control study that was conducted in MUCH in compliance with Department of Clinical Pathology and Insurance outpatient clinic, from February 2012 through May 2013. The study included 100 Egyptian children with chronic hepatitis C (63% males) with mean age of 13.01 years (±2.81 SD). In addition, 100 healthy Egyptian children with matched age and sex were enrolled as control. Patients were recruited from outpatient clinic in MUCH and Health Insurance outpatient clinic. Healthy controls were selected from children attending MUCH outpatient clinic for routine follow-up care.

Diagnosis of patients relied on persistently positive anti-HCV antibody and HCV RNA by PCR for at least 6 months. Patients with decompensated cirrhosis, other causes of liver damage, autoimmune or metabolic disorders, co-infection with hepatitis B virus and/or human immunodeficiency virus and malignancy were excluded[11].

All studied groups were subjected to history taking, physical examination, laboratory evaluations and molecular diagnosis to detect TLR2 Arg753Gln and TLR4 Asp299Gly, Thr399Ile polymorphisms: DNA extraction, agarose gel electrophoresis, polymerase chain reaction of the extracted DNA, RFLP technique[12]. In addition, liver biopsy was done for all patients. Eighty two out of 100 patients were treated with Peg-IFN α-2b in a dose of 60 mcg/m2/ week given subcutaneously and ribavirin 15 mg/kg orally per day in 2 divided doses. Treatment duration was 48 weeks.

The polymorphism in TLR2 Arg753Gln locus is practically limited to G and A alleles. The presence of TLR2 alleles was identified by the presence of a restriction site for Aci I enzyme. Therefore, GG wild genotype= band at 227 bp, AA homozygous genotype= two bands at 265 bp and 75 bp, and GA heterozygous genotype= 3 bands at 227 bp, 265 bp and 75 bp.

The polymorphism in TLR4 Asp299Gly locus is practically limited to A and G alleles. The presence of TLR4 G allele was identified by the presence of a restriction site for Nco I enzyme, which cuts the TLR4 G allele into two fragments, 223 and 26 bp, and leaves the TLR4 A allele without cut giving a band at 249 bp. Therefore, AA wild genotype= one band at 249 bp, GG homozygous genotype= two bands at 223 bp and 26 bp, and AG heterozygous genotype= 3 bands at 249 bp, 223 bp and 26 bp.

The polymorphism in TLR4 Thr399Ile locus is practically limited to C and T alleles. The presence of TLR4 T allele was identified by the presence of a restriction site for Hinf I enzyme, which cuts the TLR4 T allele into two fragments, 377 and 29 bp, and leaves the TLR4 C allele without cut giving a band at 406 bp. Therefore, CC wild genotype= one band at 406 bp, TT homozygous genotype= two bands at 377 bp and 29 bp, and CT heterozygous genotype= 3 bands at 406 bp, 377 bp and 29 bp.

Statistical Analysis:

Data was analyzed using SPSS 16. Normality of data was first tested by one sample K-S test. Parametric data were expressed in mean ± standard deviation. Non parametric data were expressed in median. In addition, Mann-Whitney was used to compare continuous variables in two different groups. Chi-square tests were used to compare the categorical variables between both cases and controls. P value <0.05 was considered as statistically significant.

Results

In our patients, there were no clinical stigmata for chronic hepatitis. Liver function tests including, SGPT, SGOT, bilirubin and albumin were nearly normal. They were normoglycemic. Serum alkaline phosphatase, uric acid, creatinine and alpha fetoprotein were average.

Of 100 patients, 52 showed polymerase chain reaction less than 10^5 IU/ml, 41 were between 10^5 - 10^6 IU/ml, and only 7 were more than 10^6 IU/ml. As regard histologic activity grading within liver biopsy, most of them were grade A1 (63 patients), followed by grade A2 (32 patients), then grade A3 (4

patients), lastly grade A0 (one patient). Also, most of them in hepatic fibrosis grading were grade F1 (76 patients), followed by grade F2 (17 patients), then grade F3 (4 patients), lastly grade F0 (3 patients).

Of 100 patients, 82 were treated. 49 (59.7%) of them showed sustained virological response, one (1.22%) relapsed and 32 (39%) were non responder.

We genotyped TLR2 Arg753Gln, TLR4 Asp299Gly and TLR4 Thr399Ile. We found that TLR2 Arg753Gln, TLR4 Asp299Gly were not polymorphic at all, only the wild type GG and AA were detected respectively, and no other types were found (Table 1). As regard TLR4 Thr399Ile polymorphism, the polymorphic T allele was detected in 3 out of 200 allele in patients (1.5%) but not in controls. Three patients has heterozygous CT genotype (3%), while all controls has the wild CC genotype (Table 2) (Figure 1, 2).

Table (1): Genotype and allele frequency of TLR2 Arg753Gln and TLR4 Asp299Gly among patients and controls.

	Patient n=100 n (%)	**Control n=100 n (%)**
TLR2 Arg753Gln		
Genotypic frequency		
GG (wild type)	100 (100%)	100 (100%)
Allelic frequency		
G	200 (100%)	200 (100%)
TLR4 Asp299Gly		
Genotypic frequency		
AA (wild type)	100 (100%)	100 (100%)
Allelic frequency		
A	200 (100%)	200 (100%)

Table (2): Genotype and allele frequency of TLR4 Thr399Ile among patients and controls.

	Patient n=100 n (%)	**Control n=100 n (%)**
TLR4 Thr399Ile		
Genotypic frequency		
CC (wild type)	97 (97%)	100 (100%)
CT (Heterozygous)	3 (3%)	0 (0%)
Allelic frequency		
C	197 (98.5%)	200 (100%)
T	3 (1.5%)	0 (0%)

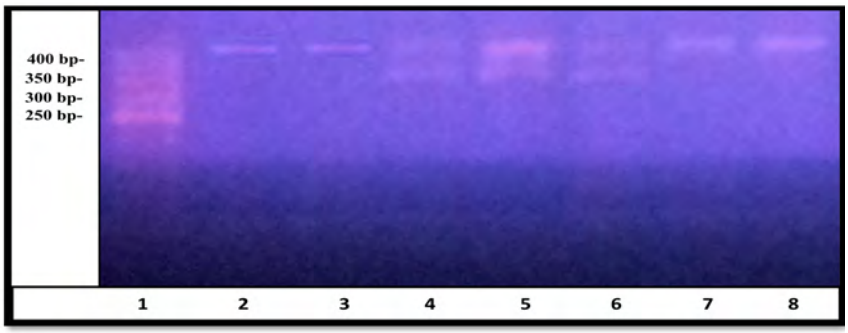

Fig. 1: TLR4 Thr399Ile polymorphism for patients by RFLP using Hinf I restriction enzyme. DNA molecular weight marker XIII (50 bp ladder) in lane 1. The presence of two bands at 406 bp and 377 bp were interpreted as C/T allele (heterozygous type) in lanes 4, 5, 6. The presence of one band at 406 bp in lanes 2, 3, 7, 8 were interpreted as C allele (wild type).

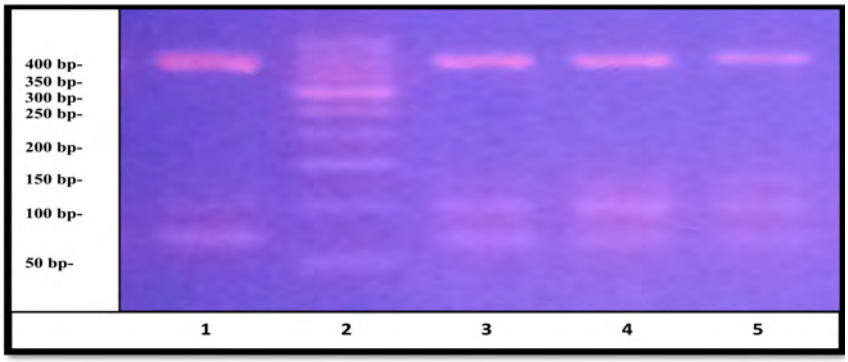

Fig. 1: TLR4 Thr399Ile polymorphism by RFLP using HinfI restriction enzyme. DNA molecular weight marker XIII (50 bp ladder) in well 2. PCR product in well 1 was detected at 406 bp. Wells 3, 4, 5 shows one band at 406 bp and were interpreted as C allele (wild type).

Discussion

In our study, TLR2 Arg753Gln, TLR4 Asp299Gly polymorphisms were absent and TLR4 Thr399Ile polymorphism was low in frequency. That was against studies done in Caucasians, and similar to other studies done in Chinese and Japanese. It should be noted that the prevalence of TLR polymorphisms varies with ethnic groups. TLRs SNPs in HCV have done predominantly in Caucasian patient cohorts. TLR2 Arg753Gln, TLR4 Asp299Gly, and TLR4 Thr399Ile were detected in approximately 10% of Caucasians, and were rare or absent in Asian populations. Frequencies of toll interleukin 1 receptor adaptor protein SNPs was rare in Africa[13].

A study found that the commonly reported TLR SNPs in the Western countries were absent in their study group[14]. Also, no heterozygous or homozygous SNPs in TLR2 Arg753Gln, TLR4 Asp299Gly, and TLR4 Thr399Ile were found in Chinese patients [15,16,17]. Of 411 Japanese subjects, no TLR4 Asp299Gly or Thr399Ile polymorphisms were detected[18].

Among Caucasian subjects, some studies had suggested that the TLR4 polymorphisms was not related to conditions such as rheumatoid arthritis, myocardial infarction, severity of atopy and susceptibility to pulmonary tuberculosis[19,20,21,22].

These conflicting results could be explained by racial difference and environmental pressures. In addition, these polymorphisms have no role in our patients with chronic hepatitis C. However, this does not mean that these polymorphisms itself does not play an important role in HCV infection.

Conclusion

The frequency of TLR2 Arg753Gln, TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms seems to be absent or rare in our population, and racial difference could explain the variable frequency of these polymorphisms.

Based on the absence or rarity of TLR2 Arg753Gln, TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms, they probably have no role in HCV infection in our population, and large scale

studies in different areas of the country on TLRs polymorphisms are recommended, to map their frequency and association with HCV infection in Egypt.

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REPRINT

BENHA MEDICAL JOURNAL

STUDY OF TOLL-LIKE RECEPTORS GENOTYPE POLYMORPHISM IN EGYPTIAN CHILDREN WITH CHRONIC VIRAL HEPATITIS C

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EFFECT OF ANTICOAGULANT (WARFARIN) ON BLOOD PRESSURE (EXPERIMENTAL AND CLINICAL STUDY)

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Abstract

Background: *While some studies in rats had linked warfarin therapy to arterial calcification, arterial stiffening and systolic hypertension, others in human reported no change in blood pressure by warfarin treatment.*

Aim: *The aim of this study was to investigate the effects of warfarin on blood pressure of experimental dogs, the isolated perfused blood vessels of rat hind limb and the isolated rabbit's heart. Also, the effect of warfarin on blood pressure in individuals with hypertension was examined.*

Methods: *The effects of warfarin on blood pressure in dogs either alone or with drugs as acetylcholine, atropine, phenylephrine, adrenaline and noradrenaline were examined. Also, the effects of warfarin on isolated perfused blood vessels of rat hind limb and the isolated rabbit's heart were investigated. Moreover, blood pressure in hypertensive individuals receiving warfarin treatment was measured.*

Results: *Warfarin decreased blood pressure in dogs when given alone or with acetylcholine, atropine, phenylephrine, adrenaline and noradrenaline. Also, warfarin increased perfusion of blood vessels of rat hind limb with no effects on isolated rabbit's heart. In human, no change in blood pressure was reported in hypertensive patients receiving warfarin treatment.*

Conclusion: *While warfarin has an antihypertensive effect in experimental animals which was neither muscarinic agonist nor alpha and beta receptors blocker, we were not able to show the same effect in humans with hypertension.*

Key Words: *adrenaline, atropine, blood pressure, warfarin.*

Introduction

Systolic hypertension is an important cardiovascular risk factor in elderly patients, wherein elevated systolic blood pressure (BP) is more reliable than diastolic BP as a determinant of cardiovascular risk[1]. The pathogenesis of isolated systolic hypertension is closely linked to arterial stiffness, which is associated with calcification of the arterial media[2]. Aging, diabetes mellitus, and kidney disease are the most prominent factors that lead to increase collagen accumulation and calcification of the vascular wall, the two key processes that lead to increased arterial stiffness[3]. These wall changes lead to faster pulse wave conduction, amplified pulse wave reflection and abnormal diastolic decay of the BP curve, resulting in higher systolic BP, lower diastolic BP, and consequently higher pulse pressure[4]. Elevated pulse pressure (PP) is an independent predictor of cardiovascular events[1].

Evidence has linked warfarin therapy to arterial calcification, arterial stiffening and systolic hypertension[5]. Warfarin causes inhibition of gamma carboxylation of matrix GLA protein (MGP), a step necessary for the activation of this vitamin K-dependent protein with important anti-calcifying activity [6]. Vascular calcification and systolic hypertension occur in rats given high doses of warfarin, a process presumed to be related to warfarin's inhibition of vitamin K regeneration and thus low levels of active MGP[7].

There is preliminary evidence from cross-sectional studies in humans indicating that chronic exposure to warfarin is associated with increased calcification of the coronary arteries and cardiac valves[5]. In order to define the blood pressure effects of warfarin in humans, a post-hoc analysis of "Stroke Prevention in Non-rheumatic Atrial Fibrillation" (SPINAF) study was performed. This study did not reveal any change in BP in patients receiving warfarin for atrial fibrillation[6]. The aim of the present work was to investigate the effects of warfarin on the blood pressure of experimental dogs, the isolated perfused blood vessels of rat hind limb and the isolated rabbit's heart. Also, the effect of warfarin on ABP parame-

ters in individuals with hypertension was examined.

Material and Methods

(I) Animal experiments:

Chemicals used: Warfarin sodium (Vial containing 5 mg lyophilized powder) was provided by Bristol-Myers Squibb Pharma Company, USA. Atropine was obtained from Macfarlan Smith Ltd Edinburg, UK. Adrenaline, noradrenaline, phenylephrine and acetylcholine were purchased from Sigma-Aldrich, (St. Louis, MO, USA).

Animals used and methods:

20 Mongrel dogs of either sex, weighing 15-20 kg, were used for recording blood pressure according to method of McLeod[8]. Each dog was anesthetized by thiopental sodium (25mg/kg) intravenously (IV) as induction, and then anesthesia was maintained by chlorase (80 mg/kg, IV). A midline incision was made on the skin starting from the lower end of the larynx up to the upper end of the thorax. Muscles were separated along the midline, trachea was exposed, tracheostomy was performed and Y shaped tracheal cannula was inserted into the trachea to allow free breathing and to provide artificial respiration with the help of a respiratory pump. The right carotid artery was exposed, cannulated and connected to a mercury manometer through rubber tubing filled with 3% sodium citrate as an anticoagulant. The other end of the manometer was provided with a floating cork carrying a painter for recording the changes in the ABP on a moving kymograph. Before the bulldog clamp was removed 50 I.U/kg heparin was injected into the arterial cannula through the rubber tubing to guard against blood clotting. The femoral vein was exposed by a midline incision on the medial surface of the upper part of the thigh. The venous cannula was inserted, and connected with a burette filled with warm saline. Heparin was also injected into the venous cannula to guard against blood clotting. Drugs were injected into the rubber tubing close to the cannula and a constant volume of saline was allowed to run every time after the injection.

30 albino rats of either sex weighing 200-250 g were used for

assessment of the effect of the drugs on the perfused blood vessels of rat hind limb, according to the method of Burn[9]. The animals were killed by blow on the head and cutting the throat. The abdominal cavity was opened, the rectum with superior and inferior mesenteric arteries was divided between ligatures and the intestine above the rectum was removed. A fine cannula was passed into the aorta distal to the kidney. The body wall of the rat was cut transversely above the point of cannulation. The hinder portion of the rat was placed on a wire mesh resting on a glass funnel. The cannula was connected though rubber tubing to a reservoir delivering the filtered warm oxygenated mammalian Ringer-Locke solution at a constant pressure. The temperature of the perfusing solution was thermostatically controlled and adjusted at 37°C. When the vessels of the rat were perfuse and the perfusate became clear as in the reservoir, the perfusate leaving the vein was counted through photoelecetric drop counter (UGO Basile, Italy) for 5 min and the mean per min was calculated. Drugs were injected into the rubber part just proximal to the cannula.

10 rabbits of either sex weighing 1.5-2 kg were used for recording the effect of the drugs on the isolated rabbit's the heart. These animals were allowed free access of food and water. The rabbit was sacrificed; its chest wall was opened. The heart was dissected and the fat and connective tissue were removed. The heart was excised along with an attached length of at least 1cm aorta and then rapidly removed[10] and placed in dish containing mammalian Ringer-Locke's solution which has the following composition in g/L (sodium chloride 9.0, potassium chloride 0.42, calcium chloride 0.24, sodium bicarbonate 0.5 and dextrose 1.0). The temperature was adjusted at 37°C and pH at 7.4. The heart was then gently squeezed several times so as to expel as much blood as possible. The aorta was dissected free and cut just below the point where it divides. The heart was then transferred to the Langendorff perfusion apparatus where the aorta was cannulated to 5ml cannula. The cannula was then connected to a reservoir delivering

oxygenated mammalian Ringer-Locke's solution at a thermostatically constant temperature (37°C). A hook was then passed through the ventricular wall and was attached to a lever for recording ventricular contractions (cm) and heart rate (beats/min), on a slowly moving smoked drum.

All animal procedures were performed in accordance with protocols approved by the Medical Research Ethics Committee of Mansoura University, Egypt. All experiments conform to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985).

Experimental protocols:

I. Effect of warfarin on blood pressure of dogs:

1. Repeated doses of warfarin were administered IV (every dose was dissolved in 100 µl distilled water) starting from 5 µg/kg to 320 µg/kg according to the dose response curve to choose the effective dose.

2. Warfarin was injected IV in a dose of 50 µg/kg and the same dose was repeated after 5 minutes followed by acetylcholine (1 µg/kg) then atropine (0.2 mg/kg) followed by acetyl choline (1 µg/kg) then warfarin (50 µg/kg).

3. Warfarin was administered IV in a dose of 50 µg/kg followed by phenylephrine (1µg/kg) then warfarin (50 µg/kg) followed by phenylephrine (1µg/kg). The same steps were done with adrenaline (1µg/kg).

4. Induction of hypertension was carried out using norepinephrine (NE) infusion in anaesthetized dog over a period of 60 minutes in a dose of 4µg/minute. After initial elevation of blood pressure (1 minute), NE infusion then stopped and warfarin (50µg/kg) IV was given, then administration of NE is continued.

II. Effect of warfarin on perfused hind limb of rats: Control group (n=6) were given 1 cc distilled water. The perfusate was counted for five minutes. Warfarin was administered in different doses ranging from 50-400 µg/kg, each dose was administered in a

separate preparation and the perfusate was collected every five minutes. The maximum effect was obtained after 1 hour and the mean was determined.

III. Effect of warfarin on isolated perfused rabbit's heart: Warfarin was administered in different doses ranging from 10 µg to 320 µg and every dose was dissolved in 100 µl acetone.

(II) Clinical part:

Design and Subjects

This is a study of 160 patients, 100 males and 60 females, 70 years or older with hypertension who had received chronic warfarin therapy for 2 years, and 160 age-matched control subjects with hypertension who never received warfarin. The indications for warfarin therapy were atrial fibrillation (75%), deep venous thrombosis and pulmonary embolism (8%) and cardiomyopathy with poor ejection fraction (3%).

We identified patients exposed to warfarin using the log of all enrollees in the outpatient clinics at Special Internal Medicine Hospital, Mansoura University, Mansoura, Egypt between January 2010 and December 2012 who had been on continued warfarin treatment, thus representing at least 2 years of exposure to warfarin. We screened 2200 records, of which 160 subjects fulfilled inclusion criteria.

Control subjects were identified from the outpatient clinics at Special Internal Medicine Hospital, Mansoura University, Mansoura, Egypt. We matched patients to the warfarin-treated individuals using a 1:1 ratio. For each warfarin subject we selected the first patient with the same age until all warfarin subjects had a matched control.

In order to be included in the study, subjects had to have at least one recorded BP value in the outpatient clinics at Special Internal Medicine Hospital between January 2010 and December 2012. Subjects were excluded if they had a history of hospitalization during the time of data collection (as the intercurrent illness could have an independent effect on blood pressure); had end-stage renal disease on dialysis or a his-

tory of cardiac valve replacement (as these conditions are associated with vascular calcification).

Measurements:

The average BP values were obtained over a period of 12 months (January 2013 - December 2013). We collected relevant information including demographics and biometric data. We reviewed progress notes and laboratory records in the medical record to abstract data focused on existing cardiovascular disease (coronary artery disease, congesting heart failure, cerebrovascular disease, peripheral vascular disease), cardiovascular risk factors (smoking, lipid profile), duration of hypertension, and the total number of visits with a recorded BP value during the 12 month period.

The use of antihypertensive medications was recorded according to the Defined Daily Doses (DDD) method based on the World Health Organization Collaborating Centre for Drug Statistics Methodology (WHO-CCDSM: http://www.whocc.no/atcddd). In this system, the most common daily dose of an agent is given the value of 1. For the sake of illustration, a DDD value of 1 for some of the most commonly used drugs in our study are: hydrochlorothiazide 25 mg/day, lisinopril 10 mg/day, felodipine 5 mg/day, and atenolol 75 mg/day. Fractions and multiples are calculated according to this value. The WHO-CCDSM website has up-to-date definitions for all antihypertensive drugs in clinical use.

We also collected data on the indication for warfarin use, duration of exposure (in years), and target international normalized ratio (INR), all of which were in the 2.0-3.5 range. Renal function was estimated based on serum creatinine, age and body weight using the Cockcroft-Gault formula[11].

Informed consent was taken from all subjects and patient anonymity is preserved. In addition, this study has been approved by Medical Research Ethics Committee of Mansoura University, Egypt and conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000).

Statistical analysis: The data were presented as mean±standard

error of mean (SEM). Student t-test was used for comparisons between groups.

Results

(I) Animal experiments:

1. Effect of warfarin on blood pressure of dog (Figure 1): Warfarin has hypotensive action on blood pressure of dog.

2. Effect of atropine on the hypotensive action of warfarin (Figure 2): when warfarin is administered IV, in a dose of 50 µg/kg in the dog and the same dose was repeated after 5 minutes, it produced a hypotensive effect. Acetylcholine in a dose of 1 µg/kg IV produced hypotension. Atropine IV, in a dose of 0.2 mg/kg produced hypotension due to cutaneous vasodilatation. A test dose of acetylcholine 1 µg/kg produced no hypotensive effect denoting full atropinization and blockade of muscarinic receptors. The same dose of warfarin produced the same control hypotensive effect after blockade of muscarinic receptors denoting that this compound is not a muscarinic receptor agonist.

3. Effect of warfarin on the hypertensive effect of phenylephrine and adrenaline (Figure 3): Warfarin given IV to the dog in a dose of 50 µg/kg produced hypotension. Phenylephrine (1 µg/kg, IV) produced hypertension indicating intact alpha receptors in the dog. Warfarin given IV to the dog in a dose of 50µg/kg given after phenylephrine produced hypotension. Phenylephrine in a dose of 1 µg/kg given IV, after warfarin produced the same hypertensive effect denoting that warfarin has no alpha blocking effect. In addition, adrenaline in a dose of 1 µg/kg after warfarin injection produced hypertensive effect due to alpha and beta agonist effects. Warfarin given IV to the dog in a dose of 50 µg/kg after adrenaline produced hypotension. Adrenaline in a dose of 1 µg/kg after warfarin injection produced the same hypertensive effect in step before, denoting that warfarin is neither alpha nor beta blocker.

4. Effect of warfarin on sustained hypertension induced by NE infusion (Figure 4): Warfarin produced antihypertensive effect on sustained hypertension induced by NE.

5. Effect of warfarin on perfused hind limb of rats (Table 1): Warfarin produced a significant increase in the rate of perfusion of hind limb of rats.

6. Effect of warfarin on isolated perfused rabbit's heart (Figure 5): Warfarin in different doses used has no effect on isolated rabbit's heart.

(II) Clinical part:

Warfarin-treated patients received two years of continuous warfarin treatment. Indications for warfarin therapy included atrial fibrillation (120 patients, 75%), recurrent thromboembolic events (16 patients, 10%), stroke (20 patients, 12.5%), cardiomyopathy or intracardiac thrombus (4 patients, 2.5%). The general characteristics of the population are listed in table 2. As is readily apparent, this is a population characterized by older age, obesity and a high burden of comorbidity. The warfarin-treated group had a substantially higher prevalence of coronary disease and congestive heart failure.

BP levels, antihypertensive drug use and numbers of visits during the observation period are displayed in table 3. No difference in BP or dose of antihypertensive drugs (both by DDD and absolute numbers) was observed between the 2 groups.

Table (1): Effect of different doses of warfarin sodium on isolated perfused hind limb of rats (number of drops in 5 minutes).

	Number of drops in 5 minutes	P value
Control (n=6)	401 ± 29.95	
50 ug kg^{-1} warfarin (n=6)	580 ± 44.8	P<0.01
100ug kg^{-1} warfarin (n=6)	637.5 ± 59.8	P1<0.01
200ug kg^{-1} warfarin (n=6)	645 ± 53.8	P2<0.001
400ug kg^{-1} warfarin (n=6)	697 ± 92.9	P3<0.001

The data were presented as mean+SEM.
P = test of significance between control and 50 µg kg^{-1} warfarin treated groups.
P1= test of significance between control and 100 µg kg^{-1} warfarin treated groups.
P2= test of significance between control mean values and 200 µg kg^{-1} warfarin sodium treated group.
P3= test of significance between control mean values and 400 µg kg^{-1} warfarin sodium treated group.

Table (2): General characteristics of the study population:

	Placebo (n=160)	Warfarin (n=160)
Age (years)	71±8	71±8
Weight (kg)	94.5±11.6	98.5±12.3
Body mass index (BMI) (kg/m^2)	31.3±3.8	32.9±4.2
Coronary artery disease	41%	63% [a]
Heart failure	5%	26% [a]
Cerebrovascular disease	12%	17%
Peripheral vascular disease	19	27%
Current smoking	14	12%
Hemoglobin ($g\ dl^{-1}$)	13.9±1.7	14.1±1.5
Cholesterol ($mg\ dl^{-1}$)	161.4±12.6	152.3±11.2
LDL ($mg\ dl^{-1}$)	45±5	42±4
HDL ($mg\ dl^{-1}$)	83±4	78±5
Triglycerides ($mg\ dl^{-1}$)	184.4±13.8	171.5±12.6
Creatinine clearance ($ml\ min^{-1}$)	75±6	76±7

a: significant ($p<0.05$) compared to placebo group.

Table (3): Blood pressure parameters and antihypertensive drug use:

	Placebo (n=160)	Warfarin (n=160)
Systolic BP (mmHg)	135.5±12.7	131.6±11.1
Diastolic BP (mmHg)	77.4±5.1	75.4±4.6
Mean arterial pressure (mmHg)	96.8±5.6	94.1±5.2
Pulse pressure (mmHg)	58.1±6.2	56.2±6.8
Drug DDD	3.5±1.2	3.8±1.3
Number of antihypertensive medications	2.3±0.7	2.6±0.9

BP: blood pressure
DDD: defined daily doses based on the World Health Organization Collaborating Center for Drug Statistics Methodology.

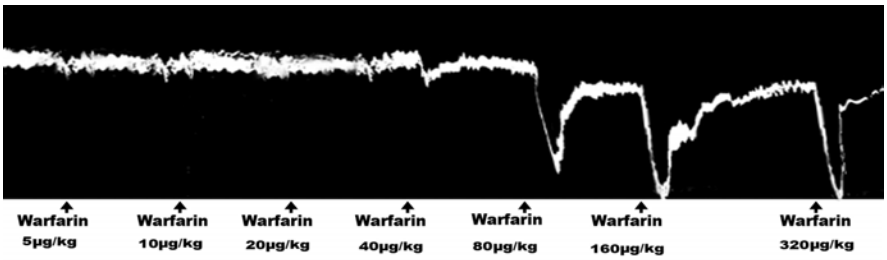

Fig. 1: Dose response curve of warfarin showing the effect on blood pressure in dogs.

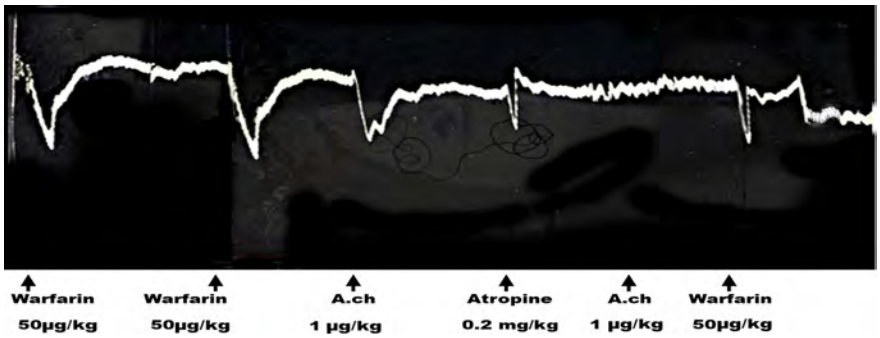

Fig. 2: Effect of warfarin on blood pressure in dogs after atropine and acetylcholine.

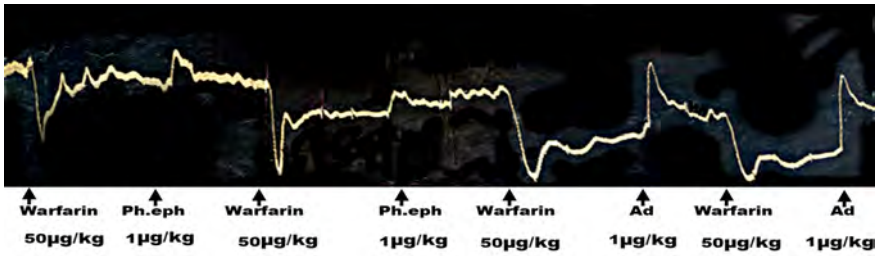

Fig. 3: Effect of warfarin on the hypertensive effect of phenylephrine and adrenaline.

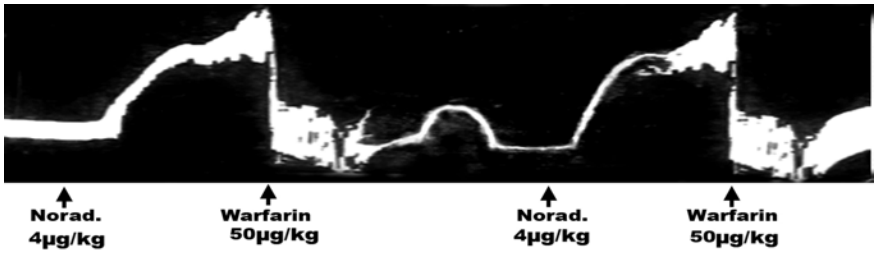

Fig. 4: Effect of warfarin on noradrenaline induced hypertension.

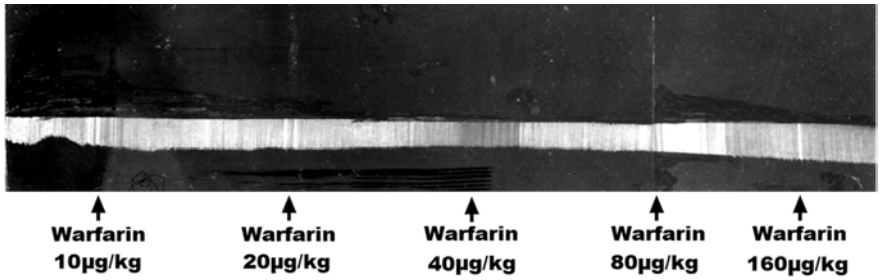

Fig. 5: Effect of different doses of warfarin on isolated rabbit's heart.

Discussion

Although the blood vessels are exposed to high pressures in hypertension, the main complications of hypertension (stroke and myocardial infarction) are paradoxically thrombotic rather than haemorrhagic[12]. In keeping with Virchow's triad, patients with hypertension do demonstrate abnormalities of vessel wall (endothelial dysfunction or damage), blood constituents (abnormal levels of haemostatic factors, platelet activation and fibrinolysis) and blood flow (rheology and flow reserve), suggesting that hypertension does confer a prothrombotic or hypercoagulable state. These abnormalities appear to be related to target organ damage and long-term prognosis and are altered by treatment. The observation that satisfactory blood pressure reduction with non-drug intervention and with various classes of antihypertensive drugs does not lead to an equal reduction in heart attacks and strokes may be due in part to unfavourable effects on the hypercoagulable state in hypertension [13]. Therefore, we examined the effect of warfarin, an anticoagulant, on blood pressure to provide a drug which is valuable in both hypertension and hypercoagulability.

The results of the present work showed that warfarin decreased blood pressure in the dogs (Figure 1). The same dose of warfarin produced the same control hypotensive effect after blockade of muscarinic receptors denoting that this compound is not a muscarinic receptor agonist (Figure 2). On the other hand, phenylephrine in a dose of 1 μg/kg given IV, after warfarin administration produced the same hypertensive effect as shown before its administration, denoting that warfarin has no alpha blocking effect (Figure 3). Also, adrenaline in a dose of 1 μg/kg after warfarin injection produced the same hypertensive effect as in step before its administration, denoting that warfarin is neither alpha nor beta blocker (Figure 3). In addition, warfarin produced antihypertensive effect on sustained hypertension induced by noradrenaline infusion (Figure 4). Moreover, warfarin produced a significant increase in the rate of perfusion of hind limb of rats (Table 1). The absence of in-

hibitory effect of warfarin on the isolated rabbit's heart (Figure 5) is useful as it will provide us with antihypertensive compound without deleterious effect on the heart. Thus, the antihypertensive effect of this compound may be due to direct vascular smooth muscle relaxant effect.

Based on the experimental findings of the current work, we investigated whether the use of warfarin causes decreased blood pressure in patients with hypertension. Our results show that long-term (average 2 years) warfarin therapy in old hypertensive patients did not result in a change in BP or Pulse pressure. Contrary to our results, Khan et al.[14] reported that warfarin has a hypertensive effect as it causes vascular calcification and arterial stiffness. They explain the effects of warfarin on the arterial wall by extracellular matrix (ECM) calcification, a process that is regulated by a number of factors. One such factor is matrix GLA protein (MGP) which, when absent in the MGP knock-out mouse, results in severe arterial calcification and death due to aortic rupture[15].

MGP is a 10kDa protein secreted by vascular smooth muscle cells, macrophages and chondrocytes. Activation of MGP involves gamma carboxylation of five glutamate residues to form calcium-binding Gla residues, a process that is vitamin K-dependent. In presence of warfarin, MGP remains inactive favoring calcification of ECM, especially vascular smooth muscle in larger vessels[16]. These procalcifying effects have been corroborated by some laboratories [7,17] and were further translated into hypertensive effects by Essalihi et al.[7] who established that treatment of Wistar rats with warfarin resulted in a phenotype of increased aortic calcification, aortic collagen-to-elastin ratio, aortic stiffness (measured by pulse wave velocity), and isolated systolic hypertension[7].

Schurgers et al[18] found more than a two-fold increase in calcium content in aortic valves removed from patients who had previously received warfarin compared with patients who had never received warfarin. Likewise, Koos et al.[5] recently reported increased coronary calcification (by

multislice spiral computer tomography) in patients treated with warfarin for at least six months compared with controls[5]. These studies did not report on BP or arterial function. In that sense, the analysis of SPINAF was the first report on the impact of warfarin on BP[6]. In that randomized, placebo-controlled clinical trial involving 525 patients with atrial fibrillation, warfarin did not result in increased BP in the overall group analysis[6]. In contrast, the results of the present work showed no changes in blood pressure in hypertensive patients receiving warfarin (Table 3).

Several possibilities may explain the different results. First, it is possible that the older age of our patients may have obscured the effects of warfarin on BP. Price et al.[19] demonstrated that older rats (42 days old) were found to have less calcification than younger animals (20 days old), and 10-month old rats did not have any evidence of warfarin-induced calcification in the carotid artery or aortic heart valves[19]. However, the average age of subjects in the Koos' and Schurgers' studies was 71 and 73 years, respectively [5,18]. Therefore, it is not clear that age is a relevant factor in humans, at least for calcification. Since we did not have an objective measure of arterial calcification or stiffness, we cannot conclude that warfarin does not cause vascular calcification leading to arterial stiffening in humans, but apparently, this does not readily translate into worse systolic BP or PP. Another possibility is that MGP might not be important in calcification of human arterial system as compared to rats. Patients with Keutel syndrome who lack MGP have extensive cartilaginous calcification but their vascular tree is not severely affected and these patients do not develop hypertension[20]. However, long- term follow-up of a patient with Keutel syndrome did reveal extensive calcification of coronary, hepatic, renal and cerebral arteries, but unfortunately there was no mention of the aorta or its major branches [21]. The precise role of MGP in humans remains to be further defined.

Dose may also have affected the results. Rat studies use a larg-

er dose as compared to humans. Also, our study included predominantly elderly Egyptian subjects, thus we cannot generalize our results to other populations. Mineral metabolism is regulated by multiple factors that include the levels of calcium, phosphorous and vitamin D. We could not look at these factors, as data were not available for the entire population. In addition, we did not exclude anyone based on the medical history significant for musculoskeletal illness e.g. Paget's disease and secondary hyperparathyroidism that have been known to affect soft tissue calcification.

In conclusion: While warfarin has an antihypertensive effect in experimental animals which is neither muscarinic agonist nor alpha and beta receptors blocker, we are not able to show the same effect in humans with hypertension.

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EFFECT OF ANTICOAGULANT (WARFARIN) ON BLOOD PRESSURE (EXPERIMENTAL AND CLINICAL STUDY)

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IMPACT OF HCV-RELATED CHRONIC LIVER DISEASE ON HEALTH RELATED QUALITY OF LIFE AMONG EGYPTIAN PATIENTS

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Abstract

Background: *Egyptian population, with the highest prevalence of HCV in the world, provides a unique opportunity to clarify this issue.*

Aim: *to assess HRQOL in Egyptian patients with HCV-related chronic liver disease and identify possible relationships between HRQOL with either demographic or laboratory parameters in these patients.*

Methods: *A cross sectional study was carried out on adult HCV patients attending the Tropical Medicine Department, Faculty of Medicine, Mansoura University. They fill the questionnaire that ask about, Socio-demographic characteristics, impact of HCV on lifestyle and HRQOL. Through clinical examination and investigations were done for all patients.*

Results: *560 patients with complete data were included in the study; their mean age was 48.9±10.7 years. The most affected domain of SF-36 score was bodily pain (97.70%). Male gender, Rural, illiterates, unemployed and low income patients were associated with a low score in most of the domains. ALT, AST and albumin were positively correlated whereas INR showed negative correlation with most of the domains. Classification and age were the most common independent predictors.*

Conclusion: *Our findings give a clear picture of the magnitude of the impact of HCV on HRQOL among Hepatitis C patients from which bodily pain and social functioning were the most affected, also strongly support the need to offer adequate information and provide educational programs for patients.*

Key words: *Quality of life, HCV, Cirrhosis, Child-Pugh classification.*

Introduction

The world Health Organization (WHO) has declared chronic liver disease as a global health problem. The prevalence of HCV infection is approximately 3% worldwide (170 million people).[1] Countries with high rates of chronic infection are Egypt (15%), Pakistan (4.8%) and China (3.2%).[2] The majority (85%) of patients infected with hepatitis C virus (HCV) develop the chronic form of the disease, which in 20-30% of cases will evolve to cirrhosis, liver failure or hepatocellular carcinoma, after several decades.[3]

QOL is a complex concept involving patient's perception of his/her ability to perform functions such as work, but also comprises the physical effects of the illness and concomitant psychological conditions, anxiety, depression, and feeling of hopelessness.[4] Other related issues are studied such as sexual problems, relationships with his/her family, friends and the healthcare team.[5] QOL is a popular term that conveys an overall sense of well-being, including aspects of happiness and satisfaction with life as a whole. It is broad and subjective rather than specific and objective.[6] The concept of HRQOL refers to the impact of health and illness on an individual's QOL.[7]

In early stages, liver disease patients show few or non-specific symptoms, therefore reporting insignificant effects on HRQOL. As the disease progresses to cirrhosis and complications arise (ascites, muscle cramps, fatigue etc.) individuals report noteworthy effects on HRQOL.[8] However several studies reported that patients with chronic HCV have a significant reduction in their HRQOL that is not related to the severity of the liver disease.[9] In fact many HCV patients have a previous or ongoing addiction and/or psychiatric problems, reflected in lower HRQOL. In addition, the majority of HCV patients have a lower social economic status compared to the general population. Others suggest that HCV may act within the central nervous system.[10] Perhaps due to HCV brain colonization or to systemic activation of cytokines which, in turn, influence brain cytokine production and neurotransmission.[11] Moreo-

ver, fatigue, malaise, depression and cognitive impairment and all neuropsychiatric disorders are among the most common complaints of patients with chronic hepatitis C and occur independently of liver disease or treatment status.[12] Recent studies have demonstrated through cerebral magnetic resonance images of abnormal cerebral metabolism and cognitive impairments in patients with chronic hepatitis C.[13] HRQOL can be measured by generic and disease specific measures. The Medical Outcomes Study Short Form-36 (SF-36) is currently the most used instrument worldwide, and a shorter version is available (SF-12).[14]

Our aim of this study was to assess HRQOL in Egyptian patients with HCV-related chronic liver disease and identify possible relationships between HRQOL with either demographic or laboratory parameters in these patients.

Subjects and Methods

This cross sectional study was carried out on adult HCV patients (≥18yrs) of both gender selected from Outpatient and Inpatient Clinics at Tropical Medicine Department, Faculty of Medicine, Mansoura University.

Sample size: was calculated online (www.dssresearch.com). A pilot study was done on 50 hepatitis patients, from which the mean of the total score was found to be 55.1±26.3 and by considering the worst expected as 56.1, the sample size was 446 with 95% confidence level and 80% study power. These study included 600 patients with chronic liver disease due to hepatitis C virus (diagnosis of HCV based on positive HCV antibodies and positive HCV-RNA by PCR), but patients with other causes of chronic liver disease (positive HBsAg, Autoimmune hepatitis, metabolic, cholestatic, alcoholic), language or cognitive difficulties that prevented reliable completion of the questionnaire were excluded. The patients were given brief explanations of the objectives of the questionnaire. Patients were also assured of their anonymity and the confidentiality of their responses. 40 of them were excluded because they had incomplete data.

Measures and data management:

All Hepatitis C patients of the study sample were subjected to a precoded interview questionnaire. The questionnaire was answered within 30 minutes and completed in the same visit. This questionnaire used to identify the following data: Socio-demographic characteristics include name, gender, age, residence, occupation, education, and family income. Through clinical examination, routine laboratory investigations, abdominal ultrasound were done for all patients. The severity of liver disease was assessed by Child-Pugh classification that is based on (two clinical data) ascites and encephalopathy and (three laboratory findings) Albumin g/dl , Bilirubin mg/dl and INR, it is classified as Child A score (5-6), Child B score (7-9) and Child C score (10-15). This Child-Pugh classification were performed for cirrhotic patients only (Group11 and Group111). Generic Health-Related Quality Of Life assessment, SF-36 was used specifically to measure eight health domains: physical functioning (PF), (10 items), role limitations due to physical problems (RP), (4 items), bodily pain (BP), (2 items), general health (GH), (5 items), Energy/ Fatigue (E/F), (4 items), social functioning (SF), (2 items), role limitations due to emotional problems (RE), (3 items), and emotional wellbeing (EW) (5 items). On the basis of these separate subscales, component summary scores can be calculated to provide a global measure of physical (Physical Component Summary score, PCS) and mental functioning (Mental Component Summary score, MCS), respectively. The scale scores range from 0 to 100, with higher scores indicating a better health status (Guyatt,1993).

A modification applied to the SF-36 such as simplify the Arabic version tool language to be better understandable for the local respondent's culture. Total generic HRQOL score was obtained by summing the scores of the eight domains, higher score indicates better Generic HRQOL.

Statistical techniques:

The completed questionnaire were subjected to revision and the collected data were coded, processed and analyzed through

SPSS (Statistical Package for Social Sciences) (Standard version release 16.0). K-S was used to test the normality of different parameters. A descriptive analysis of the collected data was done in the form of frequencies and percent. Mann Whitney U test and Kruskal Wallis test used to analyze the relation between domains of the quality of life and different variable. Correlation between domains of the quality of life, age and laboratory investigations was examined by Spearman's correlation coefficient. The multiple linear regressions were used to assess predictors of SF-36 parameters. Variables were retained in the final models if they were significantly associated with the SF-36 parameter being tested. P≤0.05 was chosen as the level of statistical significance.

Results

560 patients with complete data were included in the study; their mean age was 48.9±10.7 years. Male and female patients accounted for 55.5% and 44.5%; respectively. About 50.2% of these patients were from rural areas versus 49.8% from urban areas. 37% were illiterate, 66.8% of our group had no enough income with 35.9% of them were not working (table 1).

The most affected domain of SF-36 score was bodily pain (97.70%) followed by social functioning (94.30%) and role limitation due to emotional factor (90.40%). The least affected was energy domain (23.60%) as shown in (figure 1).

Several demographic factors were studied to see if there was any association with SF-36 scores. Male gender was associated with a low score in the following areas: PF (P=.001), RP (P=.017), RE (p=0.022), E/F (P=.001), and BP (P=.043), PCS (p=0.002), MCS (0.001). Rural patients showed significant lower scores than urban patients regarding most of the domains except E/F, EW, and GH which were significantly higher among them. Increasing level of education was associated with higher scores where illiterates achieved the lower scores in PF (P<.0001), RP (P<.0001), RE (P<.0001), SF (P<.0001), and BP (P<.0001), PCS (P<.0001), MCS (P<.0001). Employment was associated with a higher score in PF

(P<.0001), RP (P=.001), SF (P<.0001), and BP (P<.0001), PCS (P<.0001), MCS (P<.0001). Employment was associated with a higher score in PF (P<.0001), RP (P=.001), SF (P<.0001), and BP (P<.0001). Low income was associated with a low score in the following areas: PF (P<.0001), RP (P<.0001), SF (P<.0001) and BP (P<.0001), PCS (P<.0001).

Regarding the severity of liver disease based on Child's-Pugh classification for cirrhotic patients, it was evident in this study that 235 (42.3%) had no cirrhosis, 141 (25.2%) had Child's A patients, 85 (15.2%) Child's B pa tients, and 99 (17.3%) had child's C patients.

As regard SF-36 scores and severity of chronic liver disease Table (3), all domains, PCS and MCS were significantly affected among different studied groups (p<0.001), comparison of eight domains, PCS and MCS scores of SF-36 between non cirrhotic (patients chronically infected with HCV) and child's A patients did not show any statistically significant difference (p>0.05), however there was statistically significant difference between these non cirrhotic patients and both child' B and child' C (p<0.001) except for GH where the significance was detected between non cirrhotic and child' C patients. Also we found statistically significant difference in SF-36 domains, PCS and MCS between compensated cirrhotic patients (child's A) and decompensated cirrhotic patients (both child's B and child's C) p<0.001 except for EW and GH where the significance was detected between (child's A) and (child's C) patients only. At the same time between patients with advanced liver disease (child's B and child' C), PCS and the domains of SF-36 scores showed statistically significant difference (p<0.05) except EW and GH.

As regard correlation between laboratory findings and domains of SF-36. ALT was positively correlated with PF, RP, RE, SF, BP, PCS and MCS with statistical significance (p<0.001) but only negatively correlated with E/F. At the same time, AST correlated positively with all the domains except E/F, EW and GH (P<0.001). There were significant positive correlation (p<0.001) between albumin

and different domains as PF, E/F, EW, SF, and BP. Bilirubin only correlated positively with EW and BP (r=0.054 and 0.237 respectively) and negatively correlated with E/F (r=-0.345). Whereas INR showed significant negative correlation with most of the domains (p<0.001) except for RP, RE and PCS.

Age showed significant negative correlation with most of the domains except E/F, EW and GH which showed significant positive correlation (p<0.001) (Table 4).

There are independent predictors for worsening of HRQOL in different domains. Using linear regression analysis, it is evident that classification and age were the most common independent variable where classification was negatively associated with all domains in patients with HCV however age was associated negatively with most of the domains except E/F, EW and GH which was positively associated with them. Gender is an independent predictor for worsening of HRQOL only in (EW) domain. Residence is an independent predictor for worsening of SF-36 in the following domains; (BP), (SF) and (PCS) score (p<0.000). Income is a predictor for worsening of SF-36 score in (GH), (E/F) domain and MCS (p<0.000). Education is an independent predictor for worsening of SF-36 score in (RE) domain only (p<0.000). AST is a predictor for worsening of RE, E/F, EW, GH and PCS domains (p<0.000). ALT is a predictor for worsening of SF-36 score in GH and EW domain (p<0.000). INR only detected as a predictor for SF.

Table (1): Socio-demographic characteristics of the studied group.

Socio-demographic		N	Percent
Sex	Male	311	55.5%
	Female	249	44.5%
Residence	Urban	279	49.8%
	Rural	281	50.2%
Education	Illiterate	207	37.0%
	Read & write /basic	125	22.3%
	Secondary	122	21.8%
	University and more	106	18.9%
Income	Not enough	374	66.8%
	Just enough	134	23.9%
	More enough	52	9.3%
Occupation	Professional,	38	6.8%
	Managerial	49	8.8%
	Clerk &the Armed Forces,	95	17.0%
	Skilled	132	23.6%
	Unskilled,	45	8.0%
	Not working	201	35.9%

Table (2): Quality of life (SF-36) scores according to severity of HCV-related chronic liver disease.

	Chronic HCV N=237 (42.3%) (1)	Compensated HCV (Child A) N =141 (25.2%) (2)	Decompensated HCV (Child B) N =85 (15.2%) (3)	Decompensated HCV (Child C) N =97 (17.3%) (4)	Kruskal Wallis test P value	Manwittneu U test
Physical component summary (PCS)	54.8±15.4	55.2±16.1	30.6±13.5	21.45±5.1	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(3)=0.000 (2)versus(4)=0.000 (3)versus(4)=0.000
Mental component summary (MCS)	56.0±.8.9	55.9±9.6	30.6±11.8	44.9±5.28	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(3)=0.000 (2)versus(4)=0.000
Physical functioning	63.8±22.3	63.3±24.1	21.8±23.7	44.4±13.2	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(3)=0.000 (2)versus(4)=0.000 (3)versus(4)=0.000
Role limitation due to physical factor	47.5±30.6	50.7±33.7	12.1±22.4	.000±.000	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(3)=0.000 (2)versus(4)=0.000 (3)versus(4)=0.03
Role limitation due to emotional factor	44.6±31.2	47.0±37.2	16.5±27.0	1.01±7.5	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(3)=0.000 (2)versus(4)=0.000 (3)versus(4)=0.000
Energy/fatigue	67.6±15.9	68.5±14.7	52.2±12.5	47.5±7.27	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(3)=0.000 (2)versus(4)=0.000 (3)versus(4)=0.000
Emotional well-being	57.9±15.6	54.9±15.1	51.5±17.4	46.2±18.9	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(4)=0.000
Social functioning	54.0±19.8	53.1±18.7	25.4±19.1	12.6±16.8	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(3)=0.000 (2)versus(4)=0.000 (3)versus(4)=0.000
Bodily Pain	47.5±16.1	44.9±13.6	24.0±14.9	12.14±13.1	0.000	(1)versus(3)=0.000 (1)versus(4)=0.000 (2)versus(3)=0.000 (2)versus(4)=0.000 (3)versus(4)=0.000
General health	60.5±12.9	61.8±11.3	59.5±13.5	55.2±9.1	0.000	(1)versus(4)=0.000 (2)versus(4)=0.000

Table (3): Correlation between domains of quality of life, laboratory investigation and age.

	ALT		AST		Albumin		Bilirubin		INR		Age	
	r	P	r	p	R	p	r	p	R	p	r	p
Physical functioning	0.361**	0.000	0.325**	0.000	0.212**	0.000	0.111	0.22	-0.343**	0.000	-0.505**	0.000
Role limitation due to physical factor	0.257**	0.000	0.273**	0.000	0.293	0.37	0.234	0.450	-0.222	0.47	-0.515**	0.000
Role limitation due to emotional factor	0.224**	0.000	0.312**	0.000	0.168	0.052	0.215	0.06	-0.328	0.150	-0.387**	0.000
Energy/fatigue	-0.233**	0.000	-0.378**	0.000	0.234**	0.000	-0.345**	0.000	-0.242**	0.000	0.472**	0.000
Emotional well-being	-0.034-	0.422	-0.272**	0.000	0.200**	0.000	0.054**	0.000	-0.314**	0.000	0.335**	0.000
Social functioning	0.263	0.000	0.314	0.000	0.154**	0.000	-0.185	0.09	-0.317**	0.000	-0.513**	0.000
Bodily Pain	0.326	0.000	0.317	0.000	0.431**	0.000	0.237**	0.000	-0.380**	0.000	-0.527**	0.000
General health	0.015	0.732	-0.380	0.000	0.125	0.07	0.321	0.12	-0.277**	0.000	0.246	0.000
Physical component summary (PCS)	0.350	0.000	0.277	0.000	0.238	0.33	0.132	0.34	-0.194	0.23	-0.533**	0.000
Mental component summary (MCS)	0.256	0.000	0.194	0.000	0.212	0.12	0.432**	0.000	-0.273**	0.02	-0.327**	0.000

Table (4): Predictors of quality of life domains:

Domain	Predictors	Model prediction	Model p value	Unstandardized Coefficients	Sig.	95% Confidence Interval for B	
				B		Lower Bound	Upper Bound
Physical functioning	(Constant)	56.7	0.000	123.458	0.000	114.885	132.031
	Classification			-17.820-	0.000	-19.587-	-16.053-
	Age			-0.796-	0.000	-0.983-	-0.609-
Role limitation due to physical factor	(Constant)	41.3	0.000	115.558	0.000	105.216	125.899
	Classification			-12.712-	0.000	-14.843-	-10.580-
	Age			-1.104-	0.000	-1.329-	-0.878-
Role limitation due to emotional factor	(Constant)	30.9	0.000	85.147	0.000	68.138	102.157
	Classification			-20.535-	0.000	-27.579-	-13.491-
	Age			-0.738-	0.000	-.993-	-0.483-
	AST			0.126	0.001	0.049	0.204
	Education			-2.407-	0.038	-4.676-	-0.139-
Energy/fatigue	(Constant)	40.4	0.000	48.950	0.000	40.720	57.180
	Classification			6.078	0.000	4.881	7.275
	Age			0.429	0.000	0.311	0.547
	AST			-0.044-	0.015	-0.079-	-0.008-
	Income			-2.460-	0.025	-4.604-	-0.315-
Emotional well-being	(Constant)	20.8	0.000	36.494	0.000	26.523	46.465
	Age			0.417	0.000	0.278	0.556
	Classification			9.917	0.000	6.059	13.775
	AST			-0.096-	0.000	-0.139-	-0.052-
	ALT			0.036	0.001	0.014	0.058
	Sex			3.007	0.031	0.268	5.746
Social functioning	(Constant)	49.5	0.000	106.008	0.000	98.097	113.919
	Classification			-11.551-	0.000	-13.038-	-10.064-
	Age			-0.698-	0.000	-0.855-	-0.540-
	Residence			-3.665	0.021	-6.783	-0.547
	INR			-4.235	0.04	-7.432	-0.234
Bodily Pain	(Constant)	52.8	0.000	90.652	0.000	84.480	96.824
	Classification			-9.606-	0.000	-10.766-	-8.446-
	Age			-0.573-	0.000	-0.696-	-0.450-
	Residence			-3.601-	0.004	-6.034-	-1.168-
General health	(Constant)	22.3	0.000	63.458	0.000	56.301	70.615
	AST			-0.130-	0.000	-0.160-	-.0099-
	ALT			0.041	0.000	0.026	0.056
	Age			0.139	0.005	0.043	0.236
	Classification			3.824	0.005	1.153	6.495
	Income			-1.937-	0.031	-3.693-	-.0181-
	INR			-4.541	0.049	-8.55	-0.035

Continue:

Domain	Predictors	Model prediction	Model p value	Unstandardized Coefficients	Sig.	95% Confidence Interval for B	
				B		Lower Bound	Upper Bound
Physical component summary (PCS)	(Constant)	54.5	0.000	100.527	0.000	93.485	107.569
	Classification			-9.959-	0.000	-11.156-	-8.762-
	Age			-0.571-	0.000	-0.687-	-0.454-
	Residence			-2.555-	0.029	-4.850-	-0.261-
	AST			-0.035-	0.047	-0.070-	0.000
Mental component summary (MCS)	(Constant)	26.3	0.000	70.757	0.000	66.199	75.315
	Classification			-3.645-	0.000	-4.312-	-2.977-
	Age			-0.153-	0.000	-0.224-	-0.081-
	Income			-1.424-	0.031	-2.714-	-0.133-

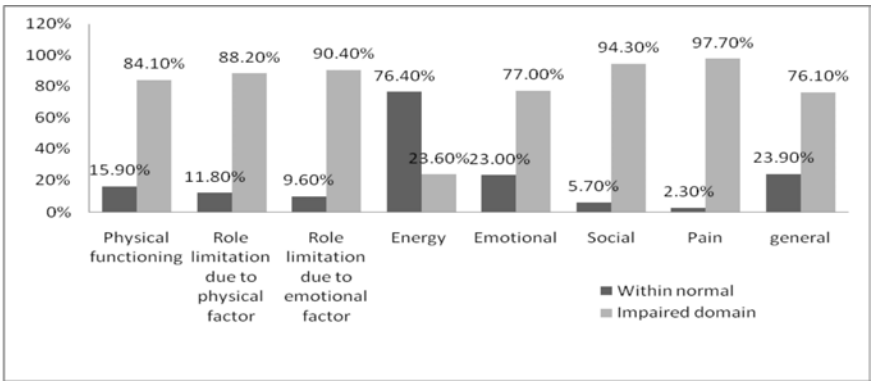

Fig. 1: Description of the normal and impaired domains among the studied group.

Discussion

Egyptian population, with the highest prevalence of HCV in the world, provides a unique opportunity to clarify this issue. We looked for potential confounding factors for SF-36, PCS and MCS score among chronic HCV infected patients. Socio-demographic variables might be predictors of SF-36 score of quality of life in individual chronically infected with HCV. In this study, rural residence showed significant association with the worsening of SF-36 score in most of the domains, PCS and MCS scores ($p<0.05$), using linear regression analysis residence is an independent predictor for worsening of SF-36 in the following do-

mains; pain, social and PCS score ($p<0.001$), these findings were in agreement with Strauss and Dias Teixeira,[15] and Almaza et al,[16] who mentioned that inhabitant of rural area in Egypt had a very low quality scores. The impairment of HRQL among patients from rural areas could be more obvious because the agricultural work required more physical performance which makes patients exhausted, also HCV chronic liver diseases treatment is expensive and most of them can't afford. Illiteracy among the studied groups showed statistically significant reduction in the majority of SF-36 scores ($p<0.001$), using linear regression analysis education is an independent predictor for worsening of SF-36 score in role due to emotional factor domain only ($p<0.000$), the results of this study were not agree with Almaza et al,16. Also El-Seoud et al,[17] found that QOL of highly educated patients was more impaired in physical functioning and role limitation attributed to physical and emotional problems. In general population, low education could be one of the most important predictor of QOL in both physical and psychosocial domains.[18]

As regard correlation between age and SF-36 score, PCS and MCS the current study showed significant negative correlation except in energy/fatigue, emotional wellbeing and general domain, using linear regression analysis, it is evident that age is a strong predictor for worsening of HRQOL in all domains ($p<0.001$). These is in agreement with Minola et al,[19] and Bezemer et al,[20] which stated that subjects infected with HCV at younger age have better outcomes. However Sinakos et al,[21] previously confirmed the positive correlation between age and emotional wellbeing.

Regarding gender HRQOL more affected in male than female, this result is in contrast to what found by Bezemer et al,[20] who stated that men and women differed in the course of HRQOL with an increase on scores for men on several dimensions. Serum aminotransferases (ALT&AST) in the current study found a significant positive correlation with SF-36 score, PCS and MCS score except Energy/ Fatigue, Emotion show negative

correlation, using linear regression analysis, ALT is a predictor for worsening of SF-36 score in general and emotional domain ($p<0.001$), our results may be explained by most of the patients included in these study were non cirrhotic "chronic HCV infection" and child's A in whom serum aminotransferases were elevated than in advanced stage of liver disease and these patients had good HRQOL score than in decompensated cirrhotic patients, these results agreement with Ayman,[22] who explained the results as when laboratory parameters showed significant correlations with SF-36 domains, most of these laboratory parameters were related to liver function (albumin, INR, and AST), suggesting that active liver disease or perhaps significant fibrosis was more likely affecting the HRQOL domain score rather than the virus itself. But disagreement by Schwarzinger et al,[23] who reported in individual chronically infected with HCV, serum ALT and AST levels did not correlate with HRQOL measures.

Regarding the severity of liver disease based on Child's-Pugh classification for cirrhotic patients, it was evident in this study that 25.2% of studied patients were classified as Child's A, 15.2% as Child's B and 17.3% Child's C but non cirrhotic "patients with chronic HCV infection" were 42.3%, these results were in agreement with the obtained results from an Egyptian study where compensated cirrhosis was 65.7% and decompensated cirrhosis (Child's B and C) was 34.3%.[24] These findings were not consistent with those of Sumskiene et al,[25] who found that Child's A and Child's C patients were 25% and 75% respectively. The variation of severity of chronic liver disease (CLD) could be explained by the difference in sociodemographic variables such as residence, etiology of CLD, co morbid diseases smoking and other substance abuse might add a more injurious effect on CLD patients and the net results is worsening of QOL.

This study found a significant reduction of HRQOL by SF-36 Questionnaire in individual chronically infected with HCV. The eight domains of SF-36 were impaired,

with pain domain the most affected (97.70%) followed by social (94.30%). The least affected was energy domain (23.60%). The same reported by El-Seoud et al,17 who found that all QOL domains were reduced but the most affected domain was the role limitation attributed to physical problems (88.5%) and the least affected one was the mental health domain (26.7%).

By comparing between different classifications of the studied group, the current study show that as liver disease becomes more severe patient's HRQOL. All domains of SF-36 Questionnaire, PCS and MCS scores showed statistically significant affection among different studied groups ($p<0.001$) except between patients with chronic HCV infection "non cirrhotic" and child's A "compensated cirrhotic "patients, there was difference but statistically insignificant ($p>0.05$), one possible explanation of this findings is that there is little difference in the degree of liver impairment between both groups.

Using linear regression analysis, severity of CLD is a strong predictor for worsening of SF-36 score in all domains ($p<0.001$), these results of the current study did not agree by Hauser et al,[26] who reported that there was no significant association of QOL with severity of liver disease, on the other hand these results were in partial agreement with Simone et al,[27] which concluded that physical domain was more affected than the other domains in patients with Child's C compared to those with Child's A and Child's C. In another study, poor health perception was increased significantly with the severity of liver disease Sobhonslidsuk et al,[28].

Conclusion and Recommendations

Our findings give a clear picture of the magnitude of the impact of HCV on HRQOL among Hepatitis C patients from which bodily pain and social functioning were the most affected, also strongly support the need to offer adequate information and provide educational programs for patients. Though educating patients is a time-consuming and consequently expensive procedure, there is no

doubt, that it would have a favor. Training of physicians about QOL and its related issues in daily practice is crucial for patients with CLD, also doctors must look at HCV infection as not just a cause of chronic liver disease but as a factor that affect the total quality of life that mostly impaired in these patients.

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IMPACT OF HCV-RELATED CHRONIC LIVER DISEASE ON HEALTH RELATED QUALITY OF LIFE AMONG EGYPTIAN PATIENTS

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ROLE OF THE NO/cGMP PATHWAY IN THE EFFECT OF L-ARGININE AND SODIUM NITROPRUSSIDE ON THE SPONTANEOUS CONTRACTILITY OF RAT NON-PREGNANT UTERUS

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Abstract

The effect of the endogenous and exogenous nitric oxide (NO) upon the spontaneous uterine contractility in non-pregnant rats was investigated in this work. The possible involvement of intracellular guanosine 3,5-cyclic monophosphate (cGMP), a second messenger for NO, was also studied. Spontaneously contracted uterine strips were obtained from thirty virgin albino rats. Uterine tissue was incubated with L-arginine in the absence or presence of L-NAME or methylene blue (MB) and with sodium nitroprusside (SNP) in the absence or presence of MB. The effect of the cGMP analogue, 8-bromocyclic guanosine monophosphate (8-br-cGMP) on the spontaneously active uterine strips was also investigated. Uterine tissue homogenates were prepared for estimation of the intracellular cGMP quantity in all experiments.

The NO synthase substrate, L-arginine, produced a significant reduction in the spontaneous uterine activity, as well as an increase of the intracellular cGMP. Incubation of the uterine strips with the NO synthase inhibitor L-NAME prior to L-arginine not only abolished L-arginine-induced uterine relaxation, but also significantly increased the strength of spontaneous contractions, which was associated with a significant decrease of cGMP quantity in uterine tissue homogenates. The loss of the L-arginine inhibitory effect on the spontaneous rat uterine contractions by L-NAME pretreatment confirms the role of the NO in the regulation of the contractile activity. Incubation of the rat uterine strips with

the NO donor, SNP, induced a significant reduction of the spontaneous contractions, and in a significant elevation of the intracellular cGMP. Also, incubation of uterine strips with the cGMP analogue, 8-br-cGMP, showed a significant reduction in spontaneous uterine contractility. However, complete inhibition of the guanylyl cyclase by MB partially inhibits the relaxing effect of both L-arginine and SNP on uterine contractility, but did not abolish it. This indicates a role of cGMP in the mechanism of action of L-arginine and SNP. However, the failure of complete inhibition of the uterine relaxing effect, after suppression of cGMP synthesis suggests the involvement of other mediators in the NO-dependent relaxation pathway beside the accumulation of cGMP.

It is concluded that both L-arginine and SNP inhibit the spontaneous contractions of the non-pregnant rat uterus through the involvement of both cGMP dependent and independent pathways.

Keywords: *nitric oxide; cGMP; contraction; uterus; non-pregnant rats.*

Introduction

Nitric oxide (NO) has been found to be of great importance throughout the body as a vasodilator, neurotransmitter and relaxant of vascular and non-vascular smooth muscles. Endogenous NO production from its precursor L-arginine occurs in uterine smooth muscle cells via NO synthases[1]. Recent interest has developed in determining the role of NO in the control of uterine contractility. While the majority of previous studies focused upon the possible role of endogenous NO in the maintenance of uterine quiescence during pregnancy, there have been only a few studies on a role for NO in the regulation of function in the non-pregnant uterus. Kuenzli et al.[2] investigated the existence of functionally relevant NO synthase in the non-pregnant monkey uterus, and suggested a role for NO in the moment-to-moment regulation of monkey uterine contractile activity. The potential efficacy of NO as a uterine relaxant has also been demonstrated in rats, in which NO acts as a smooth muscle relaxant in the myometrium[3].

The NO/guanosine 3,5-cyclic monophophosphate (cGMP) signaling cascade is of importance in the

cardiovascular system, where it controls smooth muscle relaxation [4]. The role of an intracellular cGMP-dependent mechanism in the regulation of uterine contractility is, however, a matter of debate. Despite the number of studies which have established the presence of NO synthase in vivo, only a few studies have explored the possible mechanism of action of NO in the uterus. It has been proposed that NO affects uterine smooth muscle tone via elevations in cGMP[5,6]. The hypothesis that cGMP action in myometrium is compartmented has been also proposed[7]. On the other hand, other investigators suggested that cGMP plays a minor role, if any, in the regulation of rodent and human uterine contractility[8,9,10].

The aim of the present study was not only to investigate the effect of the endogenous NO production by L-arginine and the exogenous NO donation by sodium nitroprusside (SNP) on the spontaneous uterine contractions in the non-pregnant rat, but also to explore the role of the NO/cGMP cyclase pathway in the relaxant contribution by NO.

Material and Methods

Source and preparation of isolated uterine strips:

In this study, thirty virgin adult female albino rats, weighing 150-200 g each and aging 10 weeks old to ensure sexual maturity[11], were used. Animals were fed with standard laboratory chow and water was available ad libitum, and housed in the animal house of Menofiya Faculty of Medicine under artificial light/dark cycle of 12 hrs. All animals were allowed to acclimatize for at least 7 days prior to experimental manipulations. Experimental procedures were carried out in compliance with the guidelines of Institutional Animal Care Committee, and were in accordance with the Ethical Committee of The Faculty of Medicine, Menofiya University.

To prepare isolated uterine strips, rats were sacrificed by neck elongation and dislocation. The abdomen was rapidly opened and the bicornuate uterus was identified behind coils of the intestine. One uterine horn was then carefully excised to avoid excessive stretching, and placed on a petri dish containing freshly prepared Kreb's solu-

tion at 37 ^{0}C. The Kreb's solution had the following composition[12]: NaCl (118.0 mM), KCl (4.7 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1.0 mM), KH_2PO_4 (1.0 mM), glucose (11.0 mM) and $NaHCO_3$ (25.0 mM). The attached loosely adhering connective tissue, fat and ovary were rapidly removed and the uterine horn was cut longitudinally in strips, 15 mm in length and 5 mm in width. A uterine muscle strip was then fixed from its lower end by a silk thread to a glass tissue holder, and vertically suspended in a "50 ml-capacity, thermostatically controlled 37 ^{0}C organ bath" containing freshly prepared Kreb's solution, gassed with 95% oxygen and 5% carbon dioxide. The upper end of the uterine strip was connected via another silk thread to an isometric strain-gauge tension transducer (Myograph, Narco Biosystems, UK) coupled to a four channel ossilograph (Harvard, England) for recording the uterine contractions from the longitudinal axis under a resting tension of 1 g. The organ bath was covered with aluminum foil to prevent light-induced degradation of the chemical agents.

An equilibrium period of 30 min was allowed for the preparation to recover from the trauma of dissection and for the establishment of steady spontaneous rhythmic uterine contractions. The preparation was washed thrice with 50 ml of Kebs' solution between experiments, and was allowed a 15 min rest period in bathing solution for complete recovery of the uterine strip and returning to the basal spontaneous contraction level. The strength of the uterine contractions was determined by dividing the sum of the peak tensions in a 10 min by the number of contractions during that time. Statistical calculations were done by comparing the mean value during 10 min after addition of active agent with that during the 10 min before its addition, except when otherwise is mentioned. The strength of the basal spontaneous contractions was shown to be not significantly different during the course of the experiments. All experiments were completed within 6 hours of tissue collection. As far as possible, experiments were performed with strips from the same uterus.

Studying the effect of the NO synthase substrate, L-arginine,

and the NO synthase inhibitor, L-NAME, on the spontaneous uterine contractility:

Spontaneously active uterine strips (n=10) were treated with the NO synthase substrate, L-arginine in this experiment. L-arginine (Sigma-Aldrich, St. Louis, MO, USA) was added to the bathing solution for 10 min, and the change in the strength of isometric uterine contraction was monitored. Non-cumulative concentration-response curves were performed by logarithmic increments of the concentration of L-arginine from 1 μM to 10 mM. Only one concentration-response relationship was performed in the uterine strips derived from each rat. L-arginine solution was always buffered with 25 mM Tris-HCl and brought to a pH of 7.4 before addition to the organ bath.

Other uterine strips (n=10) were first pretreated with the NO synthase inhibitor, nitro-L-arginine methyl ester (L-NAME), before addition of L-arginine to assess the influence of the NO synthase inhibition upon L-arginine-induced effect on spontaneous contractility. L-NAME (Sigma-Aldrich, St. Louis, MO, USA) was added firstly to the bathing solution with an end-concentration of 25 μg/ml[13] for 30 min, followed by exposure to the maximal effective concentration of L-arginine detected by the concentration-response curve. Evaluation of the effect of L-arginine treatment upon L-NAME-pretreated strips was performed by comparing the contractile activity in the presence of L-NAME with that seen following L-arginine addition in each strip, and the % change was calculated.

Studying the effect of the exogenous NO donor sodium nitroprusside (SNP) on the spontaneous uterine contractility:

The effect of the exogenous NO on the spontaneous contractile activity of the uterus was evaluated by incubation of the rat uterine strips with SNP (Sigma-Aldrich, St. Louis, MO, USA). Non-cumulative concentration-response curves were performed by log increments of the concentration of SNP, from 10 nM to 1 mM. Only one concentration-response relationship was performed in the uterine strips derived from each rat.

Assessment of the role of the

NO/cGMP pathway in the mediation of the action of L-arginine and SNP:

The involvement of the NO/cGMP pathway in the action of L-arginine and SNP on spontaneous uterine contractions was assessed by prior incubation of the uterine strips (n=10) with the guanylyl cyclase inhibitor methylene blue (Sigma-Aldrich, St. Louis, MO, USA) in a concentration of 1 μM[14] for 30 min, then adding the maximal effective concentration of L-arginine or SNP to the bathing medium. Spontaneous activity was evaluated both before and following methylene blue pretreatment to determine the effect of methylene blue upon spontaneous contractile activity, as well as after addition of L-arginine or SNP to the bathing medium to evaluate the effect of the inhibition of guanylyl cyclase enzyme on their action. The % changes in spontaneous uterine contractions after L-arginine or SNP addition to strips pretreated with methylene blue were calculated.

Effect of the cGMP analogue, 8-bromocyclic guanosine monophosphate (8-Br-cGMP), on the spontaneous uterine contractility:

Non-cumulative concentration-response curves of the effect of the cGMP analogue, 8-br-cGMP (Sigma-Aldrich, St. Louis, MO, USA), on rat uterine activity were performed by incubation of uterine strips with log increments of concentrations from 10 nM to 10 μM, and the strength of the uterine contractions were monitored.

The cGMP quantification in uterine smooth muscles:

Tissue strips were flash-frozen at time points during the contractile cycle of basal tension, of maximal L-arginine-induced relaxation in the presence and absence of L-NAME and methylene blue and of SNP-induced relaxation in the presence and absence of methylene blue. Maximum relaxation always occurred within 30 sec after SNP addition and within 50 sec after L-arginine addition. Frozen samples were homogenized in 6% trichloroacetic acid in acetone while immersed in a dry ice: methanol slurry. Acetone was removed by lyophilization. Samples were resuspended in water and protein was removed by microcentrifugation. Acid was removed by triplicate extraction with three volumes

of diethyl ether, and residual ether was evaporated by heating at 70^0C for 10 min. Lyophilates were re-suspended in 1 ml phosphate-buffered saline and assayed in duplicate for cGMP by enzyme-linked immunoassay (Cayman Chemical Co) according to the manufacturer directions. Tissue cGMP levels were calculated in pmol/mg protein. The remaining pellet was stored at -80^0C for protein estimation. Frozen pellets from the cGMP extraction were assayed for protein content by the method of Lowry et al.[15] as modified by Markwell et al.[16]. Pellets were resuspended in 1 ml of a buffer containing (in mM): Na_2CO_3 1.9, NaOH 600, sodium tartrate 0.07, sodium lauryl sulphate 0.35, and vortexes to distribute evenly the protein contents. Thirty µl of this suspension was used in the protein determination. Protein in the cGMP-dependent protein kinase soluble fraction was estimated by use of a commercially available assay (Bio-Rad), based on the method of Bradford[17].

Statistical analysis:

All data were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Fisher multiple comparisons were used to detect significant differences among individual means of all groups. Differences with $P<0.05$ were considered statistically significant[18]. Statistical analysis was generated using Stat View software, V 4.53 (Abacus concepts, Inc., USA).

Results

Effect of L-arginine on the spontaneous uterine contractions and on the contractions of uterine strips pretreated with L-NAME:

The uterine strips of non-pregnant rats have shown spontaneous contractions, without any addition of agonists, referred to as "basal contractions" in this study. Table (1) and Figure (1A) demonstrate the concentration-response relationship of the effect of different concentrations of the NO substrate, L-arginine, on the spontaneous contractions of uterine strips. The incubation of the spontaneously contracted uterine strips with logarithmic increments from 100 µM to 10 mM of L-arginine induced a concentration-dependent significant decrease ($P<0.001$) in

the strength of contractions compared to those of the basal contractions. The spontaneous contractions were significantly ($P<0.001$) reduced by 68.3% upon exposure of the uterine strips to the maximal effective concentration of L-arginine (1 mM).

The incubation of the spontaneously contracted uterine strips with L-NAME resulted in a significant increase ($P<0.01$) of the strength of contractions versus those of the basal spontaneous contractions. Addition of L-arginine (1 mM) to the bathing medium of spontaneously contracted uterine strips pretreated with L-NAME showed an insignificant variation in the strength of contractions from the strength of contractions with L-NAME alone (table 2). Incubation of 1 mM of L-arginine with uterine strips in the presence of L-NAME resulted in a significantly less % decrease of the strength of the uterine contractions, when compared to that induced by L-arginine in the absence of L-NAME (figure 2A).

Effect of SNP on the spontaneous uterine contractions:

Table (3) and Figure (1B) demonstrate the concentration-response relationship of the effect of different concentrations of the NO donor SNP (log increment from 10 nM to 1 mM) on the spontaneous contractions of uterine strips. A concentration-dependent significant reduction ($P<0.001$) of the strength of spontaneous isometric uterine contractions from the basal values was observed after incubation with 1 μM to 1 mM of SNP. The spontaneous contractions were significantly ($P<0.001$) reduced by 82.9% upon exposure of the uterine strips to the maximal effective concentration of SNP (100 μM).

Effect of methylene blue on the relaxing effect of L-arginine and SNP upon uterine smooth muscles:

Incubation of the guanylyl cyclase inhibitor, methylene blue, with the spontaneously contracted uterine strips resulted in a significant increase ($P<0.001$) in the strength of the isometric uterine contractions compared to that of the basal contractions (tables 2 and 4). A significant decrease ($P<0.001$) in the strength of isometric uterine contractions was

still observed after addition of L-arginine (1 mM) or SNP (100 µM) to the bathing solution of uterine strips pretreated with methylene blue (tables 2 and 4). The % reduction of the strength of uterine contractions after addition of L-arginine or SNP to uterine strips in the presence of methylene blue was significantly less ($P<0.001$) than that in the absence of methylene blue (figure 2A).

Effect of 8-br-cGMP on the spontaneous uterine contractility:

Table (5) and figure (2B) demonstrate the concentration-response relationship of the effect of the logarithmic increments of the concentration of the cGMP analogue, 8-br-cGMP (from 10 nM to 1 mM) on the spontaneous contractions of rat uterine strips. The incubation of the spontaneously contracted uterine strips with 10 µM to 1 mM of 8-br-cGMP induced a concentration-dependent significant decrease ($P<0.001$) in the strength of contractions compared to the initial strength of basal contractions. The spontaneous contractions were significantly ($P<0.001$) reduced by 43.9% upon exposure of the uterine strips to the maximum effective concentration of 8-br-cGMP (100 µM).

Estimation of cGMP in uterine smooth muscles:

Figure (3 A and B) shows the mean values of cGMP quantity in homogenates of different uterine strips. The mean values of cGMP were found to be significantly higher ($P<0.001$) in the homogenates of the uterine strips incubated with L-arginine (1 mM) or SNP (100 µM), when compared with the basal levels in non-treated strips. A significant decrease ($P<0.01$) in the uterine cGMP quantity was observed after incubation with both L-NAME and methylene blue, when compared with those of the non-treated uterine strips. The addition of L-arginine after pretreatment with L-NAME or methylene blue showed no significant variation in uterine tissue cGMP level, when compared with that in strips incubated with L-NAME or methylene blue alone. Therefore, L-arginine failed to significantly increase uterine tissue cGMP quantities after pretreatment with either L-NAME or methylene blue; the mean values of homogenate cGMP after L-arginine treatment

were significantly much less (P<0.001) in the presence of L-NAME or methylene blue than in their absence (figure 3A). The incubation of SNP with uterine strips pretreated with methylene blue have shown insignificant change of the uterine tissue cGMP production compared to strips treated with methylene blue without addition of the NO donor. Again, addition of SNP after pretreatment with methylene blue failed to increase the uterine tissue cGMP, where their mean values were significantly much less (P<0.001) in presence of methylene blue than in its absence (figure 3B).

Table (1): Effect of incubation of logarithmic increments of concentration of L-arginine with spontaneously contracted rat uterine strips on the strength of the spontaneous uterine contractions (g tension):

	1 µM	10 µM	100 µM	1 mM	10 mM
basal contractions	2.61±0.28	2.57±0.28	2.62±0.25	2.63±0.29	2.62±0.28
after L-arginine addition	2.52±0.26	2.28±0.24	1.69±0.17	0.83±0.88	0.79±0.85
P1 value	> 0.05	> 0.05	< 0.001	< 0.001	< 0.001

Values are presented as means±SD, and ten rat uterine strips were used in each experiment (n=10).

Table (2): Effect of pretreatment of spontaneously active uterine strips with L-NAME or methylene blue (MB) on the inhibitory effect of L-arginine (arg), (1 mM) on the strength of spontaneous uterine contractions (g tension) in non-pregnant rats:

	arg	L-NAME	arg+L-NAME	MB	arg+MB
initial contractions	2.63±0.29	2.71±0.28	3.34±0.30	2.65±0.24	2.97±0.27
after agent addition	0.83±0.88	3.34±0.30	3.22±0.28	2.97±0.27	2.16±0.20
P value	< 0.001	< 0.01	> 0.05	< 0.01	< 0.001

Values are presented as means±SD, and ten rat uterine strips were used in each experiment (n=10).

Table (3): Effect of incubation of logarithmic increments of concentration of sodium nitroprusside (SNP) with spontaneously contracted rat uterine strips on the strength of the spontaneous uterine contractions (g tension):

	10 nM	100 nM	1 µM	10 µM	100 µM	1 mM
basal contractions	2.63±0.28	2.63±0.28	2.61±0.25	2.60±0.29	2.58±0.28	2.57±0.28
after SNP addition	2.53±0.28	2.18±0.23	1.65±0.17	0.99±0.10	0.44±0.05	0.41±0.05
P1 value	> 0.05	> 0.05	< 0.001	< 0.001	< 0.001	< 0.001

Values are presented as means±SD, and ten rat uterine strips were used in each experiment (n=10).

Table (4): Effect of pretreatment of spontaneously active uterine strips with methylene blue (MB) on the sodium nitroprusside (SNP) inhibitory effect on the strength of spontaneous uterine contractions (g tension) in non-pregnant rats:

	SNP	MB	SNP+MB
initial contractions	2.58±0.28	2.64±0.24	2.96±0.28
after agent addition	0.44±0.05	2.96±0.28	1.62±0.17
P value	< 0.001	< 0.01	< 0.001

Values are presented as means±SD, and ten rat uterine strips were used in each experiment (n=10).

Table (5): Effect of incubation of logarithmic increments of concentration of 8-bromo-cyclic guanosine triphosphate (8-br-cGMP) with spontaneously contracted rat uterine strips on the strength of the spontaneous uterine contractions (g tension):

	10 nM	100 nM	1 μM	10 μM	100 μM	1 mM
basal contraction	2.64±0.28	2.64±0.28	2.62±0.25	2.60±0.29	2.59±0.28	2.59±0.28
after 8-br-cGMP addition	2.53±0.29	2.46±0.27	2.34±0.25	2.03±0.22	1.45±0.15	1.43±0.16
P value	> 0.05	> 0.05	> 0.05	< 0.001	< 0.001	< 0.001

Values are presented as means±SD, and ten rat uterine strips were used in each experiment (n=10).

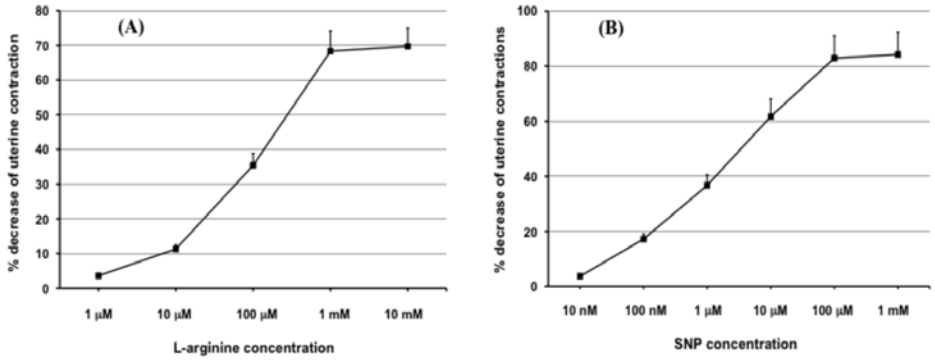

Fig.1: Concentration-response curves of **(A)** L-arginine and **(B)** sodium nitroprusside (SNP) on spontaneous uterine contractions. Logarithmic increments of concentrations of L-arginine (from 1 μM to 10 mM) or SNP (from 10 nM to 1 mM) were added to spontaneously active uterine strips (n=10 for each) in a non-cumulative manner. Results are expressed as a % decrease of spontaneous contractility, and are presented as means± SD.

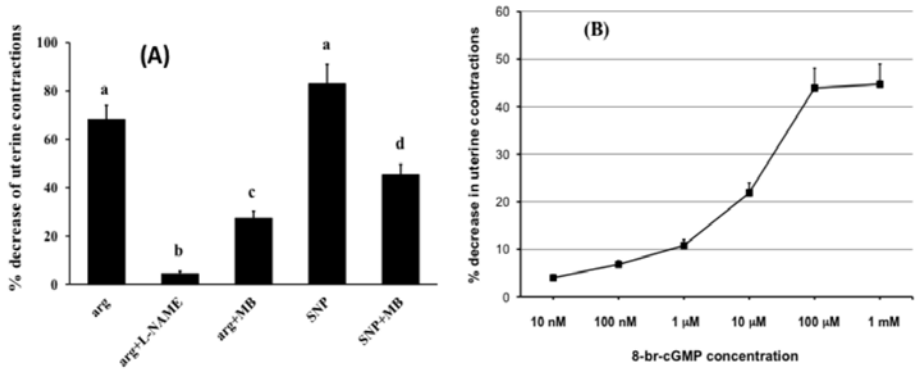

Fig.2: (A): The % decrease of the strength of uterine contractions from the basal values after incubation of uterine strips with L-arginine (1 mM) in the absence of L-NAME or methylene blue (arg), in the presence of 25 µg/ml of L-NAME (arg+L-NAME) and in the presence of 1 µM of methylene blue (arg+MB), as well as with sodium nitroprusside(100 µM) in the absence of methylene blue (SNP) and in the presence of 1 µM of methylene blue (SNP+MB). Results are presented as means± SD. Ten uterine strips were used for each experiment (n=10). Values of columns that carry different letters (a-d) are significantly variant at P<0.001. **(B):** Concentration-response curve of 8-bromocyclic guanosine triphosphate (8-br-cGMP) on spontaneous uterine contractions. Logarithmic increments of concentration of 8-Br-cyclic GMP (from 10 nM to 1 mM) were added to spontaneously active uterine strips (n=10) in a non-cumulative manner. Results are expressed as a % decrease of spontaneous contractility, and are presented as means± SD.

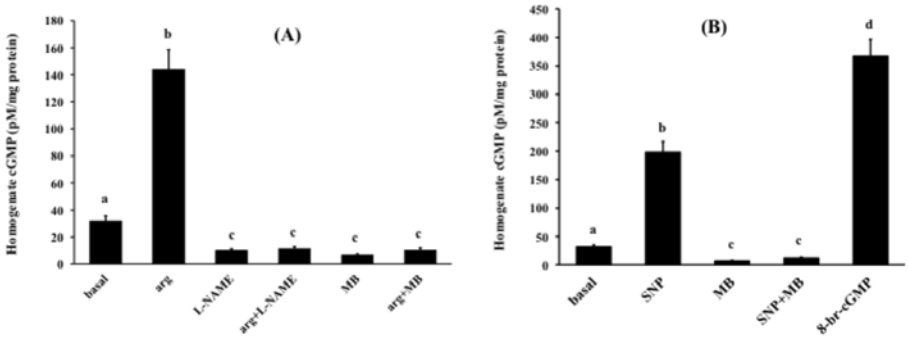

Fig.3: The homogenate cyclic guanosine monophosphate (cGMP) quantity in rat tissues obtained from non-treated uterine strips (basal) and from strips **(A):** incubated with 1 mM of L-arginine in the absence of L-NAME or methylene blue (arg), in the presence of 25 µg/ml of L-NAME (arg+L-NAME) and in the presence of 1 µM of methylene blue (arg+MB), **(B):** incubated with 100 µM of sodium nitroprusside in the absence of methylene blue (SNP) and in the presence of 1 µM of methylene blue (SNP+MB), and incubated with 100 µM of 8-bromocyclic guanosine monophosphate (8-br-cGMP). Results are presented as means± SD. Ten uterine strips were used for each experiment (n=10). Values of columns that carry different letters (a-d) are significantly variant at P<0.001.

Discussion

The present work demonstrates that both the endogenous NO liberated by the NO synthase substrate, L-arginine, and the exogenous NO, donated by SNP, are inhibitors of spontaneous contractions in the non-pregnant rat uterus. These data are consistent with those of Buhimschi et al.[6], who demonstrated that L-arginine decreased uterine spontaneous contractility in human uterus. The relaxant effect of L-arginine has also previously been demonstrated in pregnant myometrium from rats [19]. Interestingly, the NO donor S-nitroso-L-cysteine (CysNO) had no effect on spontaneous contractile activity in guinea pig myometrium, but had a relaxing activity on agonist-evoked contractions[20]. These data suggest the variability of the effect of NO donors, according to the species studied and the experimental conditions.

Our results confirm the presence of a constitutively active NO synthase in the non-pregnant rat myometrium, as the pretreatment of spontaneously active uterine tissues with the NO synthase inhibitor L-NAME actually increased the strength of spontaneous contractions. In fact, compelling evidence for the presence of NO synthase has been provided by biochemical analysis of the enzyme activity in both pregnant and non-pregnant women[21,22]. The exogenous NO donation exhibited as well a relatively high order of potency for relaxation of the uterine tissue[6]. Moreover, the failure of the NO precursor, L-arginine, to produce any significant uterine relaxation in the presence of L-NAME, observed in this work, confirms the role of NO in L-arginine- mediated uterine relaxation.

The main objective of this study was to evaluate the role of the NO/cGMP pathway in the NO-modulation of spontaneous uterine contractions. Addition of L-arginine or SNP to spontaneously contracting uterine strips was found to be associated with significant elevations in the quantity of myometrial tissue cGMP (present study). This result supports earlier findings of other investigators who worked on rat[5], human[6,23] and guinea pig[20].

Our results showed that pre-

treatment with the guanylyl cyclase inhibitor, methylene blue, before addition of L-arginine or SNP, significantly reduced the L-arginine and SNP-induced inhibition of spontaneous uterine contractions. However, contractions were still significantly lower than those of the basal spontaneous contractions in spite of our observation that the uterine tissue homogenate cGMP quantity was significantly diminished below basal levels after pretreatment with methylene blue. These data suggest that L-arginine and SNP inhibited the in-vitro spontaneous contractions of non-pregnant rat uterus, partially through elevation of the tissue cGMP and partially through a cGMP-independent pathway. The presence of a NO/cGMP dependent pathway in the human uterus was first proposed to be responsible for maintaining uterine quiescence during pregnancy by Buhimschi et al.[6]. Recently, Buxton et al.[23] discovered the ability of the guanylyl cyclase type C agonist uroguanylin to relax uterine smooth muscle in a cGMP-dependent manner. However, our findings disagree with the proposal of Yallampalli et al.[5] that elevation in cGMP was exclusively responsible for the reduction in tension observed in NO-treated uterine tissues.

To confirm the participation of the cGMP pathway in the L-arginine and SNP inhibitory effect, the cGMP analogue 8-br-cGMP was incubated with spontaneously contracted rat uterine strips in this study, and the uterine contractility was found to be significantly inhibited compared to the initial contractions. However, the inhibitory effect of the maximal effective concentration of 8-br-cGMP was significantly less than those of L-arginine and SNP, which suggests the involvement of other pathways in the mechanism of their inhibition.

The possible cGMP-independent signaling pathway of NO in the myometrium is not clear, although there have been some studies demonstrating cGMP-independent effects of NO in a variety of other tissues[24,25]. One possible mechanism involves the activation of a calcium-dependent potassium channel by NO[2], as recently, large conductance Ca^{+2}-activated

potassium channels were found to contribute to vascular function in non-pregnant human uterine arteries[26]. Recently, a role of stretch activated potassium currents in the regulation of uterine smooth muscle contractions was suggested by Buxton et al.[27]. Taken into consideration that Wink et al.[28] have found that NO can inhibit cytochrome P450A, a P450-dependent mechanism by which NO could partially relax uterine smooth muscle becomes plausible. Whether these or other mechanisms exist in the myometrium is the subject that needs further investigations.

In conclusion, the present study provides evidence that both L-arginine and SNP inhibit spontaneous uterine contractions in rat non-pregnant uterus through the involvement of both cGMP-dependent and cGMP-independent pathways. These data highlight the need for further experiments on rat uterine preparations for better understanding of the modulation of myometrium contractility.

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ROLE OF THE NO/cGMP PATHWAY IN THE EFFECT OF L-ARGININE AND SODIUM NITROPRUSSIDE ON THE SPONTANEOUS CONTRACTILITY OF RAT NON-PREGNANT UTERUS

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EFFECT OF EPIGALLOCATECHIN-3-GALLATE ONLIVER ISCHEMIA-REPERFUSION INJURY IN RATS

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Abstract

Background: *Epigallocatechin-3-gallate (EGCG), one of the famous flavonoids, comprises about one-third of green tea dry mass. The present work studies the effect of EGCG on hepatic dysfunction and oxidative stress that may associate liver ischemia-reperfusion in rats.* ***Material and Methods:*** *Fifty male albino rats, 150-200 g each, were randomized into 5 equal groups: control (C), EGCG-administered control (EC), sham-operated (S), liver ischemia-reperfusion (I/R) and EGCG-administered liver ischemia-reperfusion (E+I/R) groups. EGCG (40 mg/kg b.w.) was given by oral gavage to rats of EC and E+I/R groups, and other groups were given normal saline (vehicle), once daily for 7 weeks. At the end of experiment, 30 min ischemia followed by 30 min reperfusion of the liver of I/R and E+I/R groups was performed. Thereafter, retro-orbital blood samples were collected from all groups and plasma was separated for estimation of albumin, alanine transaminase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA) and total nitrite/nitrate levels. The liver was then removed from all groups and homogenized for measurement of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and myeloperoxidase (MPO) activities and total glutathione content.* ***Results:*** *In C, EC and S groups, non-significant variations in all the above mentioned parameters were observed. Animals of I/R group showed a decrease of albumin and a rise of ALT, AST, MDA and nitrite/nitrate contents in plasma compared to C, EC and S groups; homogenate SOD and GSH-Px activities and total glutathione contents were decreased, while homogenate MPO activity was elevated. In animals of E+I/R group, plasma albumin was signifi-*

cantly increased, while plasma ALT, AST, MDA and nitrite/nitrate contents were significantly reduced compared to I/R group. Also, homogenate SOD and GSH-Px activities and total glutathione contents were significantly higher, and homogenate MPO activity was significantly lower in E+I/R group than in I/R group. A complete correction of the homogenate SOD and GSH-Px activities was observed in E+I/R group, but other measured parameters were still significantly different from control values. Conclusion: EGCG has a partial protective effect on the oxidative insult and inflammatory processes that associate liver ischemia-reperfusion injury in rats.

Keywords: *epigallocatechen-3-gallate, green tea, liver ischemia-reperfusion, oxidative stress, antioxidant enzymes.*

Introduction

Ischemia-reperfusion injury occurs during the procedure of liver transplantation, liver resection, trauma and other surgical procedures when the liver is transiently deprived of oxygen and subsequently re-oxygenated[1,2]. The cellular damage due to hypoxia and lack of biomechanical stimulus is accentuated upon restoration of oxyge n delivery and shear stress that can be more damaging than the initial ischemia. In fact, reintroduction of blood flow brings oxygen back to the tissues, causing a greater production of free radicals and reactive oxygen species (ROS) that damage cells. The signaling events contributing to local hepatocellular damage are diverse and complex, and involve the interaction between different hepatic cells and neutrophils, macrophages and platelets[3,4]. It has been documented that ischemia-reperfusion injury is associated with excessive ROS production, and the administration of antioxidants such as ascorbic acid or α-tocopherol has been shown to be effective in reducing this hepatic injury[5,6].

Plant-based pharmaceuticals, including flavonoids, have been involved in the management of various diseases. They are essential part of human diet and are present in plant extracts that have been used in oriental medicine. Epidemiological evidences have suggested an inverse relationship between dietary intake of flavo-

noids and the risk of coronary heart disease and certain types of cancers[7,8]. Antioxidant properties and ROS-scavenging effects of the flavonoids could account for their pharmacological activity[9-11]. Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea, comprising about one-third of green tea dry mass[12], to which its pharmacological benefits are referred to. EGCG is present also in black tea, but in smaller quantities[13]. Indeed, EGCG may have therapeutic applications in the treatment of many disorders. It has been suggested that EGCG could attenuate lipid peroxidation [14], being capable of reducing the risk of type 1 diabetes[15], and exerts hypoglycemic and hypolipidemic effects[16]. Previous studies have shown that green tea extract has protective effect on the ischemia-reperfusion-induced brain injury through the inhibition of inflammatory stress-induced neuronal cell death[17,18]. EGCG has been proved to exert neuro-protective activities against neuro-degeneration and inflammatory neuronal injuries[18,19].

Whether EGCG is hepato-protective in ischemia-reperfusion conditions remains to be clarified. In the present study, the liver ischemia-reperfusion injury was induced in rats, and the possible protection provided by EGCG administration was investigated.

Material and Methods

Experimental animals and groups:

The present study followed a randomized controlled animal experiment design. Fifty male albino rats, 150-200 g each, were obtained from the animal house of Physiology Department, Faculty of Medicine, Menofiya University, Egypt. All animals received human care in compliance with the Public Health Service Policy on Human Care and Use of Laboratory Animals published by the National Institutes of Health, and were approved by the Ethical Committee of the Faculty of Medicine, Menofiya University, Egypt. Animals were fed with a standard chow diet, water ad libitum, and they were housed in the animal house of Faculty of Medicine with a 12:12-hour light/dark cycle. The animals were randomly and equally divided into five groups (n=10): (1) control (C) group,

where rats were given 1ml normal saline (vehicle); (2) EGCG-administered control (EC) group, where rats were given 40 mg/kg b.w. of EGCG[20], dissolved in 1 ml normal saline; (3) sham-operated (S) group, where rats where given the vehicle as in C group before exposure to sham operation; (4) liver ischemia-reperfusion (I/R) group, where rats were give the vehicle as in C group before exposure to liver ischemia-reperfusion; (5) EGCG-administered liver ischemia-reperfusion (E+I/R) group, where rats were given EGCG as in EC group before exposure to liver ischemia-reperfusion. EGCG or its vehicle (normal saline) was administered to the corresponding groups by oral gavage, once daily, for 7 weeks.

At the end of the experimental period, rats were fasted overnight, and animals of S, I/R and E+I/R groups were exposed to operation. Thereafter, retro-orbital blood samples were collected, and animals were killed and liver was extracted and homogenized.

Liver ischemia-reperfusion injury:

Animals of I/R and E+I/R groups were exposed to liver ischemia for 30 min, followed by reperfusion for another 30 min. The model of the liver ischemia-reperfusion injury used in this experiment was similar to that described previously by Su et al.[21]. Briefly, rats were initially anesthetized with intramuscular injection of 50 mg/kg b.w. of ketamine hydrochloride (Eipico, Egypt). The animals did not receive ventilatory support. Body temperature was monitored by a rectal probe inserted into the rectum, and were maintained between 37°C and 38°C by a thermal pad and a heating lamp. After a midline abdominal incision, hepatic vessels, including hepatic artery, portal vein and bile duct, were exposed and all vessels were occluded with microsurgical clamps for 30 min. Ischemia was confirmed by blanching of the liver. Thereafter, clamp was released for another 30 min, and reperfusion was visually verified upon the removal of clamps. During the periods of ischemia and reperfusion, the abdomen was closed. Rats of the S group were anesthetized and underwent the same operative procedures as in I/R and E+I/R groups, but without occlusion of the liver vessels.

Blood sampling:

Retro-orbital blood samples (2 ml each) were collected from fasting animals of all groups through non-heparinized capillary tubes. The samples were added to EDTA, centrifuged at 1000 rpm for 15 min for separation of plasma and stored at 4OC for estimation of various biochemical parameters.

Plasma albumin, alanine transaminase (ALT) and aspartate aminotransferase (AST) levels:

Plasma albumin level was determined using specific kits (Sigma-Aldrich, St. Louis, MO, USA). The liver enzymes ALT and AST were determined in plasma by an enzymatic colorimetric method using Randox reagent kits (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer instructions.

Plasma malondialdehyde and total nitrite/nitrate contents:

Lipid peroxidation was assessed by the measurement of the secondary product malondialdehyde (MDA) in plasma after precipitation of protein by addition of trichloroacetic acid then thiobarbituric acid (TBA), which reacted with MDA to form TBA reactive product that was measured at 532 nm. A fresh solution of MDA was made by the hydrolysis of 1,1,3,3-tetramethoxy propane and was used as a standard[22]. The method for estimation of plasma nitrite/nitrate content in plasma was done using a commercial assay kit (Oxford Biomed Research, Inc), based on the Griess reaction [23]. Plasma nitrite/nitrate content was measured after enzymatic conversion of nitrate to nitrite by nitrate reductase in the presence of NADPH. The oxidation of the coenzyme was monitored by the decrease in absorbance at 340 nm [24]. Results are expressed as micromole per liter (μM).

Preparation of liver homogenates:

Rats of all groups were lastly sacrificed by neck elongation and dislocation, and the abdomen of each rat was opened and the rat's whole liver was dissected and excised. The right liver lobe was cut into small pieces, 2-3 mm3, and equal amounts were homogenized in ice-cold buffer 0.1 M phosphate, pH 7.4, 1 mM EDTA, 10 μM indomethacin (Cayma Chemical, Ann

Arbor, MI, USA), using an Omni tissue homogenizer (Omni international, Gainesville, VA, USA). The extracts were centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was stored at -80°C for measurement of hepatic tissue homogenate antioxidant enzymatic activities and total glutathione content. Protein concentration was measured by the bicinchoninic acid (BCA) method to correct for differences between preparations[25].

Superoxide dismutase activity:

The activity of the endogenous antioxidant enzyme superoxide dismutase (SOD) in liver homogenates was assayed following the method developed by Nishikimi et al.[26] and modified by Kakkar et al.[27]. Five µg of protein were mixed with sodium pyrophosphate buffer, phenazine methosulphate (PMT) and nitro blue tetrazolium (NBT). The reaction was started by the addition of NADH. The reaction mixture was incubated for 90 seconds at 30°C. The reaction was then stopped by the addition of 1 ml of glacial acetic acid. The absorbance of the chromogenic substance formed was measured at 560 nm. Results were expressed in U/mg protein, where one unit of SOD activity is defined as the enzyme concentration required for inhibiting chromogenic substance production by 50% in one minute under the assay conditions[27].

Glutathione peroxidase activity:

The activity of the endogenous antioxidant enzyme glutathione peroxidase (GSH-Px) in the liver homogenates was assayed as described by Paglia and Valentine [28], using H_2O_2 and NADPH as substrates. The conversion of NADPH to $NADP^+$ was followed by recording the changes in absorption intensity at 340 nm (Ransel kit, Randox, UK). Results were expressed in U/g protein, where one unit (U) of GSH-Px activity is defined as the amount of the enzyme required to consume 1 nM of NADPH per minute per mg protein[28].

Myeloperoxidase activity:

The myeloperoxidase (MPO) activity in hepatic tissue was determined by the spectrophotometric method. This method uses 3, 3', 5, 5'-tetramethyl benzidine (TMB) as an oxidizable dye, and the reaction was started by adding hydrogen peroxide (H_2O_2) in the medi-

um[29]. The assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Results are expressed as U/mg protein.

Total glutathione assay:

Total glutathione content of equal weights of liver tissue samples was measured according to a modified method by Griffith[30], which was based on conversion of 5,5´-dithiobis-2-nitrobenzoic acid to 5-thio-2-nitrobenzoate by nicotinamide adenine dinucleotide phosphate in the presence of GSH reductase. Formation of 5-thio-2-nitrobenzoate was measured by spectrophotometry at 412 nm and comparing that to a GSH standard curve. Results are expressed as mg/g protein.

Statistical analysis:

The data were expressed as mean ±standard deviation (SD). The data were processed and analyzed using the SPSS version 10.0 (SPSS, Inc., Chicago, Ill., USA). One-way ANOVA was done followed by Tukey's post hoc test. Statistical analysis was performed for each group (n=10) to the other groups within the experiment. The results were considered significant if $p \leq 0.05$.

Results

The presented table demonstrates the mean values of fasting levels of plasma albumin and plasma ALT and AST liver enzymes in various groups. Non-significant variations in the mean values of plasma albumin, ALT and AST were observed in C, EC and S groups. Animals of I/R group showed a significant decrease ($P<0.001$) of plasma albumin and a significant increase ($P<0.001$) of plasma ALT and AST, compared to those of C, EC and S groups. In E+I/R group, a significant improvement was observed in plasma albumin, ALT and AST levels, where plasma albumin was significantly higher ($P<0.001$) and plasma ALT and AST were significantly lower ($P<0.001$) than those in I/R group; however, values did not return to the basal levels, where plasma albumin level was still significantly less ($P<0.001$), and plasma ALT and AST levels were still significantly more than those in C, EC and S groups.

Figure (1) demonstrates the changes of fasting plasma MDA and total nitrite/nitrate contents in different experimental groups of this study. Both EC and S groups

showed an insignificant change in plasma MDA and total nitrite/ nitrate contents, when compared with the corresponding values in C group. In I/R group, both plasma MDA and total nitrite/nitrate contents were significantly increased ($P<0.001$), when compared with the corresponding values of C, EC and S groups. E+I/R group showed a significant reduction ($P<0.001$) of plasma MDA and total nitrite/ nitrate contents compared to those of I/R group; however, both plasma MDA and total nitrite/nitrate contents in E+I/R group were still significantly higher ($P<0.001$) that those in C, EC and S groups.

Figure (2) shows the SOD and GSH-Px activities in liver homogenates of all studied groups. Non-significant variations of the SOD and GSH-Px activities were observed between C, EC and S groups. I/R group showed a significant reduction ($P<0.001$) of the enzymatic activities of both SOD and GSH-Px, when compared with those in C, EC and S groups. E+I/R group showed a significant improvement of the SOD and GSH-Px activities, as their mean values were significantly increased ($P<0.001$) in comparison with those of I/R group. The SOD and GSH-Px activities recovered completely to the normal values by EGCG administration to ischemia-reperfusion rats, as their mean values in E+I/R group were insignificantly changed ($P>0.05$) from those in C, EC and S rats.

Figure (3) illustrates the liver homogenate total glutathione content and MPO activity in various experimental groups. Both EC and S groups showed an insignificant change of liver homogenate glutathione content and MPO activity, when compared with the corresponding values in C group. In I/R group, MPO activity was significantly elevated ($P<0.001$), while glutathione content was significantly decreased ($P<0.001$), when compared with the corresponding values in C, EC and S groups. E+I/R group showed a significant increase ($P<0.001$) of glutathione content and a significant reduction ($P<0.001$) of MPO activity, when compared with those of I/R group. However, glutathione content was still significantly lower ($P<0.001$), while MPO activity was still significantly higher ($P<0.001$) in E+I/R group than in C, EC and S groups.

Table (1): Effect of epigallocatechin-3-gallate (EGCG) on plasma albumin, alanine transaminase (ALT) and aspartate aminotransferase (AST) in control (C), EGCG-administered control (EC), sham-operated (S), liver ischemia-reperfusion (I/R) and EGCG-administered liver ischemia-reperfusion (E+I/R) groups:

Group	Albumin (g/L)	ALT (U/L)	AST (U/L)
C	48.1±3.6[a]	49.02±4.47[a]	133.28±11.94[a]
EC	47.9±3.4[a]	47.97±4.85[a]	132.69±10.97[a]
S	48.4±3.9[a]	51.69±4.66[a]	140.35±12.25[a]
I/R	17.9±1.6[b]	312.28±26.41[b]	337.55±27.74[b]
E+I/R	29.2±2.7[c]	248.11±19.79[c]	239.78±20.04[c]

Results are expressed as mean±SD (n=10). Values of the same column that carry different letters (a-c) are significantly variant at P<0.001.

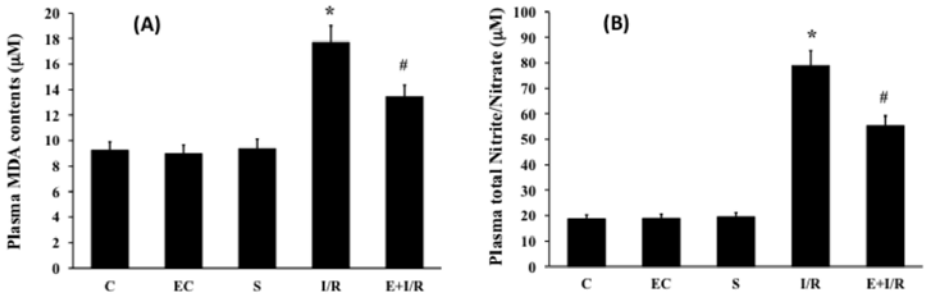

Fig.1: Effect of epigallocatechin-3-gallate (EGCG) on plasma malondialdehyde (MD) and nitrite/nitrate contents in rats. Plasma MDA (panel A) and nitrite/nitrate (panel B) contents in control (C), EGCG-administered control (EC), sham-operated (S), liver ischemia-reperfusion (I/R) and EGCG-administered liver ischemia-reperfusion (E+I/R) groups were assessed as described in Material and Methods. Data are expressed as mean±SD for each group (n=10). The marks "*" and "#"on top of the column indicate that the corresponding group is significantly different from all other groups at P<0.001.

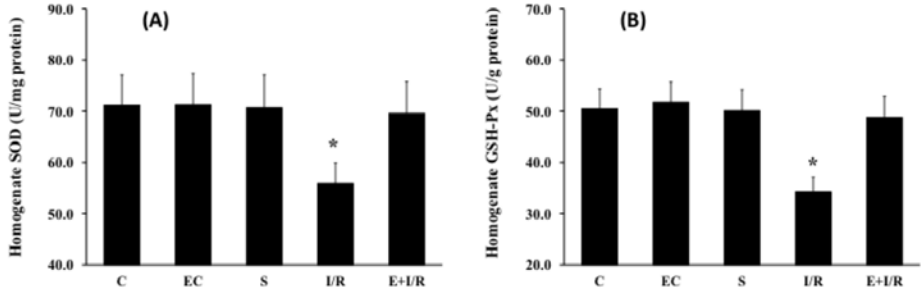

Fig.2: Effect of epigallocatechin-3-gallate (EGCG) on liver homogenate superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in rats. SOD (panel A) and GSH-Px (panel B) activities in control (C), EGCG-administered control (EC), sham-operated (S), liver ischemia-reperfusion (I/R) and EGCG-administered liver ischemia-reperfusion (E+I/R) groups were assessed as described in Material and Methods. Data are expressed as mean±SD for each group (n=10). The mark "*" on top of the column indicates that the corresponding group is significantly different from all other groups at P<0.001.

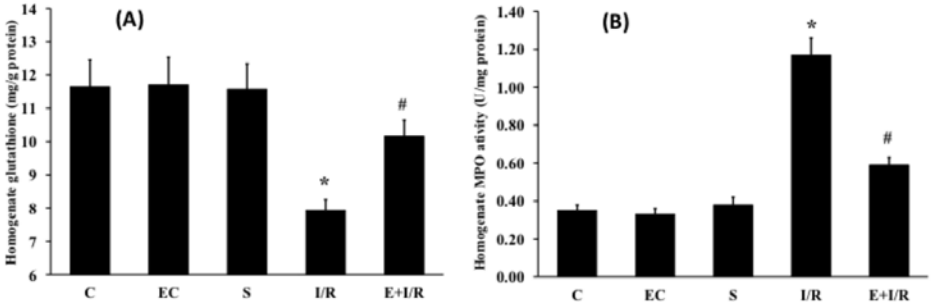

Fig.3: Effect of epigallocatechin-3-gallate (EGCG) on liver homogenate glutathione content and myeloperoxidase (MPO) activity in rats. Glutathione content (panel A) and MPO activity (panel B) in control (C), EGCG-administered control (EC), sham-operated (S), liver ischemia-reperfusion (I/R) and EGCG-administered liver ischemia-reperfusion (E+I/R) groups were assessed as described in Material and Methods. Data are expressed as mean±SD for each group (n=10). The marks "*" and "#" on top of the column indicate that the corresponding group is significantly different from all other groups at P<0.001.

Discussion

Oral administration of EGCG to normal rats resulted in insignificant variations in all the parameters measured in this study, namely plasma albumin, ALT, AST, MDA and total nitrite/nitrate levels and liver homogenate SOD, GSH-PX and MPO activities and total glutathione content. The absence of any significant effect of EGCG administration to normal rats is explained by the presence of an appropriate pro-oxidant antioxidant balance under normal unstressful conditions, with loss of the need for supplementation with other antioxidants beside that normally available.

The development of hepatic injury in our model of liver ischemia-reperfusion in rats was proved, as ischemia-reperfusion of the liver resulted in a significant decrease of the plasma albumin level and a significant increase of the plasma ALT, AST, MDA and total nitrite/nitrate levels versus those in normal rats. Also, liver tissue homogenate SOD and GSH-PX enzymatic activities and total glutathione content were significantly reduced, while homogenate MPO activity was significantly elevated. Su et al.[21] observed the increase of plasma ALT and AST activities and both plasma and hepatic MDA concentrations after liver ischemia-reperfusion in rats. The enhanced lipid peroxidation in liver ischemia-reperfusion is evidenced in our study by the rise in plasma MDA content[31]. In fact, overproduction of ROS and oxygen free radicals in ischemia-reperfusion conditions may also lead to excessive lipid peroxidation, and protein and DNA damage. The DNA damage secondary to the oxidative stress is accompanied by nuclear fragmentation, a phenomenon characterizing the apoptotic type of cell death[32]. The increased total nitrite/nitrate observed in this study is explained by accumulation of superoxide during the reperfusion phase following ischemia, and its rapid reaction with NO to form peroxynitrate[33]. In consistency, previous studies demonstrated the post-ischemic nitrosative damage in liver[34], heart [35], brain[36] and lung[37]. The reduction in the activities of the endogenous antioxidant enzymes and total glutathione content after ischemia-reperfusion injury, ob-

served in this study, is in agreement with many research workers. Kizilgun et al.[38] demonstrated a decrease in the SOD and GSH-Px activities in renal ischemia-reperfusion. Turrens et al.[39] has shown that myocardial mitochondrial Manganese-SOD activity was decreased after ischemia. In temporary testicular ischemia, oxidative stress with a sudden induction of lipid peroxidation and a concomitant suppression of endogenous antioxidant activities, including those of SOD and GSH-Px, was observed by Unsal et al.[40]. Recently, a decreased SOD and GSH-Px enzymatic activities in the liver of rats exposed to ischemia-reperfusion was also observed by Su et al.[21].

The MPO activity is considered a biomarker of inflammation that estimates neutrophil infiltration in inflamed tissues[41]. The elevation of MPO activity, after induction of liver ischemia-reperfusion injury in our experiment, is in agreement with Sims and Muyderman[42], who found that the restored blood flow exaggerates the inflammation response of damaged tissues, causing neutrophil infiltration that destroys damaged hepatic cells. Moreover, Kulmacz and Wang[43] suggested a creation of a vicious cycle between oxidative stress and inflammatory mediators. In fact, Free radicals, particularly reactive oxygen and nitrogen intermediates, are thought to be involved in the inflammatory processes, exacerbating inflammation, and causing tissue damage[44]. In contrast to our findings, Portakal and Inal-Erden[45] reported an increase of SOD and GSH-Px activities during ischemia reperfusion of the liver, as compared with controls. This contradictory result may be explained by different techniques of ischemia-reperfusion injury with a resultant different rates of production of ROS, where in the present work, the extent of the possible compensatory mechanisms of the antioxidant enzymes synthesis to face the oxidative insult was less than that of their depletion, with a subsequent final reduction in their measured enzymatic activities in liver homogenates.

The main finding in our study is that chronic EGCG administration, at a dose of 40 mg/Kg b.w. for 7 weeks, partially improves the

liver dysfunction and oxidative stress associated with ischemia-reperfusion, as demonstrated by a significant increase of plasma albumin level and a significant decrease of the plasma ALT, AST, MDA and total nitrite/nitrate contents, compared to animals exposed to liver ischemia-reperfusion without pretreatment; however, values did not return to the basal values measured in normal control rats. A complete correction of the enzymatic activities of SOD and GHS-Px in liver homogenates of ischemia-reperfusion rats after pretreatment with EGCG was observed in our study, however, the improved homogenate total glutathione content and MPO activity did not return to normal values.

The beneficial effect of EGCG in this research could be attributed to the attenuation of oxidative stress in liver ischemia-reperfusion rats. In consistency, numerous studies have shown that green tea flavonoids have antioxidant and iron chelating activities and thus, can prevent and/or reduce the deleterious effects of oxygen-derived free radicals associated with various chronic diseases[46,47]. Kondo et al.[48] demonstrated a scavenging effect of EGCG on peroxyl radicals. Guo et al.[14] described a protective mechanism of EGCG against lipid peroxidation in synaptosomes. Although the mechanisms of EGCG's antioxidant activity still remain unclear, pharmacological studies have identified several antioxidant properties such as blockade of constitutional and induced nitric oxide synthases[14,49,50] and scavenging of free radicals or attenuation of lipid peroxidation[51,52].

In conclusion, oral administration of EGCG, the main active ingredient in green tea, could attenuate the liver I/R injury in rats, and this may be of potential benefit during surgical procedures such as hepatic resection and liver transplantation.

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EFFECT OF EPIGALLOCATECHIN-3-GALLATE ONLIVER ISCHEMIA-REPERFUSION INJURY IN RATS

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COULD RESVERATROL AND COLCHICINE REVERSE LIVER FIBROSIS INDUCED EXPERIMENTALLY BY CCL_4 IN RATS?

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Abstract

Liver fibrosis is the result of chronic liver injury, and it represents a widespread medical problem. In this study, we aimed to investigate the potential antifibrotic effects of natural an antioxidant anti-inflammatory drug (resveratrol), and a known anti-inflammatory drug (colchicine) on already established liver fibrosis induced by one of the commonest hepatotoxic drugs (CCL4). The study was carried out in 50 rats weighing 150-200 g subdivided into five groups each of which consisted of 10 rats. Group1: Rats were given olive oil intragastric and served as control group. Group2: Rats were given CCL4 in a dose of 0.4gm/kg body weight three times a week, for 8 weeks. Group3: Received resveratrol received resveratrol intragastric suspended in 0.7% of carboxymethyl cellulose in a dose of 100mg/kg body weight 8 weeks after carbon tetra chloride injection and together with it for further 2 weeks. Group4: received colchicine intragastric suspended in 0.7% of carboxymethyl cellulose in a dose of (100mg/kg body weight) 8 weeks after carbon tetra chloride injection and together with it for further 2 weeks. Group5: Received both resveratrol and colchicine for the same period. At the end of experiment, animals were kept for overnight fasting and sacrificed by bilateral decapitation. Liver sections were collected and prepared for histopathology and for estimation of maondialdehyde, glutathione peroxidase and hydroxyproline content. Serum were collected for AST, ALT, TGF-β1, TNF-α and IL6.

The study showed that resveratrol but not colchicne could ameliorate liver fibrosis induced by CCL4 as represented by improvement of serum transaminases, inflammatory markers, fibrosis markers and antioxidant parameters together with improvement in histopatholgical features of liver fibrosis.

Introduction

Chronic liver diseases are major global health problems causing approximately 800,000 deaths per year worldwide[1]. Liver fibrosis is the common pathologic process of all chronic liver diseases, regardless of the cause, which results from excessive accumulation of extracellular matrix[2].

Regardless the initial cause of injury, fibrosis represents the final common pathway of chronic hepatic inflammation. The traditional view of liver disease as an irreversible process is obsolete and it is now evident that the development of liver fibrosis is a dynamic and potentially bidirectional process[3]. There is overwhelming evidence that activated hepatic stellate cells (HSC, Ito, fat storing cell, or lipocyte) are the major producers of the fibrotic neomatrix[4,5]. HSCs normally reside in the perisinusoidal space where their principal function is the storage of retinoids[6]. Following chronic liver injury, HSC proliferate, lose their vitamin A and undergo a major phenotypical transformation to smooth muscle -actin positive myofibroblasts (activated HSC) which produce a wide variety of collagenous and non-collagenous ECM proteins. Cirrhotic liver contains approximately six times more ECM overall than normal liver, and in the space of Disse collagen types III and V and fibronectin accumulate in early injury. Activated HSCs up-regulate the expression of potent inhibitors of metalloproteinases (TIMPs)[7].

Carbon tetrachloride (CCl4), has been considered as one of the best characterized experimental model for chemically induced rat liver damage. CCl4 is a classical hepatotoxicant causing liver cirrhosis, fibrosis, and necrosis by producing highly reactive trichloromethyl free radical, initiating lipid peroxidation and causing centrilobular necrosis. High levels of reactive oxygen species (ROS) induce cell damage and are involved in several human pathologies, including liver cirrhosis and fibrosis[8,9]. At the molecular level, a series of studies have shown that oxidative stress is commonly induced in all forms of chronic liver injury and plays a crucial role in hepatic fibrogenesis[10,11]. Therefore, the use of compounds

with antioxidant properties may prevent or alleviate many diseases associated with ROS.

Experimental models have confirmed the possibility of reversion of established liver fibrosis[12]. Resveratrol has been shown to exert antioxidant effects in experimental models of liver injury induced by ischemia/perfusion and in ethanol by induction of enzymatic activity of super oxide dismutase and catalase and to attenuate fibrosis development when co-adminstered with CCL4 to rats[13].

Rats injured with carbon tetrachloride for 4 to 8 weeks develop fibrosis, but after cessation of the injury are able to revert to virtually normal morphology. On the other hand, when the injury is protracted for longer time, (12 weeks) cirrhosis develops and in the absence of ongoing injury there is only partial reversal with remodeling of a micro-nodular to a macronodular architecture[14]. Colchicine is an anti-inflammatory and anti-fibrotic medication. Several randomized clinical trials have addressed the question whether colchicine has any efficacy in patients with alcoholic as well as non-alcoholic fibrosis and cirrhosis.

Materials and Methods

Fifty rats obtained from a local animal house, each weighing 150-200 gram were used throughout the experiment. They were kept under similar housing condition and offered food and water ad-Lib. They were subdivided into five groups each of 10 rats as follow:

Group 1: received olive oil orally and served as normal control group.

Group2: received carbon tetrachloride intraperitoneal (2ml/kg, 20% v/v in olive oil) twice a week for 8 weeks and served as CCL4 control group[15].

Group3: received resveratrol intragastric suspended in 0.7% of carboxymethyl cellulose. in a dose of 100mg/kg body weight[16]. 8 weeks after carbon tetra chloride injection and together with it for further 2 weeks.

Group 4: received colchicine intragastric suspended in 0.7% of carboxymethyl cellulose in a dose of (100mg/kg body weight) 8

weeks after carbon tetra chloride injection and together with it for further 2 weeks.

Group5; received resveratrol and colchicine intragastric 8 weeks after carbon tetra chloride injection and together with it for further 2 weeks.

At the end of experiment, animals were kept for overnight fasting and sacrificed by bilateral decapitation. Liver sections were collected and then divided into two portions, one for histopathology where liver slices were cut in equal parts, fixed in 10% formaldehyde and embedded in paraffin, the 5μm thick sections were staines with hematoxylin and eosin (H&E) before observation under light microscope. The otherpart of liver sections were homogenized and prepared for estimation of Liver malondialdehyde, glutathione peroxidase, transforming growth factor-β (TGF-β1) and hydroxyproline content.

Blood samples were collected from all studied groups and lifted for clotting at 37˚C and then centrifuged for separation of plasma for estimation of aspartate transaminase (AST), alanine transaminase (ALT), tumor growth factor β (TGF-β1), TNF-α, IL6

Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with routine laboratory methods using an autoanalyzer. Serum hyaluronic acid (HA) was determined by radioimmunoassay, hydroxyproline (HYP) content in liver, malondialdehyde (MDA) and glutathione peroxidase (GPx) levels in liver homogenates were assayed by spectrophotometry. Tumor necrosis factor-alpha (TNF-alpha), IL and transforming growth factor-beta1 (TGF-beta1) levels were determined by ELISA.

Statistical analysis: Results were expressed by mean ± SEM. Statistical significance of difference between groups was determined by ANOVA followed by t-test. $p<0.05$ was considered significant.

Reults

1- Biochemistry findings:

a- Administration of CCL4 three times a week for 8 weeks,

induced significant (P<0.05) increase in serum AST, ALT, hyauronic acid, IL-6 and TNF-α when compared to normal control group (Table1, Fig.1, 2, 3, 4, 5).

b- Administration of resveratrol alone or in combination with colchicine 8 weeks after carbon tetra chloride injection and together with it for further 2 weeks, produced significant (P<0.05) decrease in serum AST, ALT, hyauronic acid, IL-6 and TNF-α when compared to ccl4 treated group (Table1, Fig.1, 2, 3, 4, 5).

c- Administration of colchicne 8 weeks after carbon tetra chloride injection and together with it for further 2 weeks, produced insignificant (P>0.05) changes in serum AST, ALT, hyauronic acid, IL-6 and TNF-α when compared to ccl4 treated group (Table1, Fig.1, 2, 3, 4, 5).

11- Tissue homogenization findings:

A- Administration of CCL4 induced significant (P<0.05) increase in hepatic malodialdehyde, hydroxyproline contents and TGF-B1) while produced significant ((P<0.05) decrease in hepatic glutathione peroxidase when compared to normal control group (Table2, Fig.6, 7, 8, 9).

b- Administration of resveratrol either alone or in combination with colchicne produced significant (P<0.05) decrease in hepatic malodialdehyde, hydroxyproline contents and TGF-B1) while produced significant ((P<0.05) increase in hepatic glutathione peroxidase when compared to ccl4 treated group (Table2, Fig.6, 7, 8, 9).

c- Administration of colchicne produced insignificant (P>0.05) changes in hepatic malodialdehyde, hydroxyproline contents, TGF-B1 and hepatic glutathione peroxidase when compared to ccl4 treated group (Table2, Fig.6, 7, 8, 9).

111- Histopatholgical findings:

a- Histological findings of normal control group clearly showed that the normal architecture of liver with normal hepatocytes, normal sinusoids and normal central vein (Figure10).

b- Histological findings of ccl4 treated group showed that the normal architecture of the hepatic tissue was altered due to administration of ccl4 causing degeneration in hepatocytes, extensive centrilobular necrosis around the central vein of liver (Figure 11).

c- Administration of resveratrol either alone or together with colchicne showed noticeable improvement in histopathological parameters. There was no sign of necrotic region and dilation of central vein (Figure12).

d- Administration of colchicne alone produced histopathological findings more or less similar to that introduced by ccl4 (Figure 13).

Table (1): Effects of resveratrol and colchicine on serum parameters in rat with CCL4 induced liver fibrosis.

Groups	AST (U/L)	ALT (U/L)	Hyaluronic acid (ng/l)	IL-6 (pg/ml)	TNF- α (pg/ml)	(TGF-β1) µg / l
Control	42.2±6.5	48.4±12.7	75.5±10.5	60.4±3.5	12	90.5±8.4
CCL4	215.3±16.4*	200.4±15.9*	590±30.8*	580.6±22.2*	45±4.3*	850.8±42,4*
Resveratrol	72±9#	90.2±10.2#	190±15.1#	220.5±15.3#	20.2±2.5#	240.2±25.7#
Colchicne	220±13.1	203.8±16.4	583±28.2	601±19.5	43.8±7.6	860.5±38.3
Resveratrol+ Colchicne	75.3±5.5#	87.5±7.4#	200.6±10.4#	200±10#	19.1±1.7#	250.7±31.3#

Each value represents Mean ± SEM
*** Significant when compared to control group (P<0.05).**
 # Significant when compared to CCL4 group (P<0.05).

Table (2): Effects of resveratrol and colchicine on liver parameters in rat with CCL4 induced liver fibrosis.

Groups	Hepatic malondialdehyde (nmole/g liver) tissue)	Glutathione peroxidase (u/gm tissue)	Hydroxyproline content (µg/mg liver tissue)
Normal control	270.8±7.5	80.5±13	2.2±0.4
CCL4	1400±48*	10.2±1.1*	10.2±0.7*
Resveratrol	580±31#	60.5±5.5#	5.3±0.3#
Colchicne	1390±51	11.5±0.8	10.5±0.4
Resveratrol+ Colchicne	570±43#	65.6±3.2#	5.1±0.2#

Each value represents Mean ± SEM
*** Significant when compared to control group (P<0.05).**
 # Significant when compared to CCL4 group (P<0.05).

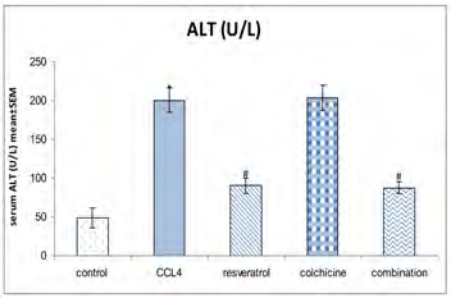

Fig. 1: Effects of resveratrol and colchicine on serum ALT in rat with CCL4 induced liver fibrosis.

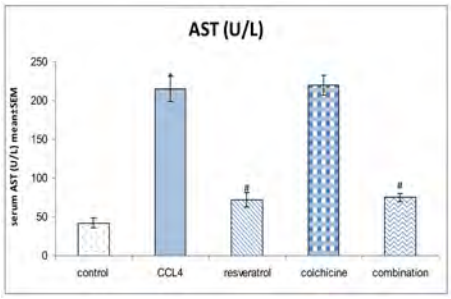

Fig. 2: Effects of resveratrol and colchicine on serum AST in rat with CCL4 induced liver fibrosis.

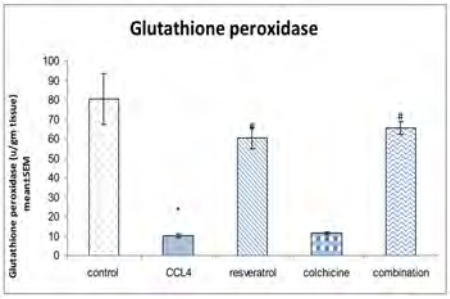

Fig. 3: Effects of resveratrol and colchicine on glutathione peroxidase (u/gm) in rat with CCL4 induced liver fibrosis.

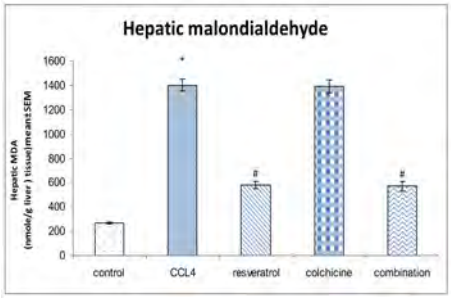

Fig. 4: Effects of resveratrol and colchicine on hepatic MDA(nmol/g liver tissue) in rat with CCL4 induced liver fibrosis.

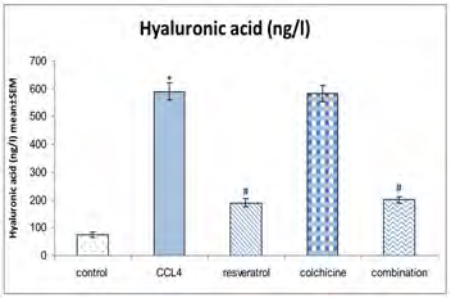

Fig. 5: Effects of resveratrol and colchicine on hyaluronic acid (ng/l) in rat with CCL4 induced liver fibrosis.

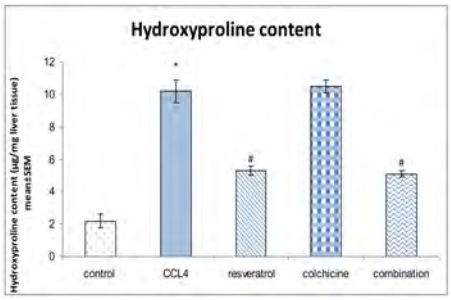

Fig. 6: Effects of resveratrol and colchicine on hydroxyprolinel content (µg/ng) in rat with CCL4 induced liver fibrosis.

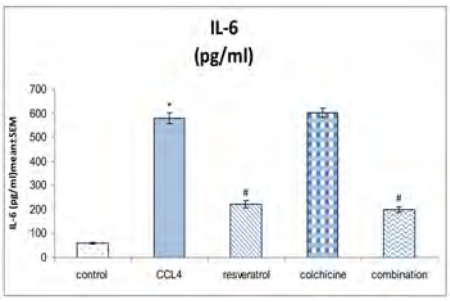

Fig. 7: Effects of resveratrol and colchicine on IL-6(pg/ml) in rats with CCL4 induced liver fibrosis.

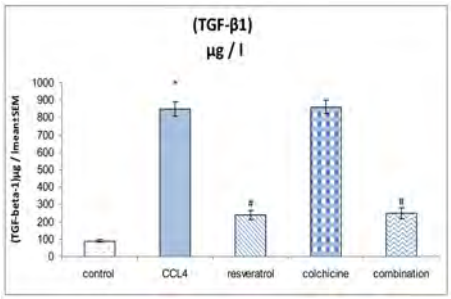

Fig. 8: Effects of resveratrol and colchicine on TGF-β-1(µg/l) in rat with CCL4 induced liver fibrosis.

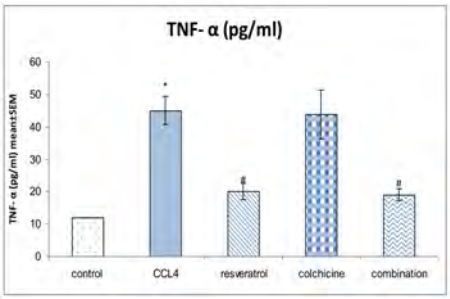

Fig. 9: Effects of resveratrol and colchicine on TNF-α (PG/ML) in rat with CCL4 induced liver fibrosis.

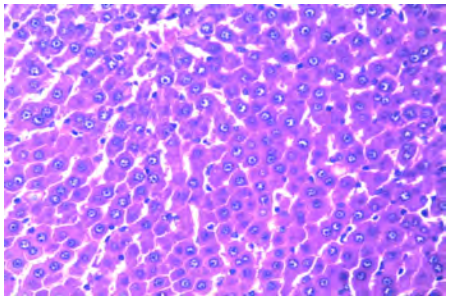

Fig. 10: groupI (received olive oil) normal liver architecture and hepatocytes (H.&E.x400).

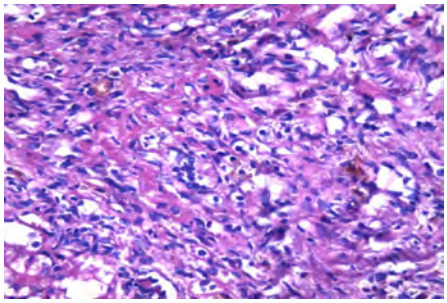

Fig. 11: group II(received ccl4) showing loss of liver architecture, destruction of liver cells, diffuse infilteration of mono nuclear cells, fibroblast, lymphocytes with cholestasis(Arrow) (H.&E.x400).

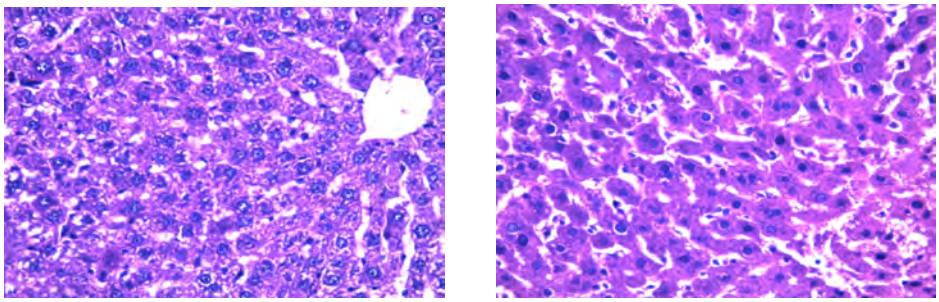

Fig. 12: groupe111,V(received resveratrol alone or with colchicine) showed noticeable improvement in histopathological parameters. There was no sign of necrotic region and dilation of central vein.

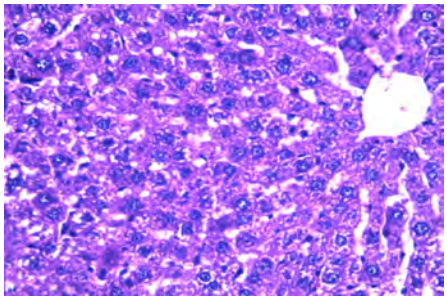

Fig. 13: group V (rceived colchicine and ccl4) showing mild diffuse vacuolar changes in hepatocytes with mild to moderate dilated centeral viens and blood sinusoids with hypertrophy of kupffers cells (H.&E.x400).

Discussion

CCL4 is widely used to induce hepatic fibrosis and cirrhosis in animal models. In this study, hepatic fibrosis was successfully induced by intraperitoneal injection of CCL4 in a dose of (0.4 gm/kg body weight) three times a week, for 8 weeks. Through this hepatic model of fibrosis, the effects of resveratrol and colchicine on hepatic fibrosis induced by ccl4in rats were examined.

It is well known that oxidative damage can induce hepatic fibrogenesis[17]. Moreover, ROS, such as H202, O2 and OH, are implicated in the development and progress of hepatic fibrosis[18]. Oxidative stress leads to lipid peroxidation, impaired mitochondrial

and peroxisomal oxidation of fatty acids, and cytokine release[19]. Lipid peroxidation accelerates collagen synthesis by stimulating stellate cells[20].

In the present study, administration of carbon tetra chloride induced significant increase in serum transaminases level. This is in accordance with several studies [15,21,22]. The elevation of serum transaminases induced by ccl4 could be attributed to the release of these enzymes from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage[23]. Regarding to the effects of ccl4 on inflammatory serum markers, it was founded that, ccl4 induced significant elevation of TNF-_ and IL-6. This is in accordance with other clinical studies[24-26]. They postulated that IL-6 is a multifunctional cytokine along with TNF-_ activates hepatic stellate cells and increases the production of extracellular matrix proteins that finally leads to cirrhosis.

In the present study administration of ccl4 induced increase in serum fibrotic and liver markers represented by elevation of serum hyaluronidase TGF-_1 and hydroxyproline liver content This is in agreement with previous studies who stated that ccl4 markedly increase TGF-β and also increase hydroxyproline contents of liver up to five times when compared to control group[27,28].

Results of the present study revealed that exposure of rats to CCl4 resulted in increase lipid peroxide level manifested by increase in MDA level together with decrease in glutathione peroxidaes level. This s in accordance with large scale studies[25,29-31]. The hepatotoxic effect of CCl4 is reported to be initiated as a result of its reductive dehalogenation by hepatic cytochrome P450 2E1 forming highly reactive trichloromethyl radical (CCl3). In the presence of oxygen CCl3 gets converted into a trichloromethyl peroxyl radical (CCl3OO).These metabolites of CCl4 in turn results in hepatic oxidative stress, severe damage to mitochondria and nuclei, hepatic fibrosis lead to necrotic cell death[32]. Abstraction of hydrogen atoms from poly unsaturated fatty acids of cell membrane

by CCl3 and CCl3OO initiates the process of lipid peroxidation (LPO) Further, CCl3 is also capable of reacting with the sulfhydryl group of reduced glutathione (GSH) resulting in abnormal function [33,34].

HA known to be good serum markers of hepatic fibrogenesis[35] HYP in liver is an important index reflecting the degree of hepatic fibrosis and hepatic fibrosis can be quantified by measuring hepatic hydroxyproline[36].

In the present study, administration of resveatrol induced significant reduction of serum transaminases levels together with significant decease in inflammatory markers, TNF-α, TGF-β1 and IL-6. This in accordance with [13,26,28,37]. They stated that resveratrol decreases the immunopositivity of TNF-α and IL-6 that were profoundly expressed in experimental rats injected with ccl4, and restored the altered architectural structure of challenged hepatic tissue by suppressing oxidative stress and apoptosis.

Administration of resveratrol in the present work significantly decreased serum fibrotic and liver markers represented by reduction of serum hyaluronidase TGF-β1 and hydroxyproline liver content This is in agreement with previous study stated that resveratrol markedly decrease TGF-β and hydroxyproline contents of liver. They interpretated the strong antifibrotic effect of resveratrol, by it ability to reduce NF-kappaB activation and TGF-beta content[27].

Colchicne administration to rats produced insignificant effects on serum transaminases, inflammatory markers, lipid peroxide level or serum and liver fibrotic markers

Cedillo et al., (1996)[38] have shown that colchicine is capable of preventing CCl4 induced cirrhosis in the rat but colchicine was unable to reverse fibrosis significantly when compared with ccl4 group. It is possible that colchicne acts by inhibiting the mechanism of toxicity of the etiological agent. For example there is evidence that colchicine inhibits CYP450IIE1[39], responsible for the bioactivation of CCl4[40] and that colchicine prevents lipid peroxidation induced

by CCl4[38] and BDL[40]. These sorts of mechanisms, very useful to prevent damage[40] are irrelevant when damage is established and the toxic stimuli removed.

Although liver fibrosis in rats is reversible, the implications for recovery from cirrhosis in humans remain to be clarified.

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COULD RESVERATROL AND COLCHICINE REVERSE LIVER FIBROSIS INDUCED EXPERIMENTALLY BY CCl_4 IN RATS?

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PREVALENCE OF OBESITY AND ASSESSMENT OF OBESITY COMPLICATION IN PRIMARY SCHOOL CHILDREN IN MENOUFYIA GOVERNORATE (BERKET EL-SABEA DISTRICT)

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Abstract

Objective: *Determination the prevalence Of Obesity and assessment of obesity complication in primary school children in Menoufyia Governorate (Berket El-Sabae District).*

Background: *Childhood obesity is one of the major public health problems in many industrialized and developing countries of the world in addition to that it is considered as the most important nutritional disorder.*

Methods: *A cross sectional study was carried out on 3500 Egyptian children (1654 males & 1846 females) to calculate prevalence of overweight & obesity among primary school children at age of 6-12 year live in Berket EL-Sabae district(January 2013 - January 2014). Two schools were randomly selected from rural areas & 3 schools from urban areas including male & female children. An approval from Menoufyia faculty of medicine ethical committee was taken before beginning of the study. An approval from the manger of each school authorities in the education ministry. Informed consent was signed by the child &.his parent.*

Results: *The prevalence of obesity among studied group was 8.4%.*

- The prevalence of overweight among studied group was 16.8%.

Conclusion: *The prevalence of obesity among studied group was 8.4% among children aged 6 to 11 years is 8.4% (in Berket El-Sabea District).*

Key Words: primary, *complication, children, obesity, prevalence.*

Introduction

An obese child: is defined as one with body mass index (BMI) more than 2SD of the reference median, An overweight child is defined as one with BMI more than 1SD of the reference median, Child with thinness is defined as one with BMI less than -2SD of the reference line and Child with severe thinness is defined as one with BMI less than -3SD of the reference line.[1]

The causes of obesity are multifactorial and include genetic predisposition, sedentary life style, over eating, fast food diet, and lack of adequate nutritional education, school environment and advertising and marketing of unhealthy foods[2].

We must encourage children to engage in health promoting behaviors while also protecting their positive feelings about their bodies, promoting dietary changes in school or community policies may be less likely to make individual children feel stigmatized or punished[3].

The aims of this study were determination of the prevalence of obesity and assessment of the effect of obesity on blood lipids and hemoglobin percentage in primary school children in Menoufyia Governorate (Berket El-Sabae District).

Methods

A cross sectional study was carried out on 3500 Egyptian children (1654 males & 1846 females) to calculate prevalence of overweight & obesity among primary school children at age of 6-12 year live in Berket EL-Sabae district (January 2013 - January 2014). Two schools were randomly selected from rural areas & 3 schools from urban areas including male &female children. An approval from Menoufyia faculty of medicine ethical committee was taken before beginning of the study. An approval from the manger of each school & authorities in the education ministry. Informed consent was signed by the child & His parent.

Inclusion criteria:

Children aged 6-12 years, both sexes and live in Berket El-Sabae district.

Exclusion criteria:

- Children below the age of 6 years old.
- Children above the age of 12 years old.
- Children with any chronic disease.

All cases were subjected to the following:

I. personal history taking with special emphasis on:

1- Personal data: name, age, sex, and residence.

2- Assessment of socioeconomic standard of family with questions about the father's education and occupation, mother's education and occupation and family income according to (Ibrahim and Abdel-Ghaffar 1990) socioeconomic scoring system.

II. Physical examination: to exclude students with chronic diseases

Anthropometric measurements: measuring weight & height and calculating BMI compared on growth chart of children of the same age and sex.

A- Weight:

Weight was measured on a digital electronic scale. The-scale was set to zero before the patient was placed on the scale and was checked weekly with known calibration weights. Weight measurement was taken with the child wearing littler or no outer clothes and shoes. The weight was approximated to nearest 100 gm[5].

B- Height:

Height was measured by a tape measure permanently fixed to a wall or door frame, the head was held firmly at the top of the board.

C- Body mass index:

BMI is a ratio between weights measured in Kilograms to squared height measured in meters through the following equation:

B.M.I = weight in kg / height in m2

Statistical method Statistical analysis:

Collected data were complied; coded and verified then analysis was performed using SPSS v. 18 software for Microsoft Windows 7.

Data were expressed in terms of standard deviation scores (z-scores)

using WHO Anthro Plus software provided by WHO website, the Z-score- cut-off points recommended by WHO were used[1].

A Z score or standard deviation score is the difference between the actual measurement and the median of age and sex matched reference population divided by the standard deviationof the reference population.

Z score= Individual's Value-median value of reference population.

Blood pressure measurement by using blood pressure charts, and all of children were normotensive.

Laboratory Investigations:

- Laboratory investigations were done for 100 obese students and 100 controls.

Blood sample was taken from the child under a septic precaution and used to estimate:

- Total cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL).
- Hemoglobin percent.

Specimens collection and preparation:

1. Blood specimens were obtained and the usual precautions in the collection of venipuncture samples were followed strictly.
2. For accurate comparison to established normal values, fasting morning serum samples were obtained.
3. The blood was collected in a plain redtop venipuncture tube with edita(2ml).
4. Quantitative determination of hemoglobin; specimens were centrifuged to separate the serum or plasma from the cells for determination of lipid profile and blood glucose. Samples were refrigerated and stored at temperatures of -20˚C.

Spectrophotometer data:

Erba Mannheim, model chem-7, Dc input 18v/24v, 100w.

SR. No 7208. Germany.

Results

This study included 3500 primary school children in Menoufyia governorate - Berket El-sabae District, there age ranged from 6-12 years. Table (1)

The prevalence of obesity in 3500 children is 8.4%. The preva-

lence of overweight in 3500 children is 16.8 %. Over weight and obesity were relatively high among age group6- < 9 year. Obesity was more common in female than male. Obesity was more common in urban than rural. Children of high socioeconomic level more obese than other children. Table (2)

From the study of 100 obese students and 100 controls.

The following results were found:

33% of the obese children had high (Cholesterol level). 25% of the obese children had high (Triglyceride level). 4% of the obese children had high (Low density Lipoprotein). 35% of the obese children had high (High density Lipoprotein). 100% of the obese children had normal (Hemoglobin level). Table (3)

Table (1): General descriptive data of studied sample.

		No.	%
Age in (years)	6 -	527	15.1%
	7.00	508	14.5%
	8.00	595	17.0%
	9.00	610	17.4%
	10.00	655	18.7%
	11.00	605	17.3%
Sex	male	1654	47.3%
	female	1846	52.7%
Residence	rural	877	25.1%
	urban	2623	74.9%
School	public	2742	78.3%
	private	758	21.7%
Socioeconomic standard	Medium	1906	54.5%
	Low	714	20.4%
	Very low	70	2.0%
	High	702	20.1%
	Very High	108	3.1%
	Total	3500	100.0%

Table (2): Comparison between different demographic factors regarding BMI z-score classes.

		BMI z-score class												X^2	p-value
		< 3SD		< 2SD		Normal		>1SD		>2SD		>3SD			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%		
Sex	male	8	0.5%	84	5.1%	1153	69.7%	300	18.1%	77	4.7%	32	1.9%	41.63	<0.001**
	female	5	0.3%	110	6.0%	1259	68.2%	288	15.6%	153	8.3%	31	1.7%		
Residence	rural	11	1.3%	40	4.6%	666	75.9%	115	13.1%	35	4.0%	10	1.1%	52.16	<0.001**
	urban	2	0.1%	154	5.9%	1746	66.6%	473	18.0%	195	7.4%	53	2.0%		
School	public	10	0.4%	40	1.5%	2117	77.2%	387	14.1%	152	5.5%	36	1.3%	511.7	<0.001**
	private	3	0.4%	154	20.3%	295	38.9%	201	26.5%	78	10.3%	27	3.6%		
Socioeconomic standard	Medium	9	0.5%	154	8.1%	1708	89.6%	3	0.2%	2	0.1%	30	1.6%	1961	<0.001**
	Low	0	0.0%	37	5.2%	419	58.7%	192	26.9%	54	7.6%	12	1.7%		
	Very low	1	1.4%	0	0.0%	65	92.9%	2	2.9%	1	1.4%	1	1.4%		
	High	2	0.3%	2	0.3%	158	22.5%	390	55.6%	131	18.7%	19	2.7%		
	Very High	1	0.9%	1	0.9%	62	57.4%	1	0.9%	42	38.9%	1	0.9%		

Table (3): laboratory data of obese children sample (presented in categorial manner).

		No.	%
Cholesterol class	Normal	67	67.0%
	Abnormal	33	33.0%
Triclyceride class	Normal	75	75.0%
	Abnormal	25	25.0%
HDL class	Normal	65	65.0%
	Abnormal	35	35.0%
LDL class	Normal	96	96.0%
	Abnormal	4	4.0%
HB class	Normal	100	100.0%
	Abnormal	0	0.0%
HB % class	Normal	100	100.0%
	Abnormal	0	0.0%

Discussion

This work aimed at detection of the prevalence of obesity among primary school children. This study included 3500 primary school children in MenoufyiaGovemorate- Berket El - sabae District, their age ranged from 6-12 years, during period from January 2013 to June 2013. Each child in the study was subjected to general clinical examination after taking a precise past medical history. Anthropometric measurements were taken for each child including, weight, height and calculation of body mass index. Finally, we determined the socio- economic class of each child according to the modified socio-economic scale for Egyptian families[6].

An obese child: is defined as one with BMI more than 2 SD of the reference median, An overweight child is defined as one with BMI more than 1 SD of the reference median, Child with thinness is defined as one with BMI less than -2SD of the reference line and Child with severe thinness is defined as one with BMI less than -3SD of the reference line[1].

In this study, comparing distribution of students regarding to sex 52.7% are females 47.3% are males. This in agreement with El-Shafie et al. (2009), study carried in El-Bagour district. He found that the distribution of females was higher than males.

The prevalence of overweight and obesity in this study comes in agreement with (Bahbah et al.,

2013), who found the prevalence of overweight and obesity in Elsinbellawin district was 11.5% and 8.5 % respectively.

While the percent of obesity in the present study (8.4%) is lower than values described by Shaheen et al., (2002) and El Ghadban et al., (2005) this could be explained by availability of fast food restaurants in big cities likes Cairo, plus limited activities level among children living in Cairo. Also the percent of obesity in the current work is lower than AbouGhazy study (2011) which may be due to the difference in sample size which was 500 students in AbouGhazy study and 3500 students in this study also the difference in age group between the students of both studies might be the cause of different results.

Regarding BMI- Z-score classes we found prevalence of overweight among female children (15.6%) lower than male children (18.1%), but we found prevalence of obesity among female children (10.00%) higher than male children (6.6%). This could be due to reduced activity levels of girls in the school or even outside the school. This is in agreement with most previous studies done in Egypt that showed significant increase in female percentage of obesity compared to boys it was 6.8% for the females compared to 4.4 % for the boys (Ismail, 1998), 14.7% & 15.08% for male &female respectively[7].

For regarding BMI Z-score classes we found percentage of overweight & obesity were 18.00% & 9.4% respectively in urban children higher than rural children 13.1% & 5.1% with significant difference. This could by Explained by dietary variation between urban and rural children. This agree with (Mahfouz et al., 1995) who found that percentage & obesity in urban 4.8% & rural children 3%.

In the present works the prevalence of overweight & obesity in private schools 26.5% & 13.9% respectively higher than governmental schools the prevalence of overweight & obesity (14.1% & 6.8%) respectively with highly significant difference. This agree with (Hafez et al., 2000) that reported the prevalence of overweight and obesity of 11% & 3.8% respective-

ly, among children of governmental schools in Cairo.

In our study, there was 70 children (2%) belonging to the very low social -class; 714 children (20.4%) belonging to the low social class;1906 children (54.5%) belonging to the medium class; 702 children (20.1%) belonging to the high class; and 1 child belonging to the very high class, and it was found that with regarding weight for age and BMI for age Z-score classes children from higher socioeconomic level have significant more positive deviation from mean than other children (P value <0.05) and with regarding height for age z score classes children from higher socioeconomic level have more negative deviation from the mean than other children with no significance (P value >0.05); as it was found that BMI for age Z score were increasing with the increase in the socio-economic class (21.4% of >2SD) were from the high class, (1.7% of >2SD) from the medium class, (9.3% of >2SD) were from the low class, (2.8% of>2SD) were from the very low class, and this comes in agree with (El shafie et al., 2013) who found these results in MitGhamr District, 18.08% of >2 SD were from the high class, 3.1% of >2 SD from the medium class, (12.09% of >2 SD were from the low class and 8.99% of >2 SD were from the very low class.

Our results confirm this assumption and were consistent with the study of (AbouGhazy, 2011) who found that obesity in Qalubia is more' in high socioeconomic classes. Which may be explained by excessive intake of processed meat, processed cheese, rice, artificial juices and soda drinks (coca) among high socioeconomic classes in addition to more sitting hours at home playing video and computer games and watching television advertisements of fattening food.

These results were in contrast to the study done by (Abdel-Salam, 2002) in Egypt, who found obese group was from the low socioeconomic class. And also the results of Farid (2001) who found that 67.3% of obese school children in Cairo had low socioeconomic status and 32.7% had middle and high socio-economic score.

The prevalence of overweight and obesity in this study comes in agree with (El-Shafie et al., 2011) who found the prevalence of overweight and obesity in El Bagour district was 11.5% and 8.5 % respectively.

In this study, the prevalence of obesity in male children aged 6-7 years (10.8%-14.2%) is higher than other age groups 8-11 years (5.5%, 8.3%, 8.9%, 5.4%, 7.7%) respectively, but in female children aged from 8-11 years (9.8%, 9.0%, 9.4%, 10.2%) is higher than other age groups 6-7 years (7.0%, 8.2%) respectively.

The prevalence of dyslipidemia in this study comes in agree with (El-Shafie et al. 2013), who found the prevalence of dyslipidemia in Kalin district was (34%, 23%, 4%, 34%) for cholesterol, triglycerides, LDL, HDL respectively.

Our results confirm the findings of other studies which show a higher frequency of dyslipidemia in obese children[8].

The mechanism of change in the lipid profile could be: hyperinsulinemia causes hepatic VLDL-synthesis and leads to increased TG- and LDL-C-levels. The lack of the effect of insulin on lipoprotein lipase in peripheral tissues could then result in an increase in TG and LDL-C[9].

In our study we found that 100% of the obese children had normal (Hemoglobin level) and normal (Random Blood Glucose).

These results reflect that there are still much efforts needed to motivate the parents and children to be more compliant to Breast-feeding, Proper nutrition and healthy dietary patterns and encourage young doctors to emphasize the importance of such management.

Conclusion

This study concluded that:

- The prevalence of obesity in 3500 children is 8.4%.
- The prevalence of overweight in 3500 children is 16.8%.
- Overweight and obesity were relatively high among age group 6- < 9 year.
- Obesity was more common in female than male.
- Obesity was more common in urban than rural.

- Children of high socioeconomic level more obese than other children.

- Dyslipidemia was more common in obese children.

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PREVALENCE OF OBESITY AND ASSESSMENT OF OBESITY COMPLICATION IN PRIMARY SCHOOL CHILDREN IN MENOUFYIA GOVERNORATE (BERKET EL-SABEA DISTRICT)

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ANATOMICAL STUDY OF THE NEUROVASCULAR SUPPLY OF THE VASTUS LATERALIS MUSCLE

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Abstract

Objectives: *To define neurovascular anatomical features of vastus lateralis muscle (VLM) and to define distances between definite anatomical landmarks and points of origin of these structures.*

Materials & Methods: *Neurovascular pedicle to VLM was identified and dissected in 10 adult human cadavers to determine: number of arterial branches to the muscle and their calibers, distance between the site of perforator's entry to the muscle and the tibial tuberosity (TT) and anterior superior iliac spine (ASIS), the length of the VLM and its arc of rotation, the distance between the mid-inguinal point, greater trochanter, ASIS and lateral epicondyle and TT and the site of entry of the muscular branches of the femoral nerve to the VLM.*

Results: *Blood supply of VLM was provided through three branches. The proximal branch originated from the ascending or transverse branch of the lateral circumflex femoral artery (LCFA), the middle branch arises from the ascending or descending branch of LCFA and the dominant distal vascular arises mainly from the descending branch of LCFA. Femoral nerve (FN) gave a single nerve trunk which divides into a short proximal and a long distal branch to supply VLM. The proximal division in turn divides into two short proximal and distal branches entered the proximal part of VLM just below the muscle ridge. The distal division coursed over the anterior aspect of VLM along its anteromedial border, with descending branch of the LCFA and divides distally into proximal and distal terminal branches which entered the lower third of the muscle. Additionally, FN gave two separate motor branches*

to the VLM. In few limbs, VLM was innervated by three separate motor branches originating from FN. Terminal vascular and nerve branches pierced VLM at its anterior border and subdivided inside its substance. Diameter of the proximal branch of LCFA in both right and left thighs was significantly wider compared to the distal branch, but was non-significantly wider compared to the middle branch. Diameter of the middle branch was significantly wider, on both sides, than the distal branch. Proximal neurovascular bundle was significantly nearest to ASIS, while the distal neurovascular bundle was significantly nearest to TT. The mean VLM length was 41 cm with mean arc of rotation of 20 cm, on both sides.

Conclusion: *VLM obtained its blood supply through three arterial branches of LCFA and nerve supply through a dominant branch of femoral nerve and had a wide arc of rotation. These data indicated the applicability of VLM as versatile grafting material for varied musculocutaneous defects.*

Keywords: *Vastus lateralis muscle, Neurovascular supply, Diameter, Distance, Bony landmarks*

Introduction

The quadriceps femoris muscle group is composed of the vastus lateralis, vastus medialis, vastus intermedius and rectus femoris muscles. The vastus lateralis is the largest muscle of the quadriceps femoris complex. It arises by a broad aponeurosis, which is attached to the upper half of the inter-trochanteric line, to the anterior and inferior borders of the greater trochanter, to the lateral lip of the gluteal tuberosity, and to the lateral lip of the linea aspera. The fibers form a large fleshy mass, which is continuous with a strong aponeurosis, placed on the deep surface of the lower part of the muscle. This aponeurosis converges into a thickened, flat tendon and inserts on to the lateral border of the patella, blending with the tendon of the other parts of quadriceps, and giving an expansion to the capsule of the knee joint. All four muscles of the quadriceps muscle complex are innervated by the femoral nerve [1,2,3].

Lower limb is vulnerable to high energy injuries, open fractures, radiation injuries, osteomyelitis, and large, soft tissue defect exposing bone tissue after radical resections of the tumors and complications of prosthesis used for total knee or hip joints. This vulnerability, points to the possibility of the need for soft tissue grafting anywhere in the lower half of the body[4].

The use of flaps is accepted as the standard reconstruction method especially in severe soft tissue defects. The reliability of successful graft taking relies on the proper adjustment of the appropriate grafting tissue. The characteristics of flap donor area vary according to the type of flap fashioning and depend on the knowledge of the neurovascular supply of the tissue to be transferred so as to maintain the viability and sensation of the flap. Secondly; is the possibility of moving the flap donor area to provide advancement flap and/or rotational flap without hampering vascular supply. Thirdly, is the diameter of blood vessels for reconstruction of anastomosis with the recipient area vessels on using free flaps[5,6].

Irrespective of the type of tissue grafting, the proper anatomical knowledge is mandatory for its successful taking. The muscles of the anterior thigh can provide a generous material for tissue grafting owing to their characteristic anatomical features of which its relation to both hip and knee joints, the propensity of rotation around a fixed point without hampering blood and nerve supply of the graft and because of their plentiful blood supply[7,8,9].

The current study tried to define the anatomical features of vastus lateralis muscle (VLM) with special concern to vascular and nerve supply of the muscle and to define the distances between definite anatomical landmarks and the point of origin of these structures so as to provide surgeons with a map-like design to help for fashioning VLM flaps.

Materials & Methods

The current prospective study was conducted at Anatomy Department, Faculty of Medicine, Benha and Zagazig Universities, Egypt since Jan 2012 till June 2013. After approval of the study

protocol by the Local Ethical Committee, 10 adult human cadavers; 8 male and two females with no evidence of previous trauma or surgery to the thigh were included. All cadavers were perfused with formalin 10% before dissection. Dissection was carried out in the supine position with the lower limbs still attached to the remainder of the body to simulate the normal surgical position.

A longitudinal incision was made from the iliac crest to the middle of the leg. After removal of skin and subcutaneous tissue, the deep fascia was excised to expose the whole length of the quadriceps muscle group and after incising the fascia lata, the sartorius and rectus femoris were identified, cut and retracted to expose the neurovascular pedicle to the quadriceps muscle. The anatomy of the VLM, its neural and vascular anatomy was studied. The femoral nerve was identified at the level of the inguinal ligament, the main nerve trunk supplying the VLM was dissected along its subsequent divisions and the terminal branches of femoral nerve divisions were traced distally into the muscle to identify their subsequent course; all distal branches to the muscle were preserved.

The neurovascular pedicle to the VLM was identified and dissected, the muscle was mobilized from the underlying vastus intermedius and the following data were determined: the number of arterial branches to the muscle and their caliber, the distance between the site of perforator's entry to the muscle and the tibial tuberosity (TT) and anterior superior iliac spine (ASIS), the length of the VLM and its arc of rotation, the distance between the mid-inguinal point, greater trochanter and ASIS and the site of entry of the muscular branches of the femoral nerve to the VLM and the distance between the entry's site of the muscular branches of the VLM and lateral epicondyle and tibial tuberosity. All measurements were conducted using Vernier caliper with accuracy of measuring up to 0.01 mm and a measuring strap.

Statistical analysis:

Obtained data were presented as mean±SD, ranges, numbers and ratios. Results were analyzed

using Wilcoxon; ranked test for unrelated data (Z-test) and Chi-square test (X2 test). Statistical analysis was conducted using the SPSS (Version 15, 2006) for Windows statistical package. P value <0.05 was considered statistically significant.

Results

The blood supply of the VLM was provided through three branches; proximal, middle and distal arteries, arising from the lateral circumflex femoral artery (LCFA). The proximal branch originated from the ascending or transverse branch of LCFA, passed transversely within the thigh deep to the rectus femoris muscle and is divided randomly into 2 or 3 smaller branches. The middle branch originated from the ascending or the descending branch of LCFA and ran parallel to the proximal nerve of the femoral nerve to VLM. The dominant distal vascular supply originated mainly from the descending branch of LCFA and ran parallel to the distal nervous branch, (Fig. 1). In sometime instances, the descending branch of LCFA gave the distal and middle arterial branches to VLM (Fig. 2).

The femoral nerve is the main nerve supply of VLM and gave a single nerve trunk to innervate it. This nerve trunk was divided into a short proximal and a long distal branch. The proximal division in turn was divided into two short main branches, a proximal and a distal; both entered the proximal part of VLM within 2 cm of each other at or just below the ridge of the muscle. The distal division coursed over the anterior aspect of VLM along its anteromedial border, with the descending branch of the LCFA and divides distally into proximal and distal terminal branches which entered the lower third of the muscle. The branches of both proximal and distal divisions ran within the central part of the VLM coursing from anteroproximal to posterodistal direction and divided into a number of smaller branches to supply the entire muscle (Fig. 3). In addition, the femoral nerve gave two separate motor branches to the VLM (Fig. 4). In few limbs, VLM was innervated by three separate motor branches originating from the femoral nerve (Fig. 5). The terminal

vascular and nerve branches pierced VLM at its anterior border and subdivided inside its substance (Fig. 6).

The distance between the site of distal nerve branch entry into the anterior border of VLM and lateral femoral epicondyle and TT was measured by a long measuring strap (Fig. 7). The distance between the ASIS and the termination of both proximal blood vessels and nerve branch to VLM was recorded by using a Vernier caliper (Fig. 8).

The measured diameter of the proximal branch of LCFA supplying VLM in both right and left thighs was significantly ($p<0.05$) wider compared to diameter of the distal branch, but was non-significantly ($p>0.05$) wider compared to the middle branch. Moreover, the measured diameter of the middle branch was significantly ($p<0.05$) wider, on both sides, compared to the diameter of distal branch. However, the diameters of the three arteries showed non-significant ($p>0.05$) difference between right and left sides (Fig. 9).

As regards the distance between site of origin and bony landmarks, proximal branch of LCFA was significantly ($p<0.05$) nearest to ASIS, while the distal branch of LCFA was significantly ($p<0.05$) nearest to TT with non-significant ($p>0.05$) difference compared to middle branch, (Table 1, Fig. 10). The mean VLM length was 41 cm with mean arc of rotation of 20 cm, on both sides, (Table 2). The descriptive measurements of distances between various anatomical landmarks related to VL muscles on both sides were illustrated in table 3.

Table (1): Measurements of LCFA branches supplying the VLM on both sides.

	Proximal branch		Middle branch		Distal branch	
	Right	Left	Right	Left	Right	Left
Diameter (mm)	3.0±0.1 (2.6-3.1)	2.9±0.1 (2.6-3)	2.8±0.1 (2.5-2.9)	2.7±0.1 (2.5-2.8)	2.2±0.1 (1.7-2.5)	2.3±0.1 (1.8-2.5)
Distance from TT-branches (cm)	23.5±2.5 (19-26.9)	22.5±2.5 (19- 24.9)	22.4±2.4 (18-24.5)	21.5±2.4 (17-22.5)	14.9±2.3 (12.9-18)	14±2.5 (12.9-16)
Distance from ASIS-branches (cm)	12.5±2.5 (9-14.5)	13.3±2.5 (9-14.9)	13.4±2.4 (10.5-14.9)	14.1±2.4 (10.5- 14.8)	16.9±2.3 (13.2-19.3)	16.9±2.3 (12.9-19.5)

Data are presented as mean±SD; ranges are in parenthesis; LCFA: lateral circumflex femoral artery; VLM: vastus lateralis muscle; TT: tibial tuberosity; ASIS: anterior superior iliac spine.

Table (2): Measurements of length and arc of rotation of VLM on both sides.

	Right	Left
Length of the muscle (cm)	41±2.3 (27.1-45)	41±4.9 (28.1-45)
Arc of rotation (cm)	20±2.9 (14.5- 22.2)	20±3.3 (14.2-23.1)

Data are presented as mean±SD; ranges are in parenthesis; VLM: vastus lateralis muscle.

Table (3): Measurements of distances between anatomical landmarks related to VLM on both sides.

	Right side			Left side		
	Mean±SD	Range	SE	Mean±SD	Range	SE
GT-EP	44.5±4.62	36-50	1.4625	43.5±1.64	35-50	1.6415
GT-TT	49.7±5.4	42-57	1.7065	48.6±1.91	40-56	1.9102
ASIS-EP	47.9±3.18	45-53	1.0050	46.2±3.79	40-52	1.2000
ASIS-TT	52.5±5.02	47-60	1.5864	52±5.03	46-59	1.5916
Lig.-EP	42.9±4.46	37-50	1.4099	42.7±3.95	36-48	1.2477
Lig.-TT	49±4.55	45-56	1.4376	48.6±4.45	44-55	1.4079
Lig.-M1	16.1±2.6	12-20	0.8226	14±2.58	10-17	.8165
Lig.-M2	17.4±2.76	15-22	0.8718	18.4±1.71	16-20	.5416
Lig.-M3	20	0	0	22	0	0
ASIS-M1	18.7±2.41	14-22	0.7608	16.1±2.33	13-19	.7371
ASIS-M2	20.1±1.05	16-25	1.0483	21.5±2.27	19-25	.7188
ASIS-M3	24	0	0	26	0	0
GT-M1	18.7±2.41	14-22	0.7608	14±1.05	12-15	.3333
GT-M2	20.5±4.01	16-27	1.2671	20.4±3.47	17-26	1.0975
GT-M3	24	0	0	26	0	0
M1-EP	29.8±4.49	24-37	1.4205	30±4.92	25-37	1.5563
M2-EP	25.1±3.93	20-29	1.2423	24.5±3.24	21-29	1.0247
M3-EP	21	0	0	19	0	0
M1-TT	36.2±4.8	32-45	1.5188	37.4±4.86	32-45	1.5362
M2-TT	29.9±3.87	25-36	1.2243	30.4±4.09	25-36	1.2927
M3-TT	26	0	0	26	0	0

Data are presented as mean±SD; SE: standard error; VLM: vastus lateralis muscle; GT: Greater trochanter of femur; EP: Lateral femoral epicondyle; TT: Tibial tuberosity; ASIS: Anterior superior iliac spine; Lig. : Inguinal ligament; M1: The entry's site of the 1st motor branch; M2: The entry's site of the 2nd motor branch; M3: The entry's site of the 3rd motor branch

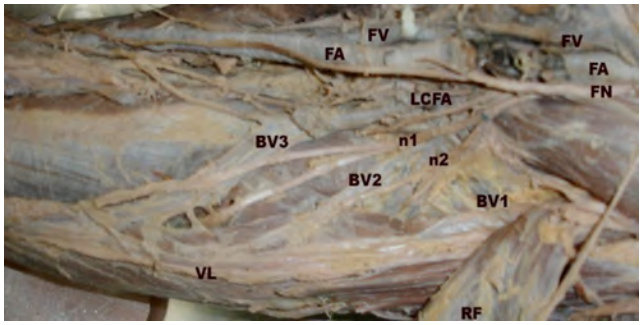

Fig. 1: Photomicrograph of the neurovascular pedicles of a left vastus lateralis (VL) muscle. The lateral femoral circumflex artery (LFCA), originating from the femoral artery (FA), divides into descending and transverse branches. The principal branch from the descending branch of LCFA is the distal branch (BV3) to the vastus lateralis muscle. This branch skirts the anterior border of the VL and is accompanied by the distal nerve branch (n1). The transverse branch of LFCA gives rise to the proximal (BV1) and middle (BV2) arterial branches to the vastus lateralis, paralleling the proximal nerve branch (n2) to the vastus lateralis. RF: the reflected rectus femoris muscle; FV: the femoral vein (FV) and FN: the femoral nerve.

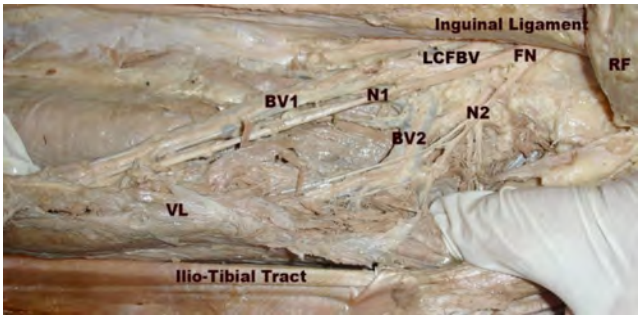

Fig. 2: Photomicrograph of a left thigh showing the distal (BV1) and middle (BV2) arterial branches originate from the descending branch of the LCFA. The femoral nerve (FN) gives distal (N1) and proximal (N2) motor branches to the vastus lateralis muscle (VL). Both vascular and nerve branches run parallel to each other and enter the vastus lateralis muscle at its anterior border. RF: The medially reflected rectus femoris muscle.

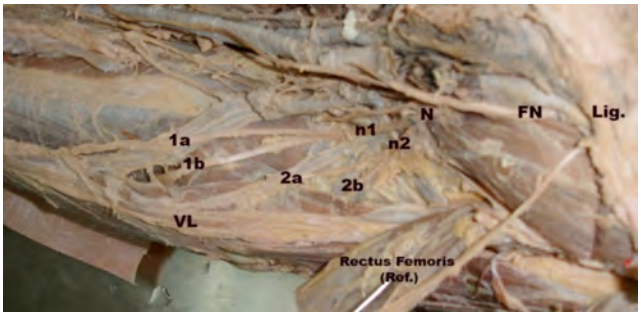

Fig. 3: Photomicrograph of a left thigh showing the innervation of the vastus lateralis muscle (VL) from the femoral nerve (FN). A nerve trunk (N) originates from the femoral nerve and divides distally into proximal (n2) and distal (n1) divisions. Each division subdivides distally into two terminal branches (1a, 1b, 2a & 2b) that enter the VL at its anterior border. The femoral nerve passes deep to the inguinal ligament (Lig.).

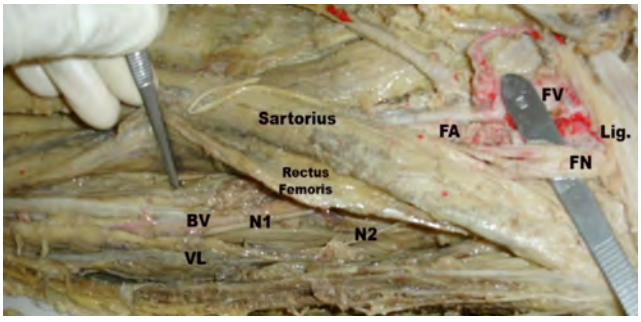

Fig. 4: Photomicrograph of a left thigh showing two separate branches (N1, N2) originating from the femoral nerve (FN) and terminate at the anterior border of the vastus lateralis muscle (VL). Blood vessels (BV) pass parallel to the motor branch (N1). The femoral nerve, the femoral artery (FA) and the femoral vein (FV) pass deep to the inguinal ligament (Lig.).

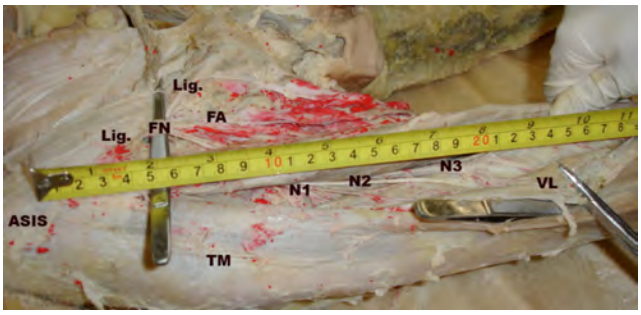

Fig. 5: Light photomicrograph of a right male thigh showing three separate branches (N1, N2 & N3) originating from the femoral nerve (FN) to supply vastus lateralis muscle (VL). A measuring strap was used to measure the distance between these branches and the anterior superior iliac spine (ASIS). The tensor fascia latae (TM) covers the upper part of VL. The femoral artery (FA) and nerve pass deep to the inguinal ligament (Lig.).

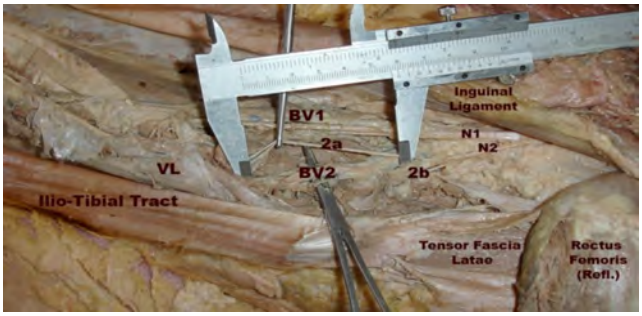

Fig. 6: Photomicrograph of a left male thigh showing the distribution of the vascular (BV1 & BV2) and nerve (N1 & N2) branches inside the vastus lateralis muscle (VL). The proximal nerve (N2) divides into two terminale branches (2a & 2b).

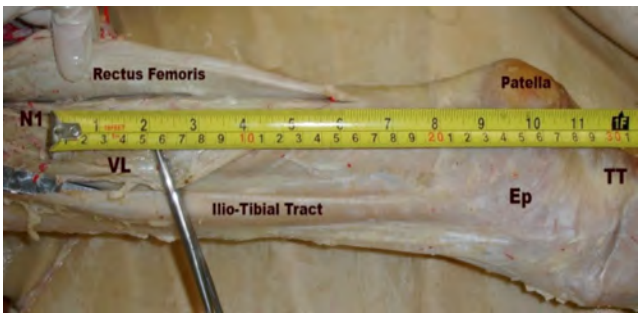

Fig. 7: Photomicrograph of a right limb showing the measurement between the site of the distal nerve (N1) entry to the vastus lateralis muscle (VL) and the lateral epicondyle of femur (Ep) and the tibial tuberosity (TT).

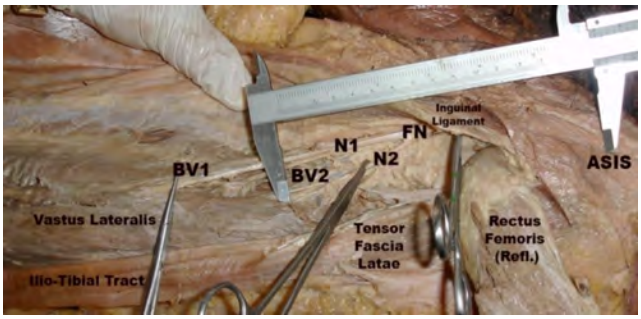

Fig. 8: Photomicrograph of a left limb showing the distribution of the two motor branches (N1 & N2) of the femoral nerve (FN) to the vastus lateralis muscle. The two nerve branches run distally parallel to the blood vessels (BV1 & BV2) of the vastus lateralis muscle. The distance between the anterior superior iliac spine (ASIS) and the termination of the proximal blood vessels and the nerve branch is measured by a Vernier caliber.

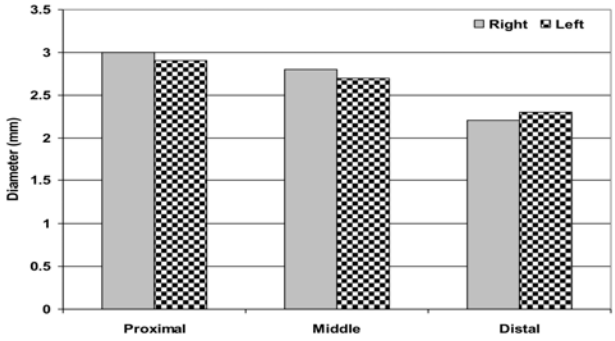

Fig. 9: Mean diameter of branches of LCFA supplyin VL muscle.

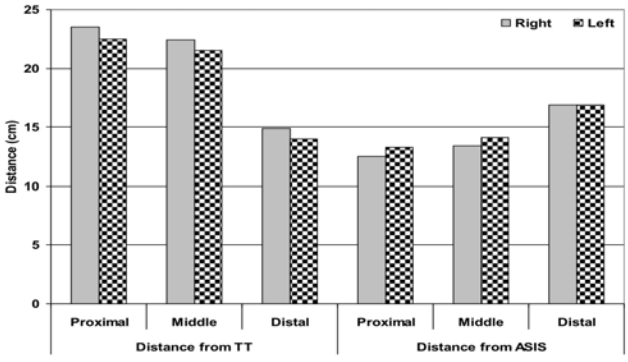

Fig. 10: Mean distance between site of origin of LCFA branches supplying VL and bony landmarks.

Discussion

The current study found the blood supply of vastus lateralis muscle (VLM) is dependent on the lateral circumflex femoral artery (LCFA) which gave three main branches; proximal, middle and distal branches with the proximal originated from the ascending or transverse branch of LCFA, the middle from the ascending or transverse branch of LCFA, while the dominant distal branch arises mainly from the descending branch of LCFA. There were non-significant differences between right and left thighs as regards the site of origin and diameter of these branches, and distances between the origin site and anterior superior iliac spine (ASIS) and tibial tuberosity (TT). However, the proximal branch was significantly nearer to ASIS and far from TT, with the distal one was in reverse. The current study additionally detected that nerve supply of VLM was dependent on terminal braches of a main branch of femoral nerve and these branches were concomitant to vascular supply.

These data go in hand that previously documented in literature wherein Malhotra et al.[10] reported that dissection of 27 cadaver thighs identified an average of four musculocutaneous perforators to the anterolateral thigh (ALT) area per thigh with the distance from the anterior border of the VLM to the point where perforators entered the muscle ranged from 0.1 to 7 cm, 64% of perforators entered the VLM within 2 cm of the muscle's anterior border and at least one perforator entered the VLM within 2 cm of the muscle's anterior border per thigh. Sananpanich et al.[11] found the arterial supply of ALT was dependent on the descending branch of the LCFA in 38 of 47 dissections and arises with a mean diameter of 3 mm (range, 2.2-4 mm) at its origin, during the descending branch's journey to the distal part of the thigh, several branches went into the vastus lateralis and vastus intermedius muscles and the diameters were tapering with the mean terminal diameter was 1.3 mm (range 0.9-1.8 mm) and the mean total length of the descending branch from its origin to terminus was 30.3 cm (range 22.5-37.1cm).

Tayfur et al.[12] studied the

VLM of 15 adult formalin-fixed cadavers and reported that the dominant pedicle was found to be a descending branch of the LCFA with mean diameter of 2.1 mm and is located 119.4 mm distal to the pubic symphysis; the muscle also had proximal pedicles from the ascending and transverse branches of LCFA and distal minor branches with origin located 83.7 mm proximal to the intercondylar line. The motor nerve of the vastus lateralis was found to be originating from femoral nerve and entered the muscle 194.6 mm from the ASIS.

Kim et al.[13] (2010) in anatomical-clinical trial studied 8 cadavers and found the descending branches of the LCFA measured 10- and 15-cm above the lateral aspect of the patella with diameters ranged from 1 to 2 mm at 10-cm and 1 to 3 mm at 15-cm sites. Then, clinically, ALT perforator free flap dependent on the descending branch of LCFA was used for reconstruction of soft tissue defects of the ipsilateral knee in two cases; both flaps survived well without major complications up to a mean follow-up period of 13 months.

Recently, Yamada et al.[14] tried to clarify the anatomy of the connection between the descending branch of the LCFA and the lateral superior genicular artery for distally-based ALT flap reconstruction around the knee and found that the proximal pivot point was 1-12.1 cm from the inguinal ligament and the distal pivot point, found under the VLM, was 4-13.6 cm from the lateral superior edge of the patella with the most distal perforator was 8.2-28 cm from the proximal pivot point, while the most proximal perforator was 3-19.5 cm from the distal pivot point.

The obtained anatomical data implied that VLM graft may be used as versatile graft donor area for the following advantages, wide arc of rotation allowed grafting defects in hip or back regions with flap dependent on the proximal branch and to graft defects of knee and legs depending on the distal branch. Additionally, the multiplicity of blood supply sources allowed for fashioning sliding or advancement flaps and allowed

successful microsurgical anastomosis and fashioning free transfer flaps.

In line with the importance of VLM from surgical point of view, Wang et al.[15] based on the anatomic study of descending branches and cutaneous perforators of the LCFA, the perforator vessels were found and used as flap pedicle for repairing the oromaxillary soft tissue defect as anterolateral and anteromedial thigh flaps, anterolateral thigh flaps and rectus femoris perforator flaps, and bilateral anterolateral thigh flaps and all the 16 flaps in 8 cases survived completely with good functional and esthetic results and with no complication or morbidity in donor or recipient sites.

Nelson et al.[16] reported that the VLM flap is an underutilized alternative for reconstruction of deep soft tissue defects of the head and neck, its advantages include its consistent anatomy and large caliber pedicle, adequate volume, a location enabling a 2-team approach and low donor site morbidity and concluded that this versatile flap should be included as an option in complex soft tissue reconstructions of the head and neck. Rodríguez-Rosales et al.[17] documented that transposition of VLM flap as a treatment for recalcitrant deep infection after arthroplasty has presented good results, provided there is appropriate antibiotic therapy and surgical debridement, thus achieving wound healing. Recently, Ensat et al.[18] conducted 41 free anterolateral thigh flaps based on the perforators of the descending branch of the LCFA which were dissected to their origin and after flap transfer microsurgical anastomoses are performed and reported total flap loss rate of 2.4% and partial flap loss rate of only 13.8%. Whiteside[19] documented that vastus lateralis and/or vastus medialis muscles used as transfer flap with advancement of tibial attachment provide adequate coverage for anterior soft tissue deficits of the knee and improve its extensor strength.

In conclusion, the VLM obtained its blood supply through three arterial branches of LCFA and nerve supply through a dominant branch of femoral nerve and

had a wide arc of rotation. These data indicated the applicability of VLM as versatile grafting material for varied musculocutaneous defects, irrespective of recipient area.

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ANATOMICAL STUDY OF THE NEUROVASCULAR SUPPLY OF THE VASTUS LATERALIS MUSCLE

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AGE-RELATED MEASUREMENTS AND INDICES OF NORMAL HUMAN BRAIN VENTRICULAR SYSTEM: A COMPUTED TOMOGRAPHY STUDY

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Abstract

Objectives: *To detect average dimensions and indices of normal brain cerebral hemispheres and lateral ventricles in relation to age and gender.*

Subjects & Methods: *135 subjects; 70 males (M) and 65 females (F) were categorized into 4 age groups (G): GM1 & GF1 included subjects <20 years old, GM2 & GF2 included subjects aged 20-40 years, GM3 & GF3 included subjects aged 40-60 years and GM4 & GF4 included subjects >60 years old. CT imaging was conducted according to Agapejev; using high resolution scanner for measuring the width of right (RAH) and left anterior horns (LAH) of lateral ventricle, the bifrontal distance (ff) and inner skull width (FF) at this level, the bifrontal index (ff//FF), the bicaudate distance (cc), and inner skull width (CC) at this level and the bicaudate index (cc/CC).*

Results: *Bifrontal distance was significantly wider in GM4 than GM3 and in GM2 and GM4 than GF2 and GF4. The FF distance increased progressively but non-significantly among groups G1-3 in both sexes, but non-significantly decreased in G4 compared to G3 in both sexes. The FF distance was significantly wider in GM1-3 compared to GF1-3. Bifrontal index showed non-significant difference between all GF groups. It showed non-significant difference between GM1-3 groups, but was significantly higher in GM4 than GM3. Bicaudate distance of GM4 was significantly wider than GM3, in GF3 than GF2, GF4 than GF3 and in GM1 and GM4 compared to GF1 and GF4, respectively. The CC distance showed non-significant increase in GM2 than GM1, but*

showed non-significant decrease in GM3 than GM2 and in GM4 than GM3. The CC distance showed non-significant increase in GF2 than GF1 and in GF3 than GF2, but was non-significantly decreased in GF4 than in GF3. The CC distance was significantly larger in males compared to females in G1-3 groups, but non-significantly in G4. Bicaudate index showed non-significant difference up GM3 but was significantly higher in GM4 compared to GM3. Bicuadate index showed significant difference between GF3, GF2 and GF4, GF3 groups. Width of RAH was significantly wider in GM4 compared to GM3 and in GM4 than GF4. Width of LAH was significantly wider in GM4 than GM3, GF2 than GF1 and in GF3 than GF2. LAH width was significantly wider in GM1, GM2 and GM4 compared to females.

Conclusion: *Cerebral and lateral ventricle dimensions showed steady increase with age and being larger in males than in females. Subjects older than 60 years showed significant reduction of bifrontal and bicaudate indices with evident difference according to gender.*

Keywords: *Cerebral hemispheres, Lateral Ventricle, Dimensions, Indices, Normal subjects, Age, Gender*

Introduction

Age-related changes in brain structure result from a complex interplay among various neurobiological processes, which may contribute to more complex trajectories than what can be described by simple linear or quadratic models. Most brain structures do not follow a simple path throughout adult life. Cerebral atrophy has long been recognized as a prominent feature of normal aging process. However, accelerated decline in high age is not the norm of healthy brain aging and sever atrophy occurred only in demented elderly individuals and not in normal aged[1,2].

Neuroimaging studies have identified several forms of age-related change in brain structure that are relevant as potential mediators of age-related changes in cognitive performance. Prominent among these is age-related decline in the volume of cerebral gray matter, particularly in prefrontal regions. Age-related decline is also

evident in cerebral white matter volume, although with a different trajectory. Whereas the age-related decline in gray matter volume is relatively linear from younger adulthood, the corresponding decline in white matter tends to be nonlinear, with a plateau in middle-age and additional decline, beyond that of gray matter, in later adulthood[3,4].

On contrary to these data, recently, Ziegler et al.[5] and Taki et al.[6] found a significant positive correlation between the annual rate of gray matter volume change and age indicating that gray matter volume shows not linear but accelerated loss with age and so documented evaluating the annual rate of the gray matter volume change with age in healthy subjects to understand how gray matter volume changes with aging in each brain region and to anticipate what cognitive functions are likely to show accelerated decline with aging.

The cerebral ventricles are a series of interconnected, fluid-filled spaces that lie in the core of the forebrain and brainstem. The presence of ventricular spaces in the various subdivisions of the brain reflects the fact that the ventricles are the adult derivatives of the open space or lumen of the embryonic neural tube. The largest of cerebral ventricles are the lateral ventricles which are located in the center of the brain one within each of the cerebral hemispheres. In frontal sections, ventral surface of lateral ventricles is usually defined by the basal ganglia, the dorsal surface by the corpus callosum, and the medial surface by the septum pellucidum [7,8,9].

The current prospective comparative study aimed to detect the average dimensions and indices of the normal brain cerebral hemispheres and lateral ventricles in relation to age and gender as a trial to provide standard schedules of the normal brain parameters.

Subjects and Methods

The current study was designed as a comparative study of CT brain scanning findings of normal subjects. Inclusion criteria included healthy subjects free from any clinical manifestations indi-

cating brain pathology as judged by clinical examination conducted at Department of Neurology, Benha University Hospital. All CT imaging were conducted and analyzed at Diagnostic Radiology Department, Benha University Hospital. Analysis of CT images and determination of points of interest were determined by the authors and then measurements were analyzed and compared at Anatomy Department.

Exclusion criteria included history of previous brain trauma, cerebro-vascular accidents, seizures, intellectual deterioration, alcoholism and dementia. CT scans showing asymmetrical head position and presence of any detectable lesion even if all the other CT of the same patient has been normal were also excluded from this study.

The study included 135 subjects fulfilled the inclusion criteria; 70 males and 65 females. Subjects were categorized into 4 age groups (G): G1 included subjects younger than 20 years of both sexes (GM1 & GF1), G2 included subjects aged 20 to 40 years of both sexes (GM2 & GF2), G3 included subjects aged 40 to 60 years of both sexes (GM3 & GF3) and G4 included subjects older than 60 years of both sexes (GM4 & GF4).

CT imaging was conducted without sedation, anesthesia or intravenous contrast administration. The procedure was performed according to Agapejev[10]; using high resolution scanner with the scanning at an average tilt of 20o from the cantho-meatal line that extends from the lateral epicanthus of the eye to the external acoustic meatus. Individuals were put in a supine position, centralized, fixed and their heads were placed in the gantry of the apparatus in a slightly hyper-extended position. About 15 serial cross sections (cuts) of the brain for every case were taken in a display field of the axial plan. The thickness of each examined contagious tissue section was 5-10 mm between each cut and the next one. The chosen parameters were measured at tow levels of the CT scan cuts[11].

Studied parameters:

1- The bifrontal distance (ff) is

the combined width of right (RAH) and left anterior horn (LAH) of the lateral ventricle and is represented by a transverse line connecting the tips of both RAH and LAH[12].

2- The inner skull width (FF) corresponds the transverse inner diameter of the skull at the same level of the birfontal distance; i.e. the width of the cerebral hemisphere at the this level and is represented by a transverse line connecting the inner skull tables at the same level of the bifrontal distance[13].

3- The bifrontal index (Evans' ratio) (ff//FF) is the ratio between the bifrontal distance and the width of the cerebral hemisphere at the same level[14].

4- The bicaudate distance (cc) is the width of the lateral ventricles between the two caudate nuclei and is represented by a line between the maximum intentation of the caudate nucleus into the lateral ventricles[13].

5- The inner skull width at the level of the bicaudate distance (CC) is the transverse inner diameter of the skull at the same level of the bicaudate distance i.e. the width of the cerebral hemisphere at this level and is represented by a transverse line connecting the inner skull tables at the level of the bicaudate distance[13].

6- The bicaudate index (cc/CC) is the ratio between bicaudate distance and the width of the cerebral hemisphere at the same level[14].

7- The width of RAH and LAH of lateral ventricle is the maximal width of each anterior horn and is represented by a line connecting the tip of the right or left horn, respectively to the point of intersection of this line with a line derived from continuation of the inner border of the septum pellucidum[15].

Statistical analysis:

Obtained data were presented as mean±SD. Results were analyzed using Wilcoxon; ranked test for unrelated data (Z-test). Statistical analysis was conducted using the SPSS (Version 15, 2006) for Windows statistical package. P value <0.05 was considered statistically significant.

Results

The bifrontal distance (ff) showed progressive increase with age in both sexes but with non-significant difference between G1, G2 and G3. In GM4 group, the bifrontal distance was significantly (P3=0.007) wider compared to that of GM3, while in females the corresponding difference was non-significant. Among both sexes, the bifrontal distance was non-significantly wider in GM1 and GM3 compared to GF1 and GF3, respectively, while was significantly wider in GM2 (P=0.042) and GM4 (P=0.015) compared to GF2 and GF4, respectively, (Fig. 1).

The width of the cerebral hemisphere (FF distance), also showed steady increase with age with non-significant difference between age groups in both sexes till age of 60 where the FF distance in G4 starts to decrease but with non-significant difference compared to FF distance of G3 in both sexes. Among both sexes, the FF distance was significantly wider in GM1 (P=0.008), GM2 (P=0.001) and GM3 (P=0.037) compared to GF1, GF2 and GF3 groups, while the FF distance was non-significantly wider in GM4 (P>0.05) compared GF4, (Fig. 2).

The bifrontal index (ff/FF ratio) as a measure of ventricular width in relation to the width of the cerebral hemisphere showed non-significant (P>0.05) difference between all age groups of females. In males the bifrontal index showed non-significant (P>0.05) difference between GM1, GM2 and GM3 groups, while the bifrontal index in GM4 was significantly (P=0.005) higher compared to GM3. There was non-significant difference between groups G1-G3 in both sexes as regards the bifrontal index, (Table 1, Fig. 3).

The bicaudate distance (cc) showed progressive increase with age in both sexes. In males, the difference between G1, G2 and G3 was non-significant, while in GM4 group, the bicaudate distance was significantly (P3=0.012) wider compared to GM3. In females, the difference between G1 and G2 was non-significant, while in GF3 group the bicaudate distance was significantly wider (P2=0.014) than of GF2 and in GF4 (P3=0.018) compared to GF3. Among both sexes,

the difference of the bicaudate distance was significantly wider in GM1 (P=0.035) and GM4 (P=0.013) groups compared to GF1 and GF3 groups, while the bicaudate distance was non-significantly longer in GM2 and GM3 compared GF2 and GF3, (Fig. 4).

In males the width of the cerebral hemisphere at bicaudate level (CC distance) was non-significantly increased in GM2 compared to GM1, but started to decrease in GM3 with non-significant difference compared to GM2 and in GM4 compared to GM3. In females, the width of the cerebral hemisphere at bicaudate level was non-significantly increased in GF2 compared to GF1 and in GF3 compared to GF2, but started to decrease in GF4 with was non-significant difference compared to GF3. However, the CC distance was significantly larger in males compared to females in G1 (P=0.015), G2 (P=0.004) and G3 (P=0.045), but the difference was non-significant in G4, (Fig. 5).

The bicaudate index (cc/CC ratio) as a measure of ventricular width in relation to the width of the cerebral hemisphere at the level of caudate showed non-significant (P>0.05) difference up to age of 60 in males but was significantly higher (P3=0.007) in GM4 compared to GM3. In females, the difference was non-significant between GF1 and GF2, but was significantly larger in GF3 compared to GF2 (P2=0.041) and in GF4 compared to GF3 (P=0.008). The difference between males and females was non-significant till G4 where the index was significantly higher (P=0.022) in males compared to females, (Table 1, Fig. 6).

Mean estimated width of RAH was non-significantly (P>0.05) wider in G2 compared to G1 and in G3 compared to G2 in both males and females. RAH width was significantly wider (P=0.013) in GM4 compared to GM3 groups, but the difference was non-significant (P>0.05) in females. RAH width was non-significantly (P>0.05) wider in GF1 and GF2 groups compared to GM1 and GM2 groups, but was non-significantly wider in GM3 compared to GF3 groups. However, males of G4 had significantly

(P=0.024) wider RAH compared to females (Fig. 7).

Mean estimated width of LAH was non-significantly (P>0.05) wider in GM2 compared to GM1 and in GM3 compared to GM2, but was significantly (P3=0.007) wider in GM4 compared to GM3. In females RAH width was significantly wider (P1=0.002) in G2 compared to G1 and in G3 compared to G2 (P2=0.018), but the difference was non-significant (P3>0.05) in G4 compared to G3. LAH width was significantly wider in males of G1 (P=0.017), G2 (P=0.01) and G4 (P=0.017) compared to females, but was non-significantly (P>0.05) wider in males of G3 compared to females (Table 3, Fig. 8).

Table (1): Mean measures of bifrontal distance (ff), the width of cerebral hemispheres (FF) and the bifrontal index (ff/FF ratio) in studied subjects categorized according to age and gender.

	MG1 (n=21)	MG2 (n=22)	MG3 (n=17)	MG4 (n=10)	GF1 (n=18)	GF2 (n=26)	GF3 (n=11)	GF4 (n=10)
ff distance (mm)	29.9±5.5	32.6±4	34±3.3	39.6±3.9	27.7±5.7	30.1±4.4	33.3±1.9	35.1±2.8
		P1>0.05	P2>0.05	P3=0.007		P1>0.05	P2>0.05	P3>0.05
					P>0.05	P=0.042	P>0.05	P=0.015
FF distance (mm)	100.5±5.2	100.7±5.9	102.7±8.5	98.1±5.6	92±11.4	93±4.6	94.2±7.8	93±4.7
		P1>0.05	P2>0.05	P3>0.05		P1>0.05	P2>0.05	P3>0.05
					P=0.008	P=0.001	P=0.037	P>0.05
ff/FF ratio	0.3±0.06	0.33±0.05	0.34±0.05	0.4±0.03	0.31±0.08	0.32±0.05	0.36±0.04	0.38±0.03
		P1>0.05	P2>0.05	P3=0.005		P1>0.05	P2>0.05	P3>0.05
					P>0.05	P>0.05	P>0.05	P=0.015

Data are presented as mean±SD; G1: age <20 years group, G2: age 20-40 years group; G3: age 40-60 years group; G4: age >60 years group; M: males; F: females; P1: significance of difference between G1 and G2; P2: significance of difference between G2 and G3; P3: significance of difference between G3 and G4; P: significance of difference between males and females of the same age group; p>0.05: non-significant difference; p<0.05: significant difference

Table (2): Mean measures of bicaudate distance (cc), the width of cerebral hemispheres (CC) and the bicaudate index (cc/CC ratio) in studied subjects categorized according to age and gender.

	MG1 (n=21)	MG2 (n=22)	MG3 (n=17)	MG4 (n=10)	GF1 (n=18)	GF2 (n=26)	GF3 (n=11)	GF4 (n=10)
Cc distance (mm)	9.5±2.4	11.2±3.4	12.8±3.7	17.6±3.4	8.2±2.2	9.15±2.1	12.3±2.5	14.6±2.7
		P1>0.05	P2>0.05	P3=0.012		P1>0.05	P2=0.014	P3=0.018
					P=0.035	P>0.05	P>0.05	P=0.013
CC distance (mm)	105.5±5.5	107±5.7	106.1±5.2	102±5.6	94±11.8	100.9±9.6	101±5.5	99.2±9.9
		P1>0.05	P2>0.05	P3>0.05		P1>0.05	P2>0.05	P3>0.05
					P=0.015	P=0.004	P=0.045	P>0.05
cc/CC ratio	0.09±0.02	0.1±0.03	0.12±0.03	0.17±0.03	0.088±0.03	0.09±0.03	0.12±0.03	0.15±0.03
		P1>0.05	P2>0.05	P3=0.007		P1>0.05	P2=0.041	P3=0.008
					P>0.05	P>0.05	P>0.05	P=0.022

Data are presented as mean±SD; G1: age <20 years group, G2: age 20-40 years group; G3: age 40-60 years group; G4: age >60 years group; M: males; F: females; P1: significance of difference between G1 and G2; P2: significance of difference between G2 and G3; P3: significance of difference between G3 and G4; P: significance of difference between males and females of the same age group; p>0.05: non-significant difference; p<0.05: significant difference

Table (3): Mean width of right and left anterior horns of the lateral ventricle estimated in studied subjects categorized according to age and gender.

	MG1 (n=21)	MG2 (n=22)	MG3 (n=17)	MG4 (n=10)	GF1 (n=18)	GF2 (n=26)	GF3 (n=11)	GF4 (n=10)
RAH (mm)	13.9±2.6	15.5±2.4	16.1±3	18.6±1.4	14.4±2.9	14.8±2.2	15.6±1.9	16±1.9
		P1>0.05	P2>0.05	P3=0.013		P1>0.05	P2>0.05	P3>0.05
					P>0.05	P>0.05	P>0.05	P=0.024
LAH (mm)	16±3.2	17.1±1.6	17.9±2.4	20.9±1.7	13.4±2.6	15.2±2.1	17.6±1.8	17±2.1
		P1>0.05	P2>0.05	P3=0.007		P1=0.002	P2=0.018	P3>0.05
					P=0.017	P=0.01	P>0.05	P=0.017

Data are presented as mean±SD; RAH: Right anterior horn; LAH: Left anterior horn; G1: age <20 years group, G2: age 20-40 years group; G3: age 40-60 years group; G4: age >60 years group; M: males; F: females; P1: significance of difference between G1 and G2; P2: significance of difference between G2 and G3; P3: significance of difference between G3 and G4; P: significance of difference between males and females of the same age group; p>0.05: non-significant difference; p<0.05: significant difference

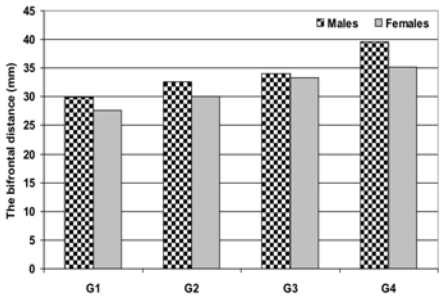


Fig. 1: Mean bifrontal distance (ff) estimated in all age groups of male and females.

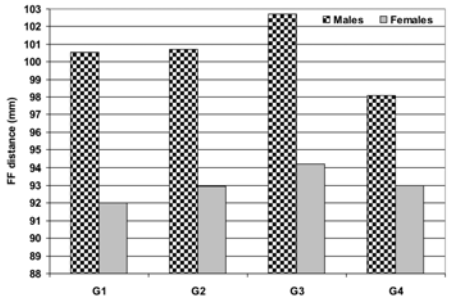


Fig. 2: Mean width of the cerebral hemisphere (FF distance estimated in all age groups of males and females.

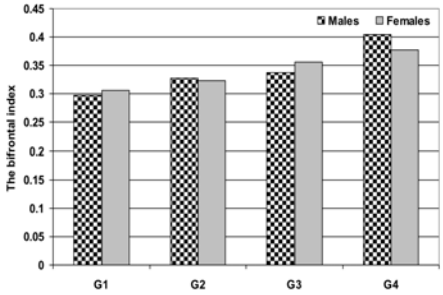


Fig. 3: Mean bifrontal index determined for all age groups of males and females.

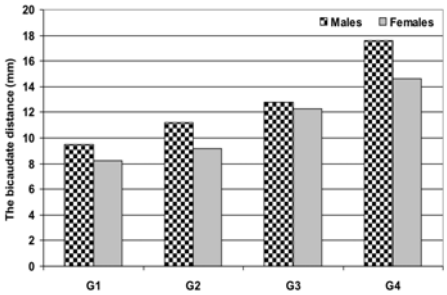


Fig. 4: Mean bicaudate distance (cc) estimated in all age groups of males and females.

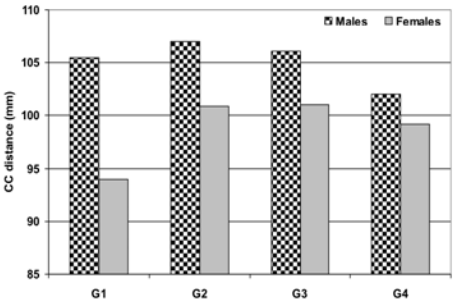


Fig. 5: Mean width of the cerebral hemisphere (CC distance) at bicaudate level estimated in all age groups of males and females.

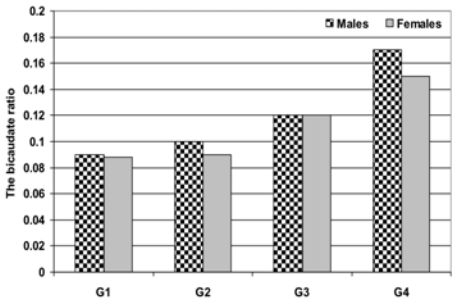


Fig. 6: Mean bicaudate ratio determined for all age groups of males and females.

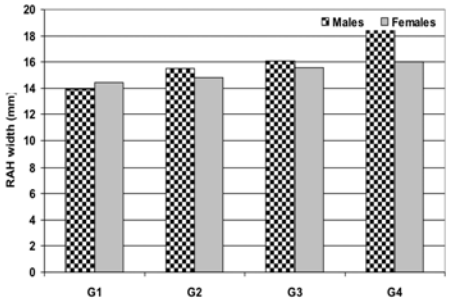


Fig. 7: Mean RAH width estimated in all age groups of males and females.

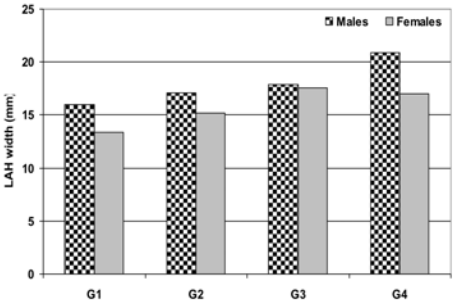
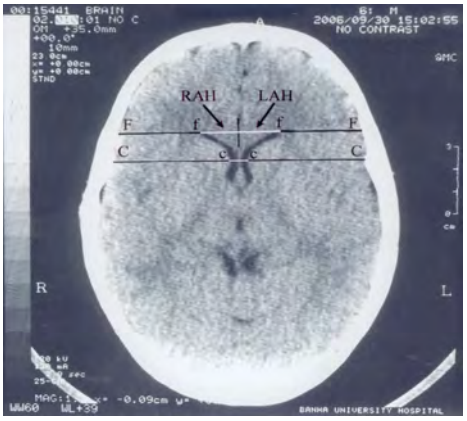
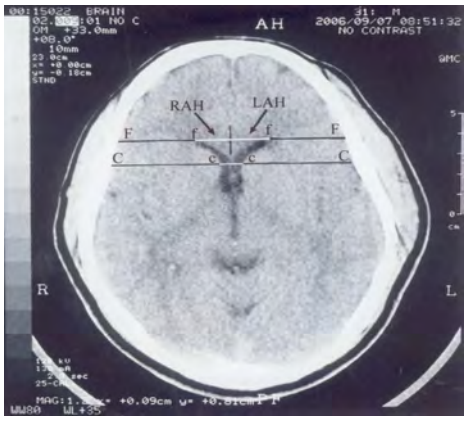


Fig. 8: Mean LAH width estimated in all age groups of males and females.



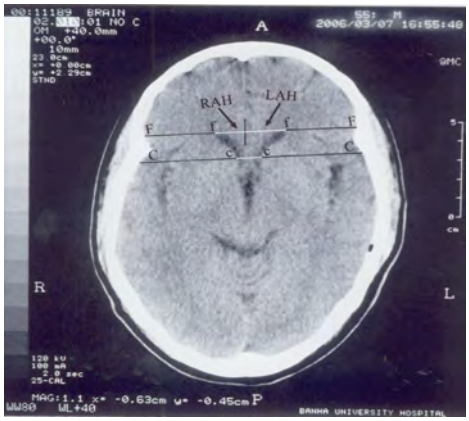
Axial CT image of anterior horn of lateral ventricles of a male-aged 6 years (MG1) showing:

- The bifrontal distance (ff)= 32.31mm
- The inner skull width at ff level (FF) = 99.23mm
- Right anterior horn width (RAH) = 15 mm
- Left anterior horn width (LAH) = 17.31 mm
- The bicaudate distance (cc) = 8.08mm
- The inner skull width at cc level (CC) = 113.18mm



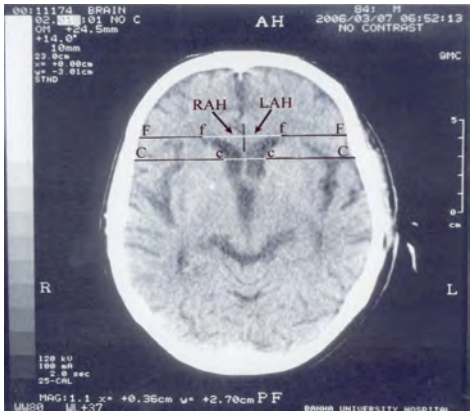
Axial CT image of anterior horn of lateral ventricles of a male-aged 31 years (MG2) showing:

- The bifrontal distance (ff)= 32.93mm
- The inner skull width at ff level (FF) = 100mm
- Right anterior horn width (RAH) = 15.85 mm
- Left anterior horn width (LAH) = 17.07 mm
- The bicaudate distance (cc) = 9.76mm
- The inner skull width at cc level (CC) = 104.88mm



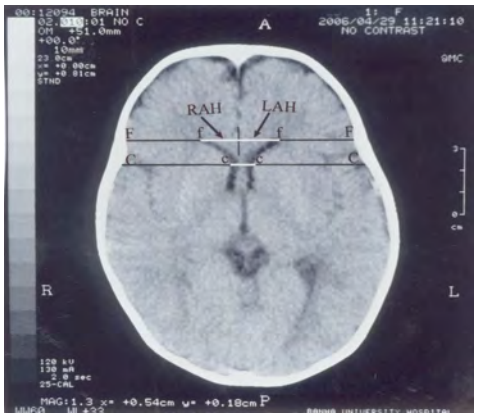
Axial CT image of anterior horn of lateral ventricles of a male-aged 55 years (MG3) showing:

- The bifrontal distance (ff)= 38.61mm
- The inner skull width at ff level (FF) = 105.82mm
- Right anterior horn width (RAH) = 17.16 mm
- Left anterior horn width (LAH) = 21.45 mm
- The bicaudate distance (cc) = 12.87mm
- The inner skull width at cc level (CC) = 114.4mm



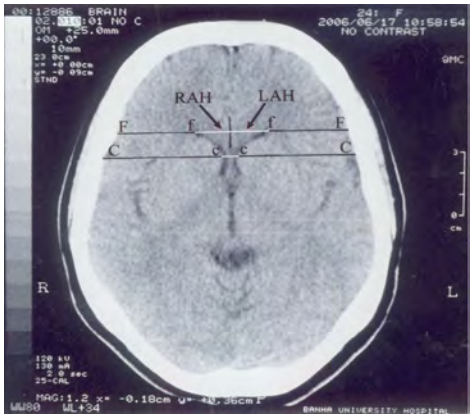
Axial CT image of anterior horn of lateral ventricles of a male-aged 84 years (MG4) showing:

- The bifrontal distance (ff)= 40 mm
- The inner skull width at ff level (FF) = 90mm
- Right anterior horn width (RAH) = 18.75 mm
- Left anterior horn width (LAH) = 21.25 mm
- The bicaudate distance (cc) = 20mm
- The inner skull width at cc level (CC) = 100mm



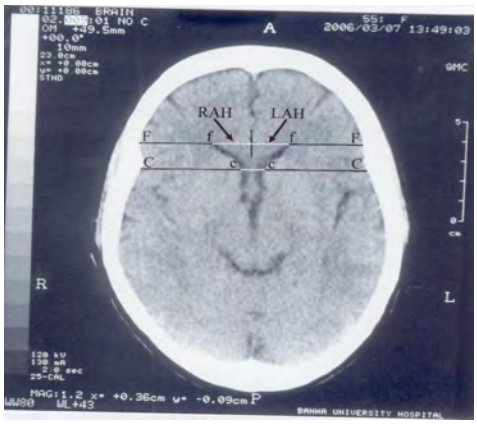
Axial CT image of anterior horn of lateral ventricles of a female aged 1-year (FG1) showing:

- The bifrontal distance (ff)= 30mm
- The inner skull width at ff level (FF) = 78mm
- Right anterior horn width (RAH) = 16 mm
- Left anterior horn width (LAH) = 14 mm
- The bicaudate distance (cc) = 10mm
- The inner skull width at cc level (CC) = 82mm



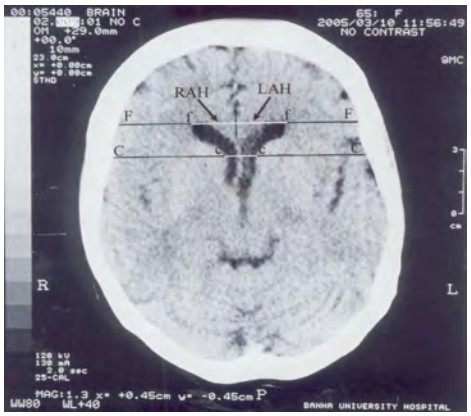
Axial CT image of anterior horn of lateral ventricles of a female-aged 24 years (FG2) showing:

- The bifrontal distance (ff)= 31.15mm
- The inner skull width at ff level (FF) = 92.30mm
- Right anterior horn width (RAH) = 13.85 mm
- Left anterior horn width (LAH) = 17.31 mm
- The bicaudate distance (cc) = 6.92mm
- The inner skull width at cc level (CC) = 99.23mm



Axial CT image of anterior horn of lateral ventricles of a female-aged 55 years (FG3) showing:

- The bifrontal distance (ff)= 32.5mm
- The inner skull width at ff level (FF) = 97.5mm
- Right anterior horn width (RAH) = 17.5 mm
- Left anterior horn width (LAH) = 15 mm
- The bicaudate distance (cc) = 17.5mm
- The inner skull width at cc level (CC) = 107.5mm



Axial CT image of anterior horn of lateral ventricles of a female-aged 65 years (FG4) showing:

- The bifrontal distance (ff)= 36.43mm
- The inner skull width at ff level (FF) = 92.14mm
- Right anterior horn width (RAH) = 17.14 mm
- Left anterior horn width (LAH) = 19.28 mm
- The bicaudate distance (cc) = 12.86mm
- The inner skull width at cc level (CC) = 187.85mm

Discussion

The current study detected a progressive steady increase of cerebral hemisphere at level of measurement of bifrontal (FF distance) and bicaudate distances (CC distance); however the differences among age groups were non-significant, irrespective of gender. However, both FF and CC distances were significantly wider in males compared to females till age of sixty, whenever, the difference became non-significant. Mean width of both horns of lateral ventricle gets wider with aging in both sexes, but the difference between age groups was non-significant till age of sixties when width of RAH and LAH became significantly wider, in males and females, respectively. Bifrontal and bicaudate indices, used as a measure of ventricular width in relation to the width of the cerebral hemisphere; showed non-significant difference between all age groups of females and between males and females in all age-groups till age of sixties. In females, the bicaudate index, the difference was non-significant between G1 and G2, but was significantly larger thereafter, while in males both indices were significantly higher in G4 compared to G3. The difference of bicaudate index between males and females was non-significant sixties when the index was significantly higher in males compared to females.

These findings illustrated the impact of aging and gender on ce-

rebral and ventricular dimensions and go in hand with that previously reported in literature; wherein Preul et al.[16] suggested that a decrease in cortical thickness and increase in ventricular width occur with normal aging. Ekinci et al.[17] detected that female subjects have less cerebral, cerebellar and brain stem volumes compared to males, although there was no statistically significant difference between genders and concluded that volumetric composition of the cerebrum, cerebellum and brain stem does not show sexual dimorphism in 24 years old people.

Akdogan et al.[18] found the ratio of total brain ventricle volume to total brain volume was comparable between the two genders, mean volume fraction of total ventricle volume to total brain volume was found to be 1.21% in the first and 3.37% in the last decades and the mean volume fraction was found to increase significantly with age. Giorgio et al.[19] reported widespread reductions in gray matter (GM) volume from middle age onwards but earlier reductions were detected in frontal cortex with widespread age-related deterioration in white matter (WM) microstructure young adulthood onwards.

Choi et al.[20] found no difference in anterior commissural (AC) volume between genders in their 20s, but in their 40s, the AC volume of males was less than that of females and no difference in AC volume between females in their 20s or 40s; while in men AC volume in their 40s was less than that in their 20s and concluded that there were gender-influenced differences in AC volume changes related to aging.

Inano et al.[21] investigated age-related and gender-related differences in the volumes of 18 neuroanatomical structures in total of 861 normal subjects and found significant gender differences in all neuroanatomical volumes which were significantly related to age, except for the caudate nucleus, pallidum, and 4th ventricle. Trimarchi et al.[22] found significant gender- and age-related volume differences and that the increase in lateral ventricle volume appears to be a constant, linear function of age throughout adult life.

Multiple studies tried to explore mechanisms underlying these changes; Kremen et al.[23] strongly suggested that genetic influences on lateral ventricular volume are increasing with age and accounted for 32-35% of the variance in lateral ventricular volume in childhood, but about 75% of the variance in late middle and older age. Shook et al.[24] using mice model of glial scarring similar to that detected along the lateral ventricle surface in aged humans found substantial ependymal cell loss resulted in reactive gliosis and the gliotic regions showed morphologic and phenotypic characteristics similar to those found in aged humans and 3D modeling together with volume measurements revealed that mice with ventricle surface scarring developed expanded ventricles, independent of neurodegeneration.

Ryan et al.[25] used MRI to measure GM, WM, hippocampal volume, corpus callosum, cerebrospinal fluid (CSF), total intracranial volume (ICV) and white matter lesions (WML) in 582 nondemented older subjects and found men and women currently on hormonal therapy, but were not past users had significantly smaller ratios of gray matter to ICV and increased atrophy (CSF/ICV ratio) compared to women who had never used hormonal therapy and that women with the estrogen receptor (ESR1 rs2234693 C) allele had significantly smaller WML.

Wåhlin et al.[26] detected significant negative relationships between the volume of several brain regions and measures of intracranial pulsatility with the strongest relationships concerned the temporal lobe cortex and hippocampus, and a positive relationship between intracranial pulsatility and ventricular volume and concluded that elderly subjects with high intracranial pulsatility display smaller brain volume and larger ventricles, supporting the notion that excessive cerebral arterial pulsatility harms the brain.

Conclusion

Cerebral and lateral ventricle dimensions showed steady increase with age and being larger in males than in females. Subjects

older than 60 years showed significant reduction of bifrontal and bicaudate indices with evident difference according to gender.

Acknowledgment

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AGE-RELATED MEASUREMENTS AND INDICES OF NORMAL HUMAN BRAIN VENTRICULAR SYSTEM: A COMPUTED TOMOGRAPHY STUDY

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MANUAL ANAL DILATATION FOR TREATMENT OF CHRONIC ANAL FISSURES: OUR EXPERIENCE

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Abstract

Objective: *In this study we aim to clarify whether the manual Anal dilatation is still viable in a modern surgical setup.*

Methods: *A total of 55 patients who underwent manual anal dilatation for chronic anal fissures from May 2012 till January 2013 were studied prospectively.*

Results: *43 of the 55 patients were male and 12 were female (male: female=3.58:1), aged between 20 to 60 years. The fissure was posterior at 6 o'clock position in 44 patients (80%) and anterior at 12 o'clock position in 8 patients (14.5%). Mean duration of hospital stay was 1.5 days. At the end of 6 weeks post op, only 37 patients (67.3%) were symptom free with the remaining 18 patients (32.7%) having complications. Among the complications, the commonest was ulcer persistence in 12 patients (21.8%). 3 patients had incontinence for flatus (5.5%), 2 had recurrence of ulcer (3.6%) and 1 had incontinenece for feces (1.8%). Mortality was 0%.*

Conclusions: *Anal fissure is a common benign ano-rectal condition. Once chronicity is established, it is difficult to treat and almost always warrants surgical management. Although manual anal dilatation is a simple and easy procedure, it is associted with significant rates of complication. For long term definitive treatment, manual anal dilatation should be abandonned completely in favour of other surgical techniques like lateral subcutaneous sphinterotomy.*

Key words: *Chronic anal fissure, fissure-in-ano, manual anal dilatation, anal stretch, Lord's anal dilatation*

Introduction

Anal fissure is a common benign ano-rectal condition associated with significant morbidity. It presents as a linear ulcer in the lower anal canal, which in 85% to 90% of cases is in the midline posteriorly, in 10% to 15% is in midline anteriorly and lateral positions being least frequently encountered[1]. When examined under anaesthesia, it appears as a longitudinal tear in the anal canal mucosa and ano-derm below the dentate line. When traction is applied on the sides of the anal canal, the fissure appears to be triangular in shape, with it's apex near the dentate line and base towards the anal verge.

Etiologically anal fissures can either be primary (idiopathic) or secondary. The primary variant is the more common presentation. Secondary fissures arise in the presence of primary pathological conditions like Crohn's disease, ulcerative colitis, other granulomatous diseases, cancers and Human Immunodeficiency Virus[1,2]. The presence of lesions in atypical positions and/or multiple lesions point towards a secondary etiology.

The first effective method found to be beneficial for treating anal fissures was manual anal dilatation. The first recorded instance of this technique was in 1838 by Récamier[3,4], thereafter there have been on and off periods of its popularity. Peter Lord performed this procedure on a large number of patients in 1968-69. He put forward this technique primarily for the treatment of second and third degree haemorrhoids and it was also used as a more agressive means of treating chronic anal fissures[5]. Watts et el showed favorable results in their case series, leading to its renewed popularity [6]. Anal dilatation is still practiced in some hospitals of Libya. There are quite a few reasons why this operation is still favored, the foremost of these being the simplicity and the relatively short learning curve.

There have been various studies done in the past with varying conclusions[6,7]. In this study we aim to present our experiences in managing chronic anal fissures by manual anal dilatation in order to clarify the role of this method in a modern Libyan surgical setup.

Patients and Methods

This prospective study was conducted in the Surgical Department of Derna Hospital.

A total of 55 patients with chronic anal fissure who underwent manual anal dilatation for a period of 9 months between May 2012 and January 2013 were included in this study. Out of these 43 were male and 12 were female patients. All clinically diagnosed patients of chronic anal fissure between 20 and 60 years of age, of either sex were included.

A detailed history was taken which included duration of pain along with other associated symptoms like constipation, bleeding per rectum, discharge and soiling, sentinel pile and previous treatment recieved. Anal examination was performed and all data about the anal fissure recorded. Criteria as set by The Standard Task Force, American Society of Colon and Rectal Surgeons to identify chronic anal fissures were used[8]. Fissures failing to heal within six weeks despite straightforward dietary measures, fissures with indurated margins and lack of granulation tissue with secondary features like sentinel skin tag, hypertrophied anal papilla were termed as chronic anal fissures.

Manual uncontrolled anal dilation was performed on all of the patients under general or spinal anaesthesia. The patients were placed in lithotomy position. Clinical examination and digital rectal examination were performed again before proceeding with the surgery. A fully lubricated index finger of each hand was placed in the anal canal after one and other. Then exerting gentle but continuous out word pressure and with gradual relaxation of the internal sphincter, the middle finger of each hand was also placed in the anal canal. During this procedure the hands repeatedly moved all around in order to relax all the segments of the lower part of the internal sphincter. The procedure was stopped when the internal anal sphincter was so much relaxed that the anal canal was accepting four fingers (two fingers of each hand) at a time without much force.

Post operatively the patients

were kept nil orally till their full recovery from the anesthesia. Effective postoperative analgesia was ensured. Warm water sitz baths two times daily were started from the next morning. After their discharge from hospital patients were followed up on weekly basis for 6 weeks. They were also advised to have high intake of vegetables, high fiber diet and 10 ml of olive oil daily. During every visit any change in symptoms and signs of patients were recorded.

Results

Out of the 55 patients, 43 (78.2%) were male and 12 (21.8%) were female (Figure1), male to female ratio was 3.58 : 1, with age range between 20 years to 60 years. The fissure was posteriorly located at 6 o'clock position in 44 (80%) patients and anteriorly located at 12 o'clock in 8 (14.5%) patients (Figure 2).The presenting complaints in these patient were painful daefecation and constipation in all 55 patients, bleeding per rectum in 49, discharge per rectum in 25, sentinel pile in 30 and pruritus ani in 13 patients (Figure 3).

Analysis of the result of the operation (anal dilatation) was based on the symptomatic improvement and the healing of the fissure (epithelialazation) of the ulcer. These were carried out on weekly basis for a duratin of six weeks, the mean stay in the hospital following anal dilatation was 1.5 days, the duration of the disappearance of the symptoms ranged from post operative day 1 to post operative day 28.

The duration of healing of the ulcer ranged from post operative day 2 to 6th post operative week, the mortality during the period of the study was zero (0%). By the end of the 6th week we note that only 37 patients (67.3%) had no symptoms with complete healing of the ulcer, in the remaining 18 (32.7%) patients, 1(1.8%) was having some degree of incontinence for stool and flatus, 3 (5.5%) patients had incontinence for flatus only, 12 (21.8%) patients had persisting ulcer and 2 (3.6%) patients developed signs and symptoms of recurrent anal fissure after healing in the 4th post operative week (Figure 4).

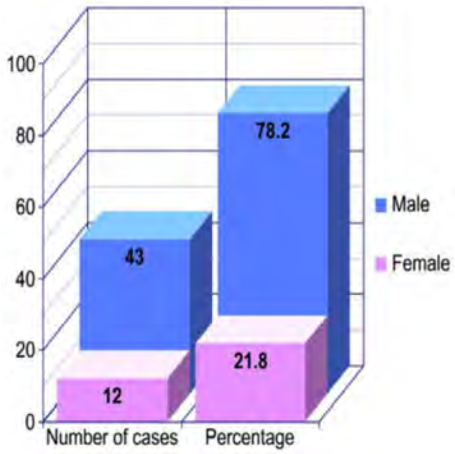

Fig. 1: Sex distribution

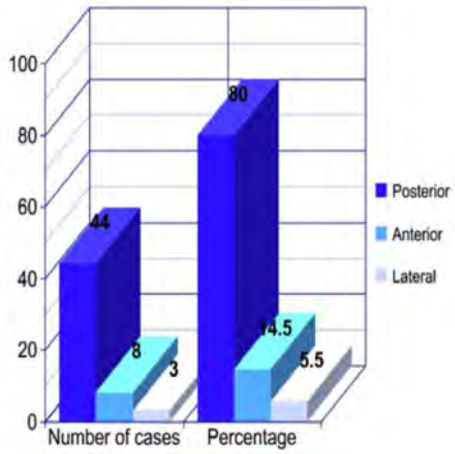

Fig. 2: Position of fissure.

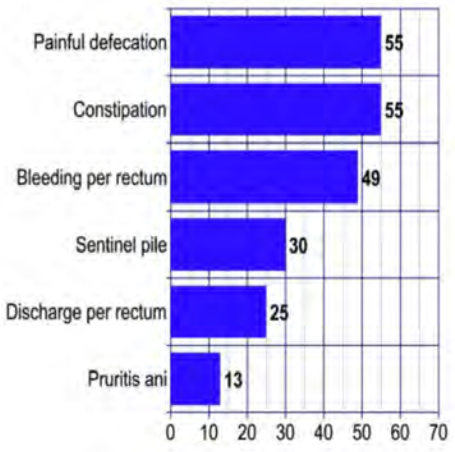

Fig. 3: Symptoms at presentation

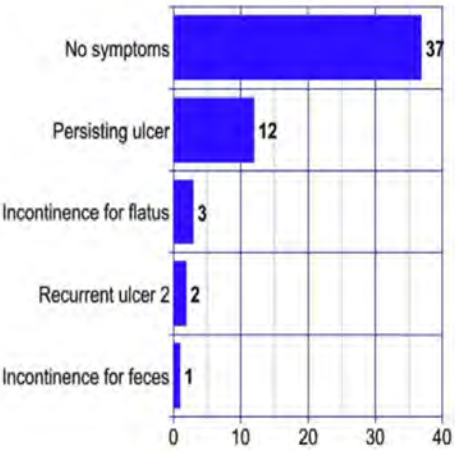

Fig. 4: Post operative complications.

Discussion

Amongst benign ano-rectal diseases, anal fissure is one of the more commonly encountered conditions. In most acute cases, complete healing could be achieved by conservative or medical treatment [9,10]. The treatment of chronic cases though, is still a very contentious topic[11]. In the vast majority of chronic cases though, surgical intervention is invariably warrented due to its immediate benefit by relief of symptoms and better healing rates[11]. These cases are associated with significant morbidity and are dificult to manage. The objective of surgical treatment is to decrease lower anal tone as a consequence of which there will be better healing.

Surgical intervention could be in the form of manual dilatation (uncontrolled and controlled) or sphincterotomy.

Manual anal dilatation gained renewed popularity in the 1960s after it showed promising results in a number of studies[6]. A randomised controlled trial done by Marby et al. showed that manual anal dilatation gave better results than lateral subcutaneous sphincterotomy (LSS)[12] Yet again in a comparitive study done in 1989 by Giebel et al., anal dilatation was stated to be superior to LSS[13]. Numerous studies were done showing consistent success rates in the range of 87% to 100%, but with inconsistent recurrence rates ranging from 0% to 56%[14].

In the present series we found that anal dilation resulted in complete healing in only 67.3% of the patients, this is much lower than what previous studies have shown[15,16,12,17]. The rates of complications are consistent with those cited in previous series [4,18,19]. The descrepency noted in literature could be due to high inter operator variability. Another point of interest to be noted is that in our series the male to female ratio was found to be much higher than what is cited in literature [19,17]. Further demographic studies need to be undertaken to find out if this is significant.

There is no single reliable way to standardize uncontrolled manual anal dilatation and both the external and internal anal sphinc-

ter can be disrupted and damaged in an irregular manner. Endosnographic studies done by Speakman et al. and by Nielsen et al. have shown that in most of the patients there would be irregular disruption of the anal sphincter [20,21].

Nowadays the most widely accepted surgical modality for treating chronic anal fissures is lateral internal sphincterotomy[22]. It was first introduced by Notaras et al. In 1969[23], since then numerous studies have shown it to be superior to uncontrolled anal dilatation [4,15], having lesser rates of post operative incontinence and recurrence.

Conclusion

Manual anal dilatation is without doubt a simple and easy procedure, and can give immediate relieve to patients suffering from chronic anal fissures. But the high rates of complications associated with this procedure increase the morbidity of this chronic malady further and in the long run increase patient re-attendence rates, discomfort, economic burden and most of all affect the patient pscychosocially. Taking into consideration the various studies done before and the findings of this series, manual anal dilatation should be completely abandoned in favour of other surgical modalities like LSS. Regular surgical audits should be undertaken in departments to ensure that practices match internationally accepted standards.

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MANUAL ANAL DILATATION FOR TREATMENT OF CHRONIC ANAL FISSURES: OUR EXPERIENCE

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HISTOPATHOLOGY OF THYROID NODULES: A STUDY OF 300 THYROID LESIONS AT DERNA, LIBYA

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Abstract

Objective: *To determine various thyroid disorders manifesting as thyroid nodule and also to evaluate the histomorphology of the lesions.*

Methodology: *A total of 300 patients with clinically thyroid nodules admitted to surgery department at Alwahda Hospital, Derna, Libya, 2006-2008 were included in the study. Thyroid function tests were initially performed. Subsequently, thyroid ultrasound and FNA of the thyroid nodules were performed exclusively for all euthyroid patients with normal TSH. Postoperative histopathological examination were done for all patients.*

Results: *Out of 300 patients with age ranging between 24 and 62 years, 80% were females. Two hundred (66.6%) patients were euthyroid, 60 (20%) were hyperthyroid and 40 (13.4%) were hypothyroid. Thyroid ultrasound showed multinodular goiter in 72 (36%), solid nodules in 70 (35%), cystic nodules in 52 (26%) and diffuse enlargement in 6 (3%) patients. FNA showed that 86% of cases were non neoplastic and 14% were neoplastic. Among the neoplasms, 2% were malignant papillary carcinoma and 12% were benign follicular neoplasms. Lobectomy were done to 169 cases (82 cases right, 41 cases were left and 46 cases were isthmusectomy), and total thyroidectomy in 6 cases. Histopathology examination showed that multinodular goiter was the commonest non-neoplastic lesion representing 36% of all cases, adenometous nodules 26%, colloid cysts 8%, Hashimoto 6%, hyperplastic nodules 5%, toxic nodules 5% and lymphocytic thyroiditis in 4% of cases. In neo-*

plastic lesions, papillary carcinoma was the commonest malignan representing 1% and benign lesions were diagnosed as follicular adenoma representing 19% of cases. A female preponderance was seen for both non neoplastic and neoplastic conditions. The nodules involved the right side more commonly than the left.

Conclusion: *Thyroid nodules are extremely common especialy multinodular goiter and frequently benign. For the most accurate diagnostic approach, fine needle aspiration biopsy should be done.*

Keywords: *Thyroidectomy. Thyroid hormones, FNAC, histopathology, Multinodular goiter, Follicular adenoma, Papillary carcinoma.*

Introduction

Palpable thyroid nodule is a common reason for seeking medical advice all over the world. It occurs in 4-7% of the population, but nodules found incidentally on thyroid ultrasound suggest a prevalence of 19-67%.[1]

The majority of thyroid nodules are asymptomatic, and they are 4 times more common in women then men.[2] The incidence increases with age, history of radiation exposure, and diet containing goitrogenic material.[3] The primary aim in investigating thyroid nodules is to exclude malignancy that occurs in 5% of nodules.[4] Fine needle aspiration (FNA) biopsy provides highly accurate cytological information from which a definitive management plan can be arranged and has similar sensitivity and accuracy levels of frozen section.[4]

Neoplasms of the thyroid have a wide spectrum of phenotype, which range from benign follicular lesions to violently aggressive anaplastic cancer.[5] Papillary carcinoma is t e most common malignancy of the thyroid, and its pathologic diagnosis is based on demonstration of characteristic cytohistologic features.[6] Total thyroidectomy is considered the preferable initial surgical approach for papillary thyroid cancers when there is no evidence of lymph node metastasis.[7]

The overall morbidity of total thyroidectomy includes temporary hypocalcemia (1%), temporary re-

current nerve palsy (3%), permanent recurrent nerve palsy (1%), hemorrhage (3%), tracheomalacia (5%), and wound infection in (3%).[5]

The aim is to determine various thyroid disorders manifesting as thyroid nodule and also to evaluate the histomorphology of the lesions.

Subjects and Methods

A total of 300 patients with clinically thyroid nodules admitted to surgery department at Alwahda Hospital. Derna, Libya, 2006-2008 were included in the study. Thyroid function tests were initially performed. Subsequently, thyroid ultrasound and FNA of the thyroid nodules were performed exclusively for all euthyroid patients with normal TSH. Postoperative histopathological examination were done for all patient s.

Results

Out of 300 patients with age ranging between 24 and 62 years, 80% were females. Two hundred (66.6%) patients were euthyroid, 60 (20%) were hyperthyroid and 40 (13.4%) were hypothyroid. Results were shown in figures 1,2,3 and 4.

Thyroid ultrasound showed multinodular goiter in 72 (36%), solid nodules in 70 (35%), cystic nodules in 52 (26%) and diffuse enlargement in 6 (3%) patients

FNA showed that 86% of cases were non neoplastic and 14% were neoplastic. Among the neoplasm: 2% were malignant papillary carcinoma and 12% were benign follicular neoplasm. Lobectomy were done to 169 cases (82 cases right, 41 cases were left and 46 cases were isthmusectomy), near total thyroidectomy in 25 cases and total thyroidectomy in 6 cases.

Histopathology examination showed that multinodular goiter was the ommonest non-neoplastic lesion representing 36% of all cases, adenometous nodules 6%, colloid cysts 8%, Hashimoto 6%, hyperplastic nodules 5%, toxic odules 5% and lymphocytic thyroiditis in 4% of cases.

In neoplastic lesions: papillary carcinoma was the commonest malignant representing 1% and benign lesions were diagnosed as

follicular adenoma representing 19% of cases.

A female preponderance was seen for both non neoplastic and neoplastic conditions. The nodules involved the right side more commonly than the left.

Fig. 1: Thyroid hormone results.

Fig. 2: Thyroid ultrasound results.

Fig. 3: Thyroid FNA results.

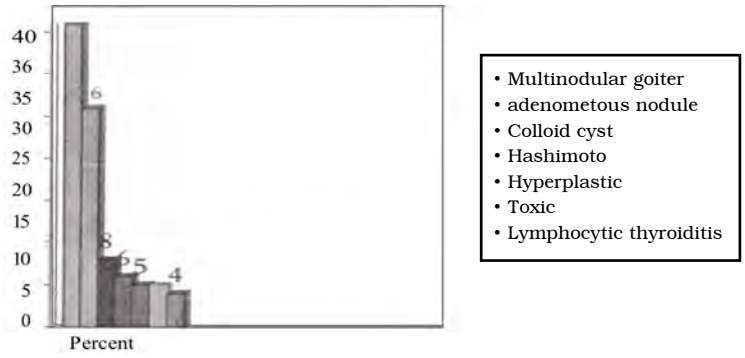

Fig. 4: Thyroid Histopatholog y results.

Discussion

Goiter is seen in general population with a frequency varying between 4-10%. The prevalence of thyroid cancer is quite rare. Between 3-11% of thyroid pathologies will be malignant.[6, 7]

In a study malignancy in thyroid gland disease detected in about 10% cases. Carcinoma of the thyroid gland is uncommon, but there is a wide geographical variation in its incidence. In the UK the annual incidence is about between 2-3/100,000 population.8 In America, 11000 cases of thyroid carcinoma are reported every year and more than 1 person die of thyroid cancer each year.[9] In India thyroid cancer is 1% of all head and neck cancers. The clinical challenge has been to identify the malignant nodules preoperatively and thus minimize the indication for surgery in benign lesions.[3,10,11]

Several procedures have been developed to obtain groups of cells or tissue from thyroid nodules. The most commonly used procedure is fine-needle biopsy (FNB), which is considered the most accurate and cost-effective tool in the preoperative investigation of thyroid nodules and has been proposed as the procedure of choice. The cytological results following FNB are divided in benign, malignant, indeterminate and non-diagnostic 1and a final result can be obtained within 24 h. The use of FNB has almost halved the percentage of patients undergoing thyroidectomy and has doubled

the yield of malignancy in patients who finally undergo surgery, thereby reducing the cost of medical care. Technically, FNB can be performed with aspiration using a syringe [fine- needle aspiration (FNA)] or without aspiration [fine-needle capillary (FNC)] and can be guided only by palpation [palpation-guided FNB (P-FNB)] or by ultrasound [ultrasound-guided FNB (US-FNB)].[13-17]

FNAC is commonly used for the diagnosis of thyroid pathology. Many authors have reported a very high accuracy. The sensitivity ofFNAC varies from 70% to 95%.[18]

According to the guidelines of the American Association of Clinical Endocrinologists, FNA is believed to be the most effective method, available for distinguishing between benign and malignant thyroid nodule, with a sensitivity and specificity approaching 96% depending on the experience of the person performing the biopsy and the skills of the cytologist interpreting the slides.[1] In a study, only 88 patients out of 100 presented with solitary thyroid nodules underwent surgery after performing FNA. Patients with benign thyroid diseases detected by FNA underwent surgery either to relieve pressure symptoms or for cosmetic purposes.[21-24]

US guidance enhances the diagnostic accuracy of FNB, as it helps the physician to direct the needle tip to the desirable site. It also helps to avoid adjacent structures, that is vessels in close vicinity to the nodule or areas of central necrosis, which often yield nondiagnostic specimens However, no study has to date compared P-FNB vs. US-FNB on the same nodules. US-FNB is usually performed on smaller nodules than P-FNB, which results in selection bias in most published studies.[25-28]

In the present study, out of 300 patients with age ranging between 24 and 62 years, 80% were females. Two hundred (66.6%) patients were euthyroid, 60 (20%) were hyperthyroid and 40 (13.4%) were hypothyroid. Thyroid ultrasound showed multinodular goiter in 72 (36%), solid nodules in 70 (35%), cystic nodules in 52 (26%) and diffuse enlargement in 6 (3%) patients. FNA showed that

86% of cases were non neoplastic and 14% were neoplastic. Among the neoplasms, 2% were malignant papillary carcinoma and 12% were benign follicular neoplasms. Lobectomy were done to 169 cases (82 cases right, 41 cases were left and 46 cases were isthmusectomy), and total thyroidectomy in 6 cases. Histopathology examination showed that multinodular goiter was the commonest non-neoplastic lesion representing 36% of all cases, adenometous nodules 26%, colloid cysts 8%, Hashimoto 6%, hyperplastic nodules 5%, toxic nodules 5% and lymphocytic thyroiditis in 4% of cases. In neoplastic lesions, papillary carcinoma was the commonest malignan representing 1% and benign lesions were diagnosed as follicular adenoma representing 19% of cases. A female preponderance was seen for both non neoplastic and neoplastic conditions. The nodules involved the right side more commonly than the left.

Routine use of US-FNB is not recommended due to cost considerations. When US-FNB is performed, care should be taken to avoid contamination of the specimens with US gel. Although US-FNB is expected to further diminish the already limited FNB complications, there is not sufficient direct evidence from the comparative studies of P-FNB vs. US-FNB. Anyway, even US guidance does not nullify the post-FNB complications.[29-32]

Thyroid fine-needle biopsy (FNB) is a simple, reliable, inexpensive and generally safe diagnostic procedure in the management of thyroid nodules. Post-FNB local pain and minor haematomas are the most common complications, while serious complications seem to be rare. Given that use of FNB minimizes unnecessary surgery and subsequent operative morbidity and mortality as well as the fact that the majority of FNB complications resolve spontaneously, the overall safety of FNB is not questioned. However, awareness of the potential complications and careful estimation of the risk-benefit ratio in an individual basis may further decrease the low morbidity of FNB. In this systematic review we tried to collect and summarize all reported clinical complications following diagnostic thyroid FNB,

aiming to make physicians aware of possible complications and to provide preventive measures to avoid them.[33-36]

A total of 184 patients with nonpalpable thyroid nodules (less than 1.5 em in diameter) were examined by means of ultrasound - guided fine-needle aspiration biopsy. Patients were included in the study based on sonographic findings implicating possible malignant nature of nodules. RESULTS. During 1997-2002, 204 ultrasound-guided fine-needle aspiration biopsies of thyroid nodules were performed; findings were nondiagnostic in 5.9o/o of cases. In 59.8% of cases, cytological examination revealed benign lesions; in 11.8%, suspected cancer; and in 22.5%, malignant lesions. Eighty-five patients underwent subsequent surgery with histological examination of specimens obtained. In 45 cases, cytological diagnosis of malignant or suspected thyroid cancer was confirmed by histological examination after surgery.[37-40]

Conclusion

Thyroid nodules are extremely common especially multinodular goiter and frequently benign. For the most accurate diagnostic approach, fine needle aspiration biopsy should be done.

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