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SEXUAL DIMORPHISM IN OXYTOCIN SECRETING NEURONS IN ALBINO RAT HYPOTHALAMUS: IMMUNOHISTOCHEMICAL STUDY

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Abstract

Background: *Sexual dimorphism in the brain is a complex phenomenon affected by the genetic factors, sex hormone and environmental inputs. Sex difference in the nervous system offers a valuable prospective to learn about the relationships between neuroanatomy and behavior.*

Aim of the work: *This work aimed to study the differences among oxytocin immunoreactive (OTIR) neurons in the hypothalamus between male and female rat and possible underlying mechanisms.*

Material and methods: *40 adult and senile albino rats of both sex were used, they were divided equally into 4 groups; adult male, adult female, senile male and senile female were used. All animals were sacrificed and specimens from anterior part of hypothalamus were prepared and stained with Hx & E and OT immunereactivity stains. Sections were subjected to image analysis for measuring the size and the number of the OTIR neurons. All data were subjected to statistical analysis.*

Results: *The number of OTIR neurons in both supraoptic and paraventricular nuclei was more in adult female than male rats. With senility, both male and female rats showed reduction in number compared to adult group in both nulei. Male rat showed reduction in number of neurons, while female showed hypertrophied neurons. Conclusion: Data from the present study showing that sexual dimorphism in OTIR neurons may be due to hormonal changes with age.*

Introduction

Brain sexual differentiation is a complex developmental phenomenon influenced by the genetic back-ground, sex hormone secretions and environmental inputs including pollutions [1].

Oxytocin (OT) hormone is a neuropeptide of the hypothalamo-neurohypophyseal system secreted by magnocellular neurons in the supraoptic and paraventriclar nuclei of the hypothalamus. OT is known to be important for milk ejection and uterus contraction in females and for erection and seminal emission in males. These sex-specific functional properties imply differential distribution of OT in the hypothalamus[2]. Sexual dimorphism in the nervous system offers a valuable prespective to understand the way in which steroid hormones affect neural function and behavior [3].

The present study was designed to show differences among oxytocin immunoreactive neurons (OTIR) in the hypothalamus between male and female and possible underlying mechanisms.

Material and Methods

Animals used:

Forty adult and senile albino rats of both sex with average weight 200-250gm, obtained from animal house of Mansoura faculty of medicine, were used and divided into the four groups:

A- Adult animals (8 ws-6ms).

I- 10 male rats.

II-10 non pregnant female rats.

Detected by vaginal opening, in proestrous stage of estrous cycle and confirmed by vaginal smear.

B- Senile animals (24 months and over).

I- 10 male rats.

II-10 female rats.

Histological Assessment:

Animals were sacrified and heads were dissected. The brain was carefully dissected and removed[4]. The anterior part of the forebrain at the level of optic chiasma, contained supraoptic and paraventricular nuclei of hypothalamus were taken. The specimens were processed for paraffin sections. Coronal sections at thickness 5 µm were cut and stained with hematoxylene and eosin[5], and at thickness 10 µm

for oxytocin immunohistochemestry[6]. All slides were examined using an Olympus microscope at X100 and X400 magnification.

Quantative evaluation of OTIR neurons & Statistical Analysis :

By using Leica Qwin 500 image analyzer computer system (England). The diameters and number of oxytocin secreting neurons in supraoptic and paraventricular nuclei in each group were measured in a five, non overlapping fixed fields. The data obtained were subjected to statistical analysis using independent samples t-test. The significance level considered was $P \leq 0.05$.

Results

The SON was related to the lateral end of the optic chiasma. It appeared as a homogeneous mass (Fig. 1). The PVN appeared in wing shaped manner on each side of third ventricle (Fig. 2).

In SON and PVN, OT immunreactivity was mainly cytoplasmic, extending to processes. OTIR neurons appeared with large perikarya, large rounded and vesicular nuclei.

In both sexes in adult and senile groups, the mean number of OTIR neurons in PVN of adult male and adult female (616 ±20.7, 823±25.6) was significantly higher than in SON of adult male and adult female (562 ± 52.1, 723±56) (Fig. 11). In the same time, The mean size of OTIR neurons in PVN of adult male and adult female (16.3±4.3, 17.6±5.1 µn) was significantly less than in SON of adult male and adult female (20±6.2, 23±5.7 µn) (Fig. 12).

In this work,OTIR neurons were more in number in adult female rat than adult male rat (Fig. 3,4,5,6). The mean number of OTIR neurons in SON and PVN in female (723 ± 56, 823 ± 25.6) was significantly higher than in male rats (562 ± 52.1, 616 ± 20.7) (Fig. 11). Also the mean size of OTIR neurons of SON and PVN was more in adult female (23 ± 5.7, 17.6 ± 5.1 µn) than adult male (20 ± 6.2, 16.3 ± 4.3 µn) with no significant difference (Fig. 12).

After senility, both PVN and SON, OTIR neurons decreased in number in male and female rats when compared with adult rats (Fig. 7,8,9,10) The mean number of OTIR neurons of SON and PVN in senile female (698 ± 21.1, 798 ± 29.3) was significantly higher than that in senile male (345 ± 30, 445 ± 23.5) (Fig. 11).

As regard the mean size of OTIR neurons of SON and PVN, the mean size of OTIR neurons of SON and PVN in senile female (28.5 ± 6.4,19.5 ± 2.4µn) µn was significantly higher than that in senile male (16.1 ± 2.8,14.8 ± 4.8 µn) (Fig. 12).

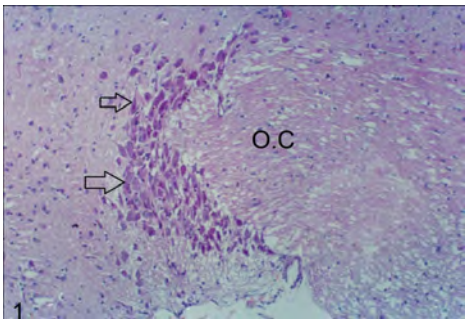

Fig (1): Photomicrograph of a coronal section in the hypothalamus of adult rat showing supraoptic nucleus (arrow) related to lateral end of optic chiasma (O.C). (H&E x 100)

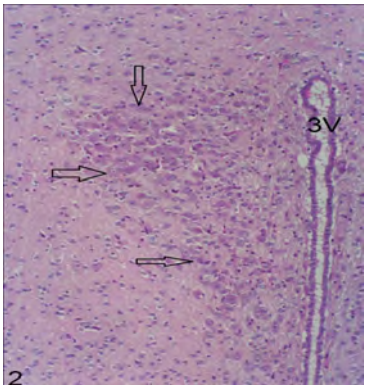

Fig.(2) : Photomicrograph of a coronal section in the hypothalamus of adult rat stage showing paraventricular nucleus (arrow). It appeare triangular in shape on each side of third ventricle (3V). (H&E x 100)

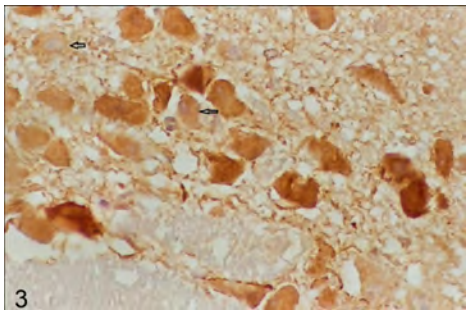

Fig. (3) : Photomicrograph of a coronal section in SON of adult male rat showing neurons with large perikarya and vesicular nucleus having OT cystoplasmic positive reaction (arrow). (OTimunoreactivity x 400).

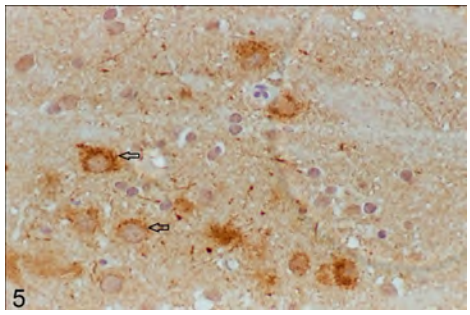

Fig. (5) : Photomicrograph of a coronal section in PVN of adult male rat showing neurons with large perikarya and vesicular nucleus having OT positive cystoplasmic reaction (arrows). (OTimunoreactivity x 400) .

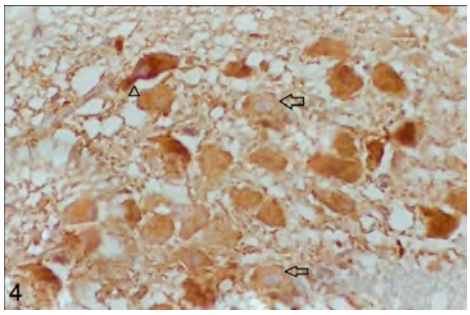

Fig. (4) : Photomicrograph of a coronal section in SON of adult female rat showing increased number of cytoplasmic OTIR neurons with large perikarya and vesicular nucleus (arrow). The positive OT reaction extends to processes (arrow head). (OTimunoreactivityx400)

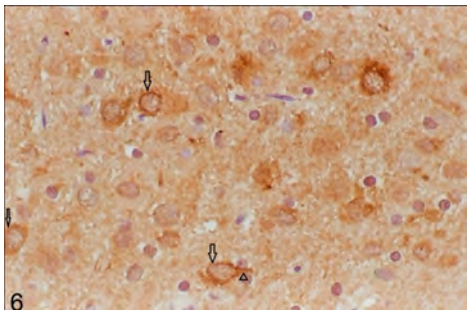

Fig. (6) : Photomicrograph of a coronal section in PVN of adult female rat showing neurons with large perikarya and vesicular nucleus having OT positive cystoplasmic reaction (arrows). The reaction extends to the processes (arrow head) (OTimunoreactivityx400)

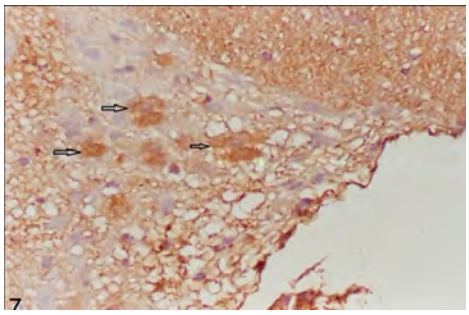

Fig. (7) : Photomicrograph of a coronal section in SON of senile male rat showing reduced number and size of cytoplasmic OTIR neurons (arrows). (OTimunoreactivity x 400) .

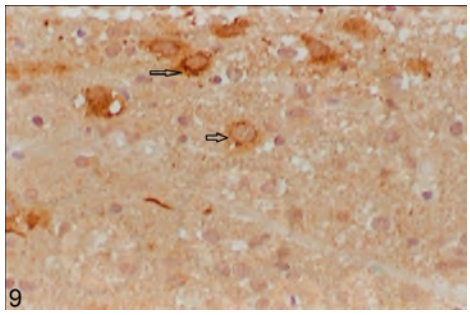

Fig. (9) : Photomicrograph of a coronal section in PVN of senile male rat showing reduced number and size of cytoplasmic OTIR neurons (arrows). (OTimunoreactivity x 400) .

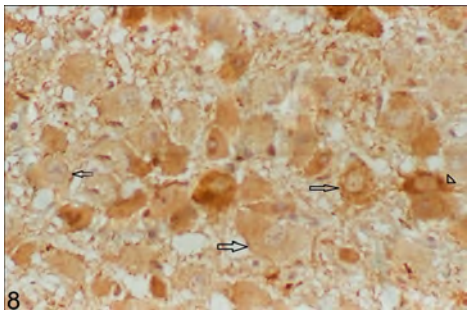

Fig. (8) : Photomicrograph of a coronal section in SON of senile female rat showing increased number and size of cytoplasmic OTIR neurons (arrows). The processes show positive reaction (arrow head). (OTimunoreactivity x 400) .

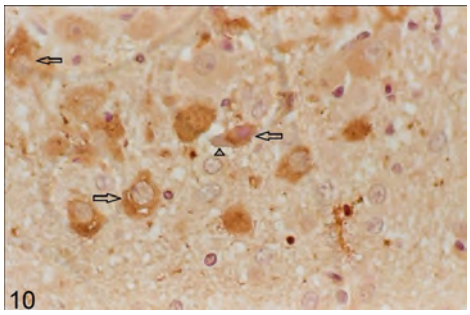

Fig. (10) : Photomicrograph of a coronal section in PVN of senile female rat showing increased number and size of cytoplasmic OTIR neurons (arrows), The reaction extends to processes (arrow head). (OTimunoreactivity x 400).

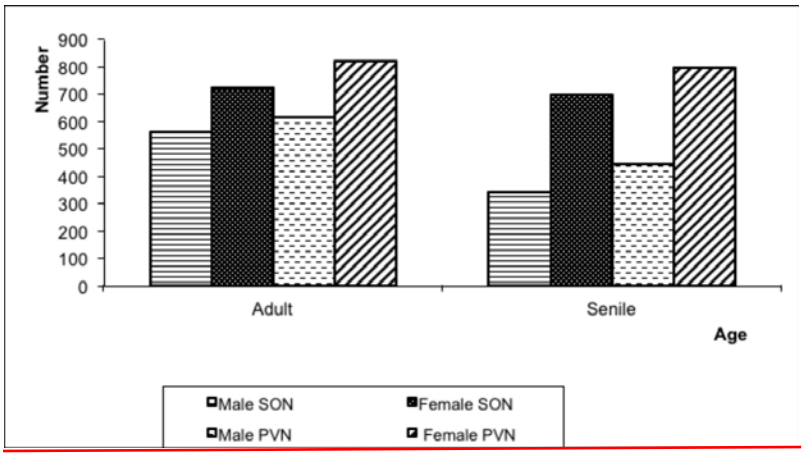

Fig. (11) : Histogram showing the mean number of OTIR cells in SON & PVN of male and female rats at different age groups.

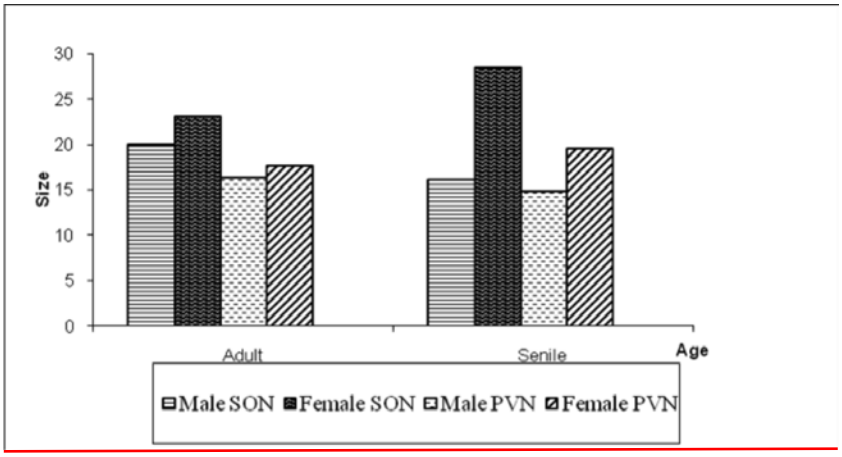

Fig. (12) : Histogram showing the mean size of OTIR cells (µn) in SON & PVN of male and female rats at different age groups.

Discussion

The current study revealed the differences among oxytocin secreting neurons in the hypothalamus between male and female in different age groups.

The histological findings of the current study revealed the location of the SON was in anterior part of hypothalamus and located lateral to optic chiasma, consisted mainly of magnocellular neurons that secrets OT hormone. This finding is in agreement with the previous results of[7]&[8], they reported that SON is related to the lateral end of the optic chiasma, and disappeard posteriorly at the level of the tuber cinereum, consisted mainly of magnocellular neurons that secrets OT,VP hormones.

The PVN nucleus of the hypothalamus was a wing-shaped structure on either side of the third ventricle. This finding is in agreement with that reported by[9] & [10].

OT immunoreactivity was in all cases confined to the perinuclear cytoplasm and to thier processes. Similar finding was reported by[2]&[11] whom stated that Long processes with numerous varicosities were considered axonal pathways.

In both sexes of all groups, OTIR neurons number in PVN was found significantly more than SON. In the same time, size of OTIR neurons was significantly more in SON than PVN. This result comes in agreement with the finding of [12] in human &[13] in rat. A possible explanation of this difference is that the volume of PVN is larger than SON. In rat, PVN is 0.5 mm^3 [10], while SON is .167 mm^3 [14].

The number of OTIR neurons was found significantly higher in adult female than male rats in both SON and PVN. Similar results were stated by[2] in rat and[15] in the mouse hypothalamus. A possible mechanism accounting for the greater number of OT secreting cells in female is that OT potentiates the stimulating effects of corticotropin-releasing factor on adenohypophysial corticotrophic cells and it enhances LH release[16]. These neuroendocrine

functions are probably more pronounced in females[17]. Also,[18] announced that estrogen modulates oxytocin gene expression in region of rat supraoptic and paraventricular nuclei that express estrogen receptor beta (ERß) in OT neurons[19].

The histomorphometric results revealed statisttically, no significant difference was found in the size of OTIR neurons between adult male and adult non pregnant female rats. This is in agreement with results of[20] in human and[2] in rat. The lack of changes in the size of the OT neurons could be explained according to[21] that it might either mean that OTIR neurons are not affected by long term changes in estrogen levels or that functional changes in this group of neurons are not readily reflected in changes in cell size in the oxytocin neurons.

After senility, both PVN and SON OT neurons decreased in number in male and female rats when compared with adult rats. Similar results were pronounced by [22] that 15-21 % neuronal loss was observed from the young adult stage to the senile stage in rat. Also, this comes in agreement with results of [23] who found statistically significant reduction in the mean number of OTIR cells in the PVN and SON with advancing male age.

As regard the size of OTIR neurons in the present study, the senile male group showed reduced size of OTIR neurons, while the senile female group showed hypertrophied OTIR neurons in both SON and PVN. These results are in contrast with[23] who documented increase in the size of OTIR neurons in SON and PVN of both sexes in the senile stage in rat, and[24] who found that no changes in size of OTIR neurons were observed in senile human male or postmenopausal females. According to[25] in the course of aging, possibly triggered by the decrease in estrogen levels in postmenopausal women, the neuronal activity in the SON and PVN increases in females and cells became enlarged. Also, In female rats, aging is associated with a decrease in both estrogen and ERβ immunoreactive cell numbers in

the hypothalamus[26]. This difference between male and female OTIR neurons in aged hypothalamus could be also explained by the result of[27] who reported that, the senile male demonstrates a 3 to 4-fold age-related increase in AR (androgen receptor) immunoreactive cells in the hypothalamus. During this same period, total serum testosterone levels decrease profoundly. Thus, there is a significant inverse relationship between ARIR cells and circulating testosterone.

Moreover, no difference in ERβ or ERβ cell numbers occurs in the aging male[26]. ERβ is responsible for mating behavior in male rat, this accounts for unchanged sexual behavior in senile male[28].

Data from the present study shows that sexual dimorphism in OTIR neurons may be due to hormonal factors i.e sex hormons, and this difference is obvious after puberty and with aging.

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ROLE OF GHRELIN HORMONE IN GASTRIC MOTILITY

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Abstract

Ghrelin is the natural ligand for the growth hormone secretagogue receptor (GHSR) and its receptors are found all over the body. It is clear that the mechanism of ghrelin induced changes in gastrointestinal motility has not been fully understood. Therefore, the principal objectives of this study were to investigate, the effect of ghrelin on the gastric motility in rat and to study the possible mechanisms of its action. Albino rats of both sexes weighing 100-180 gm were used. They were classified into 4 groups each group consists of 6 experiments. Segments from the gastric fundus and gastric antrum, were mounted in 5 ml organ bath chambers containing continuously oxygenated krebs solution. The motility was continuously recorded with a power lab recording unit and further analyzed with chart 7 software. Ghrelin (10^{-8}M) showed a significant decrease in the basal fundic motility and the addition of the nitric oxide synthase inhibitor, L-NAME (10^{-4}M) before application of ghrelin prevented its inhibitory effects. However in the antrum ghrelin (10^{-8}M) showed a significant increase in the basal antral motility and the addition of atropine sulphate (10^{-6}M) before ghrelin prevented its stimulatory effect on the antral contraction. All these finding could explain the involvement of both cholinergic and nitregeric neurons in mediating the effects of ghrelin on gastric motility.

Introduction

Ghrelin is a 28- amino acid peptide, predominantly produced by endocrine cells in the oxyntic mucosa of the stomach[1]. Ghrelin has also been found in the small

intestine, pancreas, liver, kidney, placenta, testis, ovary, pituitary gland and hypothalamus in both human and rodents[2].

Ghrelin is the natural ligand for the growth hormone secretagogue receptor (GHSR). And its receptors are found all over the body including the bowel, pancreas, stomach, heart, lungs and brain[3].

Because of the presence of ghrelin and its receptors in the stomach and small intestine, it would be reasonable to expect its participation to gastric and bowel functions. In fact, a number of studies have provided evidence of a close relationship between ghrelin and gastric motility. Some studies have indicated that ghrelin enhance gastric motility and gastric emptying in rodents, ginea pig and humans[4,5,6], stimulates small intestinal transit[7] and reverses post operative gastric ileus[8]. However, it has been also reported that ghrelin has no effect on gastric emptying in humans[9] and dose not stimulate gastrointestinal motility in dogs[10]. In addition, some studies reported that the increasing effect of ghrelin on gastric motility was completely eliminated by pretreatment with atropine or bilateral cervical vagotomy[11]. Interestingly, another study demonstrated that intravenous administration of ghrelin induced fasting like motor activity in both stomach and duodenum in vagotomised rats[12].

Aim of the study:-

It is clear that the mechanism of ghrelin induced changes in gastrointestinal motility has not been fully understood. Therefore, the principal objectives of this study was to investigate, the effect of ghrelin on the gastric motility in rat and to study the possible mechanisms of its action.

Material and Methods

Albino rats of both sexes weighing 100-180 gm were adapted to room and cage environments for 2 weeks. They were caged 3 per cage in a temperature controlled room (22°C) with a 12 hour light - dark cycle and were maintained during this period on commercial chow diet. They had free access to tap water.

Procedure:

After cervical dislocation of the rat, the abdominal and chest walls are opened and the gut from the esophagus to the rectum is dissected, removed and immediately placed in a beaker containing aerated krebs solution (118.1 mM Nacl, 4.69mM kcl, 2.5 mM Cacl2, 25 mM NaHco3, 1.2 mM KH2 Po4 1.2mM Mgso4 and 11 mM glucose). Segments from the gastric fundus and gastric antrum, were mounted in 5 ml organ bath chambers containing continuously oxygenated krebs solution. The temperature was maintained at 37°C. The lower end of the tissue segments was anchored to the bottom of the chamber and the other end connected to an isotonic transducer.

The motility was continuously recorded with a power lab recording unit and further analyzed with chart 7 software. The segments were allowed to equilibrate for 60 min; during this time the solution was changed every 15 min.

The experiments were classified into 4 groups each group consists of 6 experiments:-

Group I: to study the effect of ghrelin (10^{-8}M) on the basal motility.

Group II: to study the effect of ghrelin on the motility of after adding acetylcholine (Ach)10^{-5}M, atropine sulphate (10^{-6}M), propranolol hydrochloride (10^{-6}M), or phentolamine (10^{-6}M).

Group III: to study the effect of ghrelin on the motility after adding verapamil hydrochloride (10^{-6}M).

Group IV: to study the effect of ghrelin on the motility after adding sodium nitroprusside (10^{-6}M) or L-NAME(10^{-4}M).

Results

(1) Effects of ghrelin:

Table (1) and figure (1) show significant decrease in the amplitude and the tone of gastric fundus motility ($P<0.05$). However table (2) and figure (2) show a significant increase in the tone of gastric antral motility ($P<0.05$) and insignificant increase in the amplitude.

(2) Effects of ghrelin after Ach administration:-

Table (1) and figure (3) show a significant increase in the contraction of the fundus after addition of Ach and addition of ghrelin after Ach causes significant decrease in the fundic contraction. While table (2) and figure (4) show a significant increase in the contraction of the antrum after addition of Ach and addition of ghrelin after Ach causes significant increase in the antral contraction.

(3) Effects of ghrelin after atropine administration:-

Table (1) and figure (5) show an insignificant decrease in the contraction of the fundus after addition of atropine sulphate and addition of ghrelin after atropine causes significant decrease in the fundic contractions. While table (2) and figure (6) show a significant decrease in the contraction of the antrum ($P<0.05$) after addition of atropine sulphate and addition of ghrelin after atropine causes insignificant change in the effect of atropine on the antral contraction.

(4) Effects of ghrelin after phentolamine administration:-

Table (1) and figure (7) show an insignificant decrease in the contraction of the fundus after addition of phentolamine and addition of ghrelin after phentolamine causes significant decrease in the fundic contractions. While table (2) and figure (8) show an insignificant decrease in the contraction of the antrum after addition of phentolamine and addition of ghrelin after phentolamine causes significant increase in the antral contraction.

(5) Effects of ghrelin after propranolol hydrochloride administration:-

Table (1) and figure (9) show an insignificant increase in the contraction of the fundus after addition of propranolol hydrochloride and addition of ghrelin after propranolol hydrochloride causes significant decrease in the fundic contractions. While table (2) and figure (10) show an insignificant increase in the contraction of the antrum after addition of propranolol hydrochloride and addition of ghrelin after propranolol hydro-

chloride causes significant increase in the antral contraction.

(6) Effects of ghrelin after verapamil hydrochloride administration:-

Table (2) and figure (11) show a significant decrease in the contraction of the antrum after addition of verapamil hydrochloride and addition of ghrelin after verapamil hydrochloride causes insignificant change in the antral contraction.

(7) Effects of ghrelin after sodium nitroprosside administration:-

Table (1) and figure (12) show a significant decrease in the contraction of the fundus after addition of sodium nitroprosside and addition of ghrelin after sodium nitroprosside causes more significant decrease in the fundic contractions. While table (2) and figure (13) show an insignificant decrease in the contraction of the antrum after addition of sodium nitroprosside and addition of ghrelin after sodium nitroprosside causes significant increase in the antral contraction.

(8) Effects of ghrelin in the fundus after L-NAME:-

In table (1) and figure (14) L-NAME shows significant increase in the tone and the amplitude of the fundus motility, while addition of ghrelin after L-NAME is unable to produce its relaxing effects.

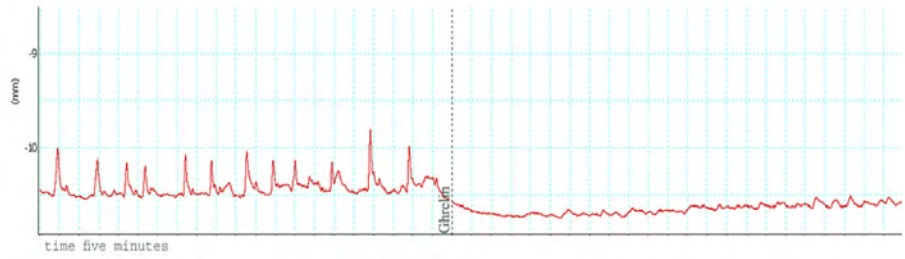

Fig (1): Effect of ghrelin (10^{-8} M) on the basal gastric fundus motility.

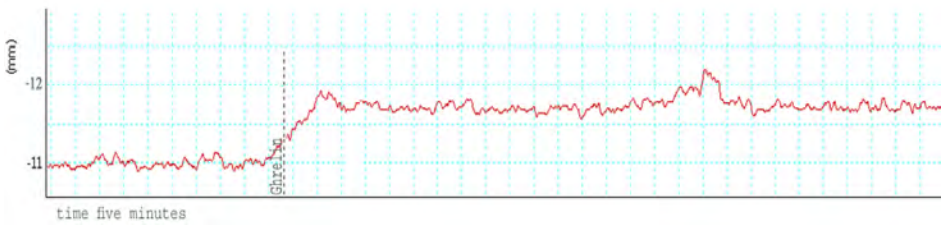

Fig (2): Effect of ghrelin (10^{-8} M) on the basal gastric antrum motility.

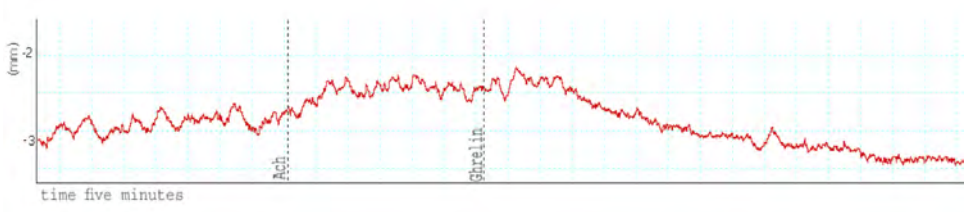

Fig (3): Effect of ghrelin (10^{-8} M) on gastric fundus motility after adding acetylcholine (10^{-5} M).

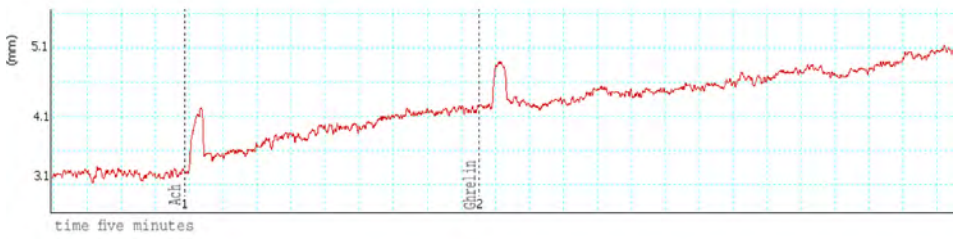

Fig (4): Effect of ghrelin (10^{-8} M) on the gastric antral motility after adding acetylcholine (10^{-5} M).

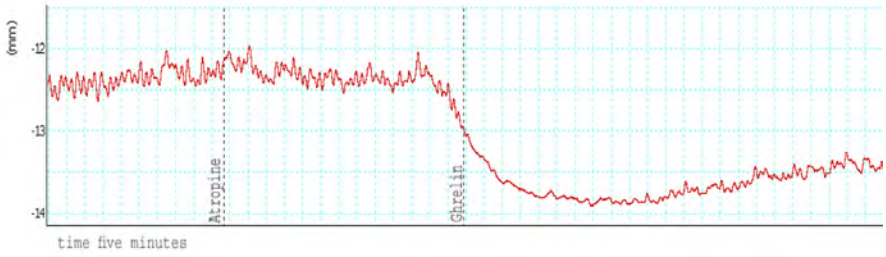

Fig (5): Effect of ghrelin (10^{-8} M) on the gastric fundus motility after adding atropine sulphate (10^{-6} M).

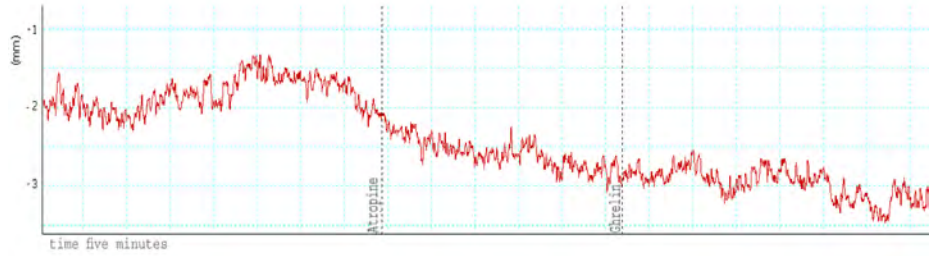

Fig (6): Effect of ghrelin (10^{-8} M) on the gastric antrum motility after adding atropine sulphate (10^{-6} M).

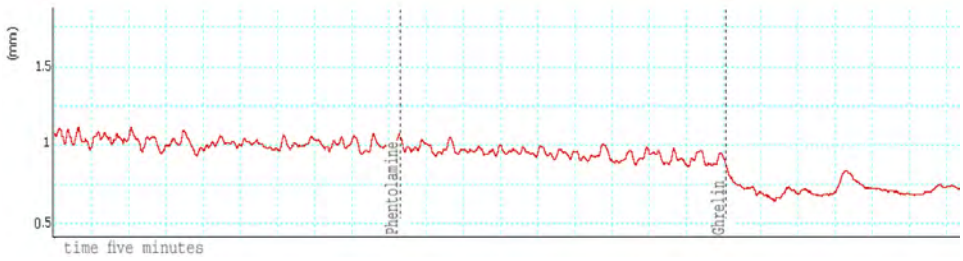

Fig (7): Effect of ghrelin (10^{-8} M) on the gastric fundus motility after adding phentolamine (10^{-6} M).

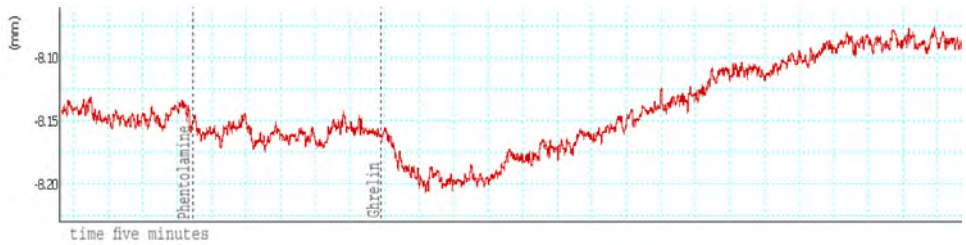

Fig (8): Effect of ghrelin (10^{-8} M) on the gastric antrum motility after adding phentolamine (10^{-6} M).

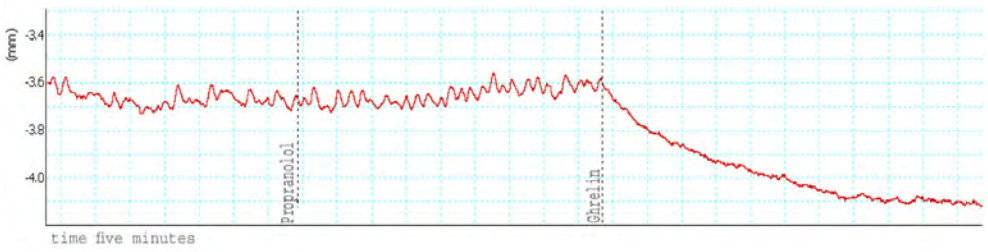

Fig (9): Effect of ghrelin (10^{-8} M) on the gastric fundus motility after adding propranolol hydrochloride (10^{-8} M).

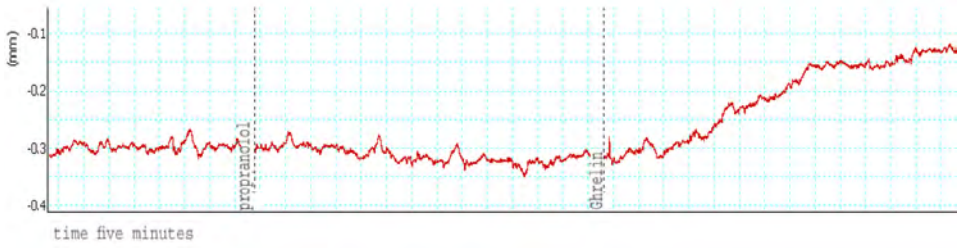

Fig (10): Effect of ghrelin (10^{-8} M) on the gastric antral motility after adding propranolol hydrochloride (10^{-6} M).

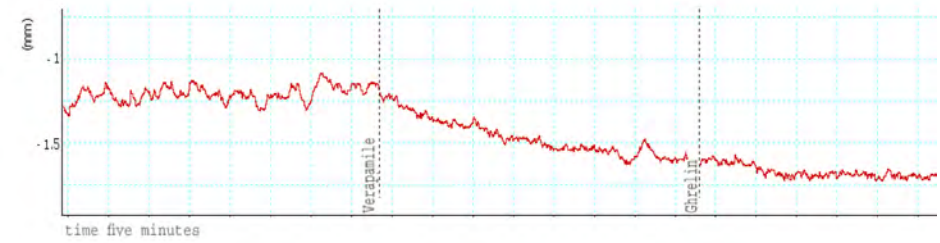

Fig (11): Effect of ghrelin (10^{-8} M) on the gastric antral motility after adding verapamil hydrochloride (10^{-6} M).

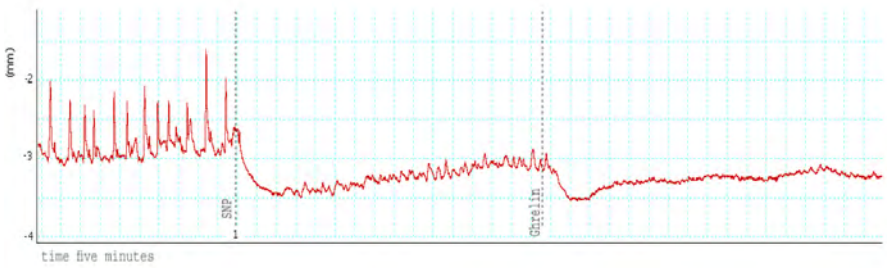

Fig (12): Effect of ghrelin (10^{-8} M) on the gastric fundus motility after adding sodium nitroprusside (10^{-6} M).

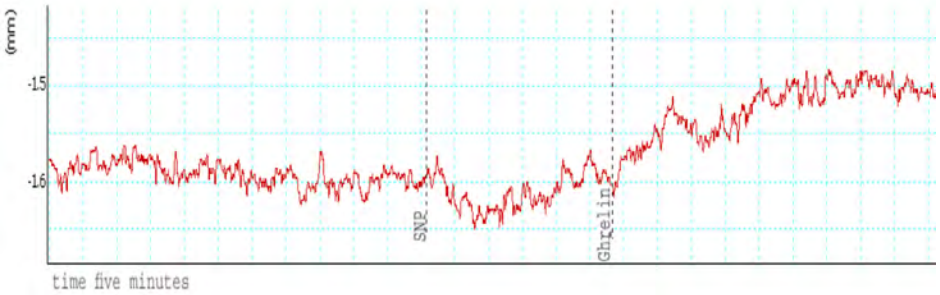

Fig (13): Effect of ghrelin (10^{-8} M) on the gastric antral motility after adding sodium nitroprusside (10^{-6} M).

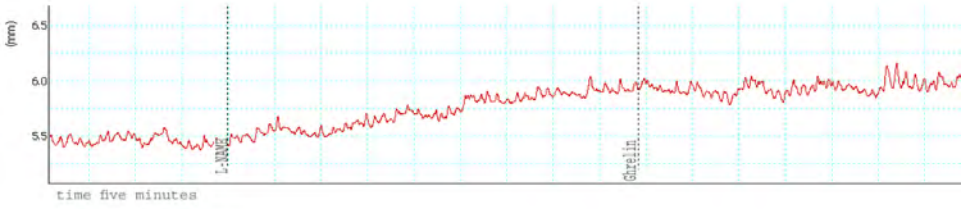

Fig (14): Effect of ghrelin (10^{-8} M) on the gastric fundus motility after adding L-NAME (10^{-4} M).

Table (1): Effects of ghrelin on the basal gastric fundus motility and its effects on motility after adding acetylcholine, atropine sulphate, phentolamine, propranolol hydrochloride, verapamil hydrochloride and sodium nitroprusside.

	Amplitude (mm)		Tone (mm)	
	Basal	Effect	Basal	Effect
Ghrelin				
Mean	0.13	0.062	- 5.26	- 7.4
Median	0.05	0.02	- 3.4	- 5
%		- 52.3		- 40.8
P1		< 0.05		< 0.05
Ghrelin after acetylcholine				
Mean	0.03	0.018	3	1.5
Median	0.03	0.015	1.25	0.15
%		- 50		-50
P2		< 0.05		< 0.05
Ghrelin after atropine sulphate				
Mean	0.17	0.06	- 5.5	- 8
Median	0.15	0.05	- 3.1	- 5
%		- 65		- 45
P3		< 0.05		< 0.05
Ghrelin after phentolamine				
Mean	0.14	0.08	- 2.33	- 3.25
Median	0.07	0.03	- 1.1	- 1.7
%		- 43		- 39
P4		< 0.05		< 0.05
Ghrelin after propranolol hydrochloride				
Mean	0.12	0.064	- 3.3	- 4.5
Median	0.053	0.03	- 3.15	- 4
%		- 47		- 36
P5		< 0.05		< 0.05
Ghrelin after L-NAME				
Mean	0.05	0.05	- 1.8	- 1.8
Median	0.02	0.02	- 2.3	- 2.3
%		Zero		Zero
P6		NS		Ns
Ghrelin after sodium nitroprusside				
Mean	0.018	0.01	- 3.4	- 4.42
Median	0.02	0.01	- 3	- 3.8
%		- 44		- 30
P7		< 0.05		< 0.05

Table (2): Effects of ghrelin on the basal gastric antrum motility and its effects on motility after adding acetylcholine, atropine sulphate, phentolamine, propranolol hydrochloride, verapamil hydrochloride and sodium nitroprusside.

	Amplitude (mm)		Tone (mm)	
	Basal	Effect	Basal	Effect
Ghrelin				
Mean	0.017	0.019	- 4.28	- 2.3
Median	0.017	0.02	- 3.1	- 1.3
%		+ 12		+ 46.3
P1		NS		< 0.05
Ghrelin after acetylcholine				
Mean	0.028	0.028	- 2	- 1.1
Median	0.025	0.03	- 1.25	- 0.25
%		Zero		+ 45
P2		NS		<0.05
Ghrelin after atropine sulphate				
Mean	0.012	0.013	0.23	0.21
Median	0.01	0.01	- 0.55	- 0.6
%		+ 7.7		- 7
P3		NS		NS
Ghrelin after phentolamine				
Mean	0.037	0.04	- 1.28	- 0.8
Median	0.025	0.025	- 1.22	- 0.15
%		+ 7.5		+ 42
P4		NS		< 0.05
Ghrelin after propranolol hydrochloride				
Mean	0.02	0.023	- 1.45	0.41
Median	0.02	0.02	- 0.65	0.43
%		+ 15		+ 128
P5		NS		< 0.05
Ghrelin after verapamil hydrochloride				
Mean	0.016	0.016	- 4.8	- 4.82
Median	0.013	0.013	- 3.7	- 3.7
%		Zero		- 0.42
P6		NS		NS
Ghrelin after sodium nitroprusside				
Mean	0.018	0.02	- 3.9	- 2.3
Median	0.018	0.02	- 2.8	- 1.8
%		+ 11		+ 41
P7		NS		< 0.05

Discussion

The present study show the effects of ghrelin on the motility of gastric fundus and antral strips in albino rats of both sexes. Ghrelin causes significant increase in the tone of the antral contractions. These findings agreed with the observations of[13,4]. Gherlin is an endogenous ligand for GHS-R[2]. Immunohistochemistry studies have demonstrated the presence of GHS-R in the stomach and colon in both rats and humans and in guinea pig ileum [3]. GHS-R is also found in the hypothalamas

and pituitary [14]. Hence it is likely that ghrelin can act at both central and peripheral levels.

In our present in vitro study ghrelin stimulates the antral motility and this stimulatory effect may be mediated through neurogenic mechanism as immunohistochemical studies have demonstrated that GHS-R is localized in the enteric nervous system (myenteric plexus) but not in smooth muscle tissue of the guinea pig, rat and human gastrointestinal tract [3].

On the other hand gherlin has a reversal effects on the fundic motility. Ghrelin causes significant decrease in the amplitude and the tone of fundus contraction. These findings are disagreed with the observations of [15] which reported a stimulatory effect of ghrelin on the rat fundic strips. The addition of the nitric oxide synthase inhibitor, L-NAME before application of ghrelin causes significant increase in the amplitude and the tone of fundic contractions and prevents the inhibitory effects of ghrelin. These observations means that, the relaxing effect of ghrelin may be most probably mediated through NO release. So, it is suggested that ghrelin acts on ghrelin receptors on nitrergic neurons and stimulates nitric oxide release which decreases the acetylcholine release from cholinergic neurons and acts on smooth muscles directly leading to its relaxation.These finding agreed with the results done by Nakamura et al, (2009) in guinea pig.

addition of Ach to the incubation media showed a significant increase the fundic and antral contractions. Ghrelin after Ach. causes more significant increase in the antral contraction as compared with the effect of Ach alone. These observations means that ghrelin potentiates the effect of Ach on the contraction of the antrum. As regard the fundic strips, ghrelin causes significant decrease in the amplitude and the tone of Ach stimulated fundic contractions.

Addition of ghrelin after atropine sulphate causes insignificant change in the amplitude and the tone of the antral segment. And these finding agreed with the

observations of[4,7,11]. Masuda et al., (2000) demonstrated for the first time that intravenous administration of ghrelin increased the number and intensity of contractions in the stomach of anesthetized rats, and that these actions of ghrelin were completely eliminated by pretreatment with atropine, or in totally vagotomized rats. Also Edholm et al (2004) and Tumer et al (2008) demonstrated that ghrelin stimulated the motility both invivo and invitro and that atropine administration blocked the effect of ghrelin. These observations means that, the peripheral effecls of ghrelin seem to be mediated via cholinergic neurons within the gut wall. Also, atropine sulphale causes insignificant decrease in the amplitude and the tone of fundic contractions but addition of gherlin after atropine causes significant decrease both in the amplitude and the tone. These observations means that ghrelin mediating its inhibitory effect on the fundus via non cholinergic mechanism.

In the present study phentolamine (receptor blocker) causes insignificant decrease in the tone and the amplitude of the contractions of the antrum. Ghrelin addition after phentolamine causes significant increase in the tone of antral contractions and significant decrease in the amplitude and the tone of fundic contractions. Also, propranolol hydrochloride (receptor blocker) causes insignificant increase in the amplitude and the tone of the contractions of the antrum. Ghrelin addition after propranolol hydrochloride causes significant increase in the tone of the antrum and significant decrease in the amplitude and the tone of fundic contractions. These observations means that ghrelin effects on the gastric motility is not mediated via adrenergic receptors mechanism.

In our study the Ca^{2+} channel antagonist, verapmil hydrochloride show a significant decrease in the tone of antral contractions Addition of ghrelin after verapamil prevents its stimulatory effects on the contractions of the antrum. Verapamil hydrochloride is a Ca^{2+} channel blocker, it blocks the voltage gated Ca^{2+} channel and thereby impede the influx of Ca^{2+} into the smooth muscle cells which

prevents the phosphorylation of myosin light chain kinase which is essential for the smooth muscle contraction.

Sodium nitroprusside (NO donor) causes significant decrease in the amplitude and the tone of fundic, contractions, but it causes insignificant decrease in the amplitude and the tone of the antral contractions. Ghrelin addition after sodium nitoprusside causes a significant increase in the tone of antral contractions. These observation support the previous mechanism of action of ghrelin in increasing the antral contractions. However, ghrelin addition after sodium nitroprasside casuses more significant decrease in the amplitude and the tone of fundic strips contractions. These observations means that ghrelin produces its effects on the fundic strips contraction via NO release from nitregeric neurons.

All these findings could explain the involvement of both cholinergic and nitrergeric neurons in mediating the effects of ghrelin on both fundic and antral contractions.

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BENHA MEDICAL JOURNAL

ROLE OF GHRELIN HORMONE IN GASTRIC MOTILITY

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REGENERATION OF SUBMANDIBULAR GLAND FOLLOWING LIGATION OF ITS DUCT RETRACES THE POSTNATAL PATHWAY OF CYTODIFFERENTIATION

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Abstract

Rat submandibular gland can regenerate following ligation-induced atrophy, eventually recovering its normal morphology and function. Previous studies have suggested that the regeneration process implies both self-proliferation of existing acini and formation of new acinar cells. Our hypothesis is that new acinar cells may differentiate from the ductal cells in a similar fashion to the process of cytodifferentiation occurring during submandibular glandular development. In this study atrophy was induced, under recovery anaesthesia, by applying ligature on the main duct of the submandibular gland. After 1 week the duct was deligated for 1, 3, 7, 14 or 21 days or 35 days and the glands collected. Tissue was prepared for histochemical study and immunohistochemstry. The histology of the regenerated glands shows several normal-looking acini, which have regained their glycoprotein content (AB/PAS positive). Regenerating tissue was characterized by the presence of branched structures ending with AB/PAS positive acinar cells. This study of rat submandibular gland regeneration suggests new acinar cells have differentiated from ducts in a similar manner to the cytodifferentiation process occurring during postnatal glandular development, furthermore, regeneration of the granular convoluted tubules from the striated ducts follows the same way as postnatal development.

Introduction

Salivary gland development represents one of the most typical example of epithelial-mesenchyme interaction[1]. It begins with the invagination of a terminal bud

from the oral epithelium into the surrounding mesenchyme which later provides the stimulus for the formation of a branched epithelial tree[2]. This process known as branching morphogenesis ,gives arise to the ductal system and marks the start of the cytodifferentiation, which in the rat submandibular gland carries on postnatally[3,4,5]. The main secretory cell type apparent at birth is not the same as in adult. In fact, the perinatal cells fall into two transient secretory cell types: TypeI (or terminal tubules cells), and TypeIII (or pro-acinar cells). The latter cell type will eventually differentiate into mature acinar cells[3,6]. During the perinatal stage of development Type III cells ultimately differentiate into mature acinar cells[7,8]. With prolonged duct ligation adult submandibular gland undergoes atrophy leading to secretory dysfunction[9-15]. The impaired function has been linked to the loss of acinar cells due to apoptosis[16,17]. Although single duct ligation may not be as effective as ligatures it has been shown that some shrunken acini are still present in the atrophic tissue created by either method but they are thought to be in a quiescent, non-functional state[9,10,11,18]. On the other hand, during early atrophy the ductal cells actively proliferate[16]. When the ligation is removed the submandibular gland is able to recover its functionregenerating the secretory tissue through both proliferation of residual acini and differentiation of new acinar cells[19,20]. In this study on the regenerated submandibular gland, we suggested that newly formed acini differentiate from unique branched structures present in this tissue. Furthermore in the same study, we followed the regeneration of the granular convoluted tubules. Therefore, the aim of this paper has been to follow the differentiation of the new acinar cells during the progression of submandibular gland regeneration following duct ligation induced atrophy, and to establish any correlation with the postnatal stage of development based on morphological evidence. The characterization of this process should provide useful information for future studies aiming to restore the glandular function.

Materials and methods

1.Experimental procedure

Twenty seven Wistar strain (250-300g) rats were used. All experimental procedures were conducted with the approval of the local ethics committee and Home Office license. Rats were divided into five groups (n = 3 each except for control = 6): one group of unoperated controls, a second group experienced 1 week of duct ligation. The third, fourth, fifth, sixth, seventh and eighth groups underwent 1 week ligation followed by deligation after 1, 3, 7, 14, 21 days or 35 days, respectively. Contralateral glands were not used as controls in this study as previously noted compensatory hyperplasia occurred when the other gland was extirpated[21], or ligated[22]. All animals were killed by an anaesthetic overdose. Atrophy was induced following the extraoral duct ligation procedure as previously described (Osailan et al., 2006a, 2006b). Under recovery anaesthesia ketamine/valpam intraperitoneally. Ketamine hydrochloride (50mg/ml, E.I.P.I.Co) and valpam (10mg/2ml, Amoun). This corresponded to about 0.5 ml/rat in the form of (0.4 + 0.1 ml of each drug, respectively), the main excretory ducts of the submandibular and sublingual glands was doubly ligated with 4/0 prolene suture less than 5 mm anterior to the hilum of the gland. A small plastic tube was applied together with the ligature in order to avoid fibrosis of the ducts (Osailan etal., 2006a, 2006b). Then washing with antibiotic was done and the incision was sutured with 4/0 prolene suture. Animals were allowed to recover from anaesthesia in a cage maintained at a warm room temperature. Aseptic conditions were used throughout the surgical procedure of duct ligation to reduce the risk of infection.

Prior to the collection of the glands following the ligation-only procedure, the presence of the ligature and the tube on the duct was confirmed in each animal. After 2 weeks the glands of the ligation-only group were removed under terminal anaesthesia (pentobarbitone 60mg / kgi.p) and weighed. In the other groups (ligation followed by deligation), the duct was de-ligated after 1 week (under recovery anaesthesia), and the glands were collected after a

further 1, 3, 5, 7, 14, 21 days or 35 days. The collection of the glands was performed under terminal anaesthesia (pentobarbitone 60mg/kgi.p.). After removal the submandibular gland was carefully dissected from the sublingual gland and weighed. Glands were fixed in 10% buffered neutral formalin 24 hours for histological purpose.

2. Histological and histochemical staining of tissue sections:

Tissue fixed in 10% neutral formalin was processed to paraffin embedding and 5 micrometers paraffin sections were cut, mounted on slides.

For general morphology tissue sections were then stained with Haematoxylin and 1% Eosin (H & E). The secretory granules inside the acinar cells were identified by Alcian Blue periodic acid Schiff's (AB/PAS) staining.

3. Immunohistochemistry on tissue sections:

The tissue sections were first de-waxed and then incubated in a solution of 3% hydrogen peroxide to inhibit the endogenous peroxidase. After being washed in phosphate-buffered saline (PBS; 0.1M), the sections were incubated with normal goat serum (DAKO, ElyUK, 1:5 dilution of PBS) to avoid non-specific binding of the primary antibody. Tissue sections were incubated with primary antibody (mouse monoclonal Anti-Actin, alpha Smooth Muscle) (DAKO clone 1A4, Ely UK, 1:50 dilution). The secondary antibody was biotinylated goat anti-rabbit polyclonal (DAKO, Ely UK, 1:400 dilution); so sections were reacted with streptavidin- biotin horse-radish peroxidase complex (DAKO, ElyUK). The peroxidase activity was visualized with diaminobenzidine tetra- hydrochloride (DAB) (0.5mg/ml) and counterstained with Mayer's Haematoxylin. A similar method was used for Ki-67 (1:50 dilution of rabbit polyclonal anti-Ki-67, ThermoScientific, Runcorn, Cheshire, UK). Sections for both antibodies were incubated at 95 1C for 15 min in citric acid buffer (pH6.0) before being incubated with the goatserum. The Ki-67 positive nuclei were counted to assess cell proliferation in the experimental tissues.

Results

1. Gland weights

Following 1 week of ligation, submandibular glands showed a reduction in weights of about 50% compared to the control (Table 1). In all deligation time points starting from 7 days after deligation till the end of the study, submandibular glands showed a significant increase in weight above the ligated glands. Overall the mean weight of submandibular glands by the end of the study was almost 95% the mean weight of normal control glands (Table 1), (Fig. 1).

2. Parenchymal elements

2.1. Acinar cells

Histological examination (H&E) of the 1 day after deligation showed the presence of several normal-looking acinar cells at the periphery of the lobules (Fig. 7). These normal-looking acinar cells, which were not apparent in the atrophic tissue (Fig. 6), exhibited normal morphology. AB/PAS histochemistry revealed that these acini had recovered their glycoprotein content, which was lost during atrophy (Fig. 20 and 21). Smooth muscle actin immunostaining revealed the presence of myoepithelial cells surrounding the shrunken acini at the periphery of the lobule 1 day after deligation. They were seen surrounding the immature acini at the 7^{th} day, then, frequent myoepithelial cells were seen surrounding mature acini and intercalated ducts at the 14th day after deligation (Fig. 28, 32 and 33).

2.2. Ducts

One of the main morphological features of atrophic glands was an increase in the proportion of ducts (Fig. 5). This characteristic was still notable in the 3 and 7 days deligated glands; however the lumen of the duct was smaller than in the atrophic gland suggesting a recovery of duct cells cytoplasm (Fig. 6, 8 and 13). However, the granular convoluted tubules started to recover 14 days after deligation, they appeared to develop from the striated ducts which well reformed at this time point (Fig. 14, 15 and 16). But the granules of the convoluted tubules started to recover 21 days after deligation to be completely recovered at 35 days after deligation (Fig.17, 18, 25 and 26). Smooth muscle actin

immunostaining revealed the presence of myoepithelial cells surrounding the dilated ducts in both ligated and 1 day deligated glands (Fig. 27 and 28).

2.3. Characterization of the branched structures :

In the 1, 3 and 7 days deligated glands, H&E staining revealed the presence of peculiar branched structures characterized by ducts ending with mature or immature acini (Fig. 8, 9 and 11). These branched structures are absent in the normal gland (Fig. 2, 3), and interestingly they displayed a configuration very similar to the structures occurring in the postnatal submandibular gland (Fig. 10, for purpose of comparison). These structures were more frequent in the 3 and 7 days deligated (12.33±2.516), (17.66±2.081) glands compared to the atrophic (1.56±0.351); however after 14 days, they disappear (Table 2). At both the deligation time points several acini at the end of the branched structures were also stained with AB/PAS revealing the presence of glycoproteins (Fig. 22 and 23). Smooth muscle actin immunostaining revealed the presence of myoepithelial cells surrounding the acini of the branched structures (Fig. 29, 30 and 31).

2.4. Cell proliferation :

Although the weight of the glands did not change significantly across the deligation time points, we detected an increase in the number of proliferating cells between day 3 and day 7 ($p \leq 0.05$), using ki-67 (Table 3). The ki-67 immunohistochemistry in all the deligation time points showed that dividing nuclei are scattered across the sections and seemed to localize mostly in the acini and only occasionally in the ducts (Fig. 37 and 38). We were able to detect proliferation in the acinar cells at the end of the branched structures (Fig. 36). The adult normal submandibular gland showed a low level of cellular proliferation within the ductal compartment (Fig. 34). The ligated gland was characterized by proliferation of several ductal cells and some non-parenchymal cells (Fig. 35).

Table (1): The weight of the submandibular gland after extraoral duct deligation compared to the control and the 7 days ligated.

Time of deligation	Number	Mean	Standard deviation	Percentage of normal
control	3	0.203	0.005	/
7 days ligation	3	0.110	0.020	55%
1 day deligation	3	0.123	0.015	60%
3 days deligation	3	0.140	0.020	70%
7 days deligation	3	0.155*	0.013	77%
14 days deligation	3	0.160*	0.020	80%
21 days deligation	3	0.173*	0.015	85%
35 days deligation	3	0.193*	0.115	95%

p (the probability) ≤ 0.05 significant in comparison to 7 days ligated.

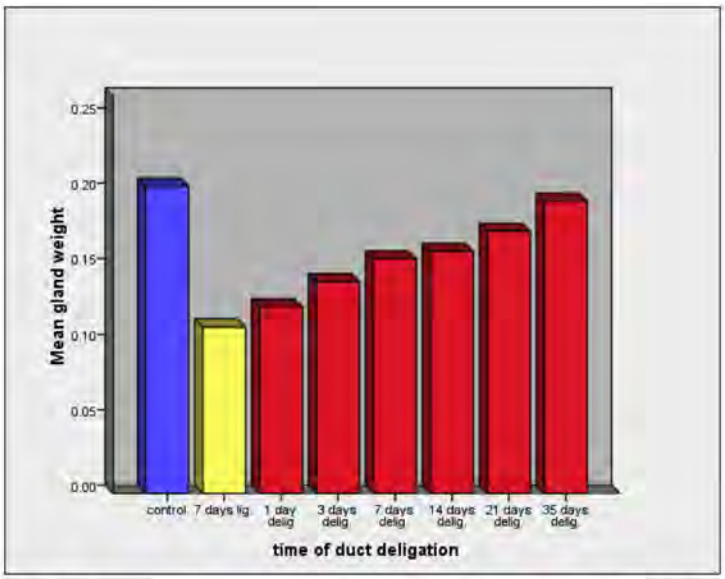
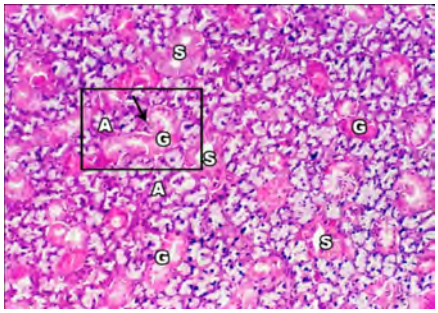
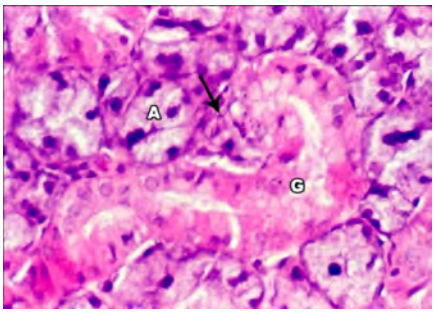


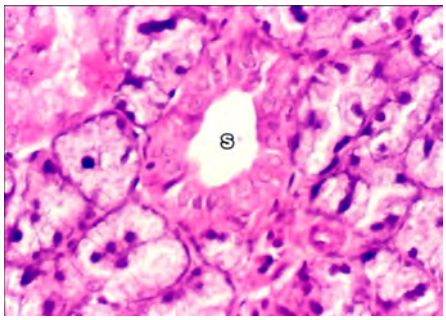
Fig.1: the weight of the submandibular gland after extraoral duct deligation compared to the control and the 7 days ligated glands.

(Haematoxylin and Eosin x 100)

Fig. 2 : Photomicrograph of the submandibular gland of control rat showing that the lobules were formed of acini (A) and a variety of ducts: the intercalated ducts (arrow), the granular convoluted tubules (G) and the striated intralobular ducts (S).

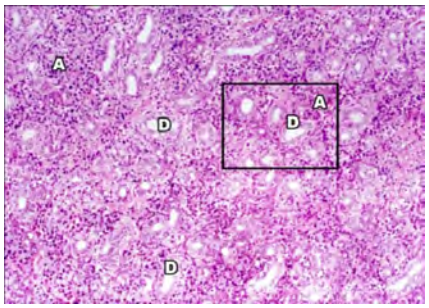






(Haematoxylin and Eosin x 400)

Fig 3,4 : Photomicrographs of the magnified area of Fig. 3 showing acini (A) and their cells were pyramidal in shape with basophilic cytoplasm, intercalated ducts (arrow) were narrow tubes lined with flat cells and extend from the acini into the granular convoluted tubules (G) which were large and filled with acidophilic granules, some cells showed apical vacuolation of the cytoplasm and their nuclei were pushed towards the base of the cells. Striated ducts (S) showed basal striations, their nuclei were rounded and situated near the centre of the cells.



(Haematoxylin and Eosin x 100)

Fig 5 : Photomicrograph of the submandibular gland 7 days after extra-oral duct ligation showing that the ducts (D) appeared to be increased in number in relation to acini (A).

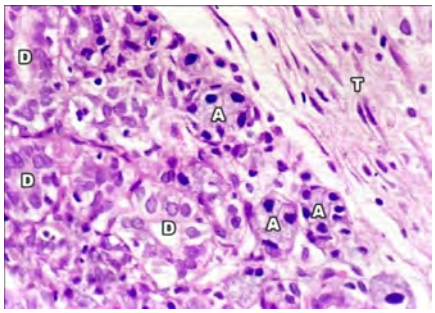
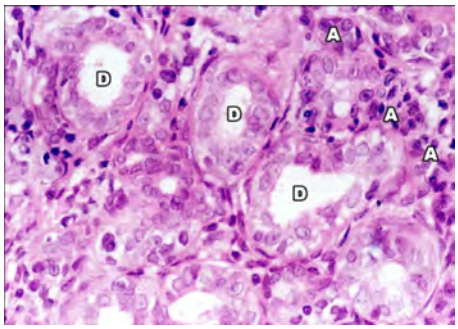
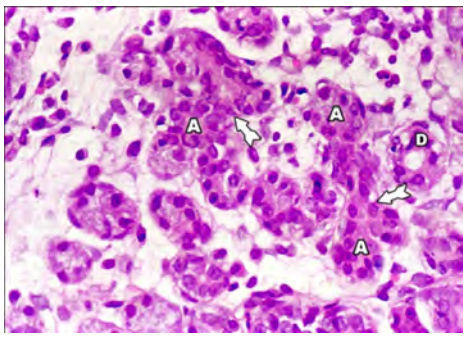


Fig. 7: Photomicrograph of the submandibular gland 1 day after extra-oral duct de-ligation showing recovery of the the acini (A) at the periphery of the lobule and large amounts of connective tissue (T) with inflammatory cell infiltration. Note: duct like structures (D) still prominent.



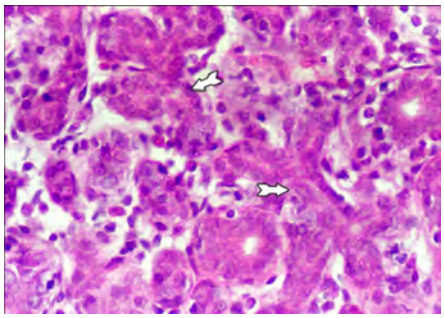
(Haematoxylin and Eosin x 400)

Fig. 6 : Photomicrograph of the magnified area of Fig. 18 showing few remaining small acini (A) with eosinophilic cytoplasm and the residual ducts had little cytoplasm with no secretory granules or basal striation so, they can be called "duct like structure" (D).

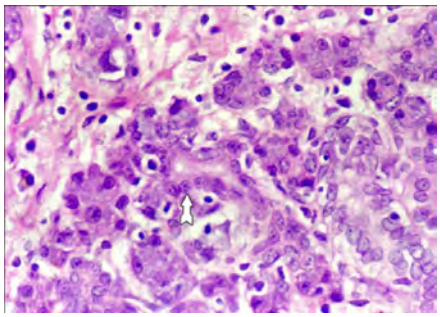


(Haematoxylin and Eosin x 400)

Fig 8 : photomicrograph of the submandibular gland 3 day after extra-oral duct de-ligation showing newly formed acini (A) arising from duct acinar structures (forked arrow).



(Haematoxylin and Eosin x 400)
Fig. 9 : photomicrograph of the sub-mandibular gland 3 day after extra-oral duct de-ligation showing increased number of duct-acinar structures (forked arrow).



(Haematoxylin and Eosin x 400)
Fig. 11 : photomicrograph of the sub-mandibular gland 7 days after extra-oral duct de-ligation showing duct-acinar (branched) structure (forked arrow) still detected.

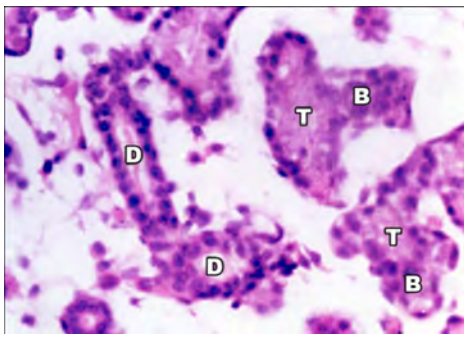
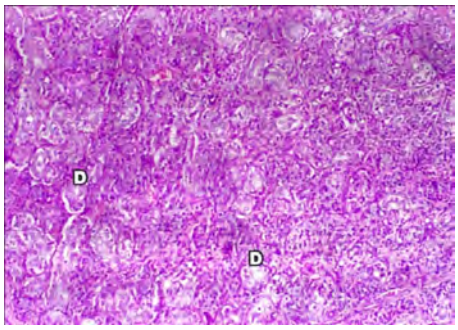
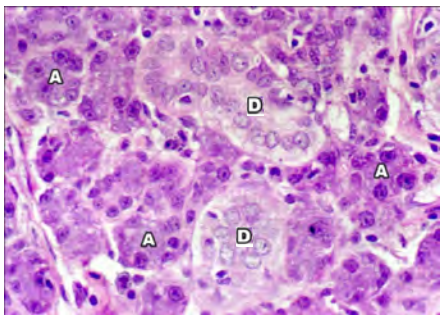


Fig 10 : photomicrograph of the sub-mandibular gland at birthv showing terminal bude (B) at the periphery of the terminal tubules (T) which arises from developing duct (D).

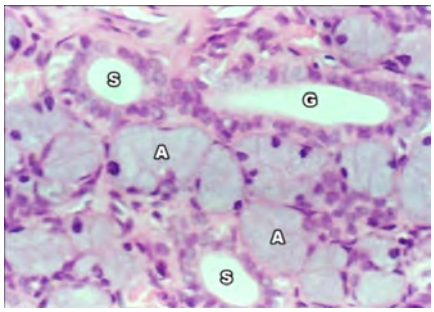


(Haematoxylin and Eosin x 100)
Fig. 12 : Photomicrograph of the submandibular gland 7 days after extra-oral duct de-ligation showing the gland became packed again and decreased ductlike structure (D).



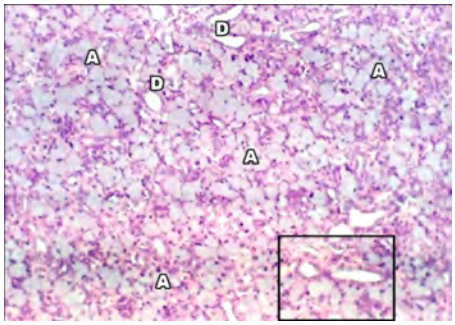
(Haematoxylin and Eosin x 400)

Fig. 13 : Photomicrograph of the submandibular gland 7 days after extra-oral duct de-ligation showing the acini (A) became more mature and the duct like structures (D) showed increased mitotic figures.



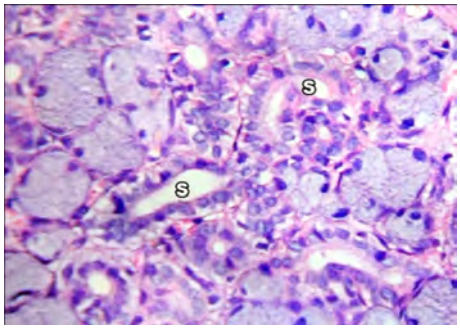
(Haematoxylin and Eosin x 400)

Fig. 15 : Photomicrograph of the submandibular gland 14 days after extra-oral duct de-ligation showing regenerating acini (A) appeared with voluminous cytoplasm and flattened nuclei dislocated basally and the acinar architecture seemed the same as that of the normal adult gland with reappearance of the striated ducts (S) and formation of the GCT (G) from the striated ducts.



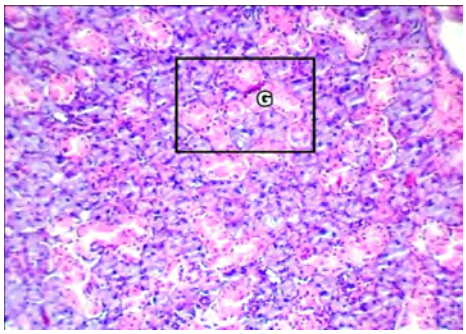
(Haematoxylin and Eosin x 100)

Fig. 14 : Photomicrograph of the submandibular gland 14 days after extra-oral duct de-ligation showing the gland was formed mainly of acini (A) with decrease duct like structures (D).

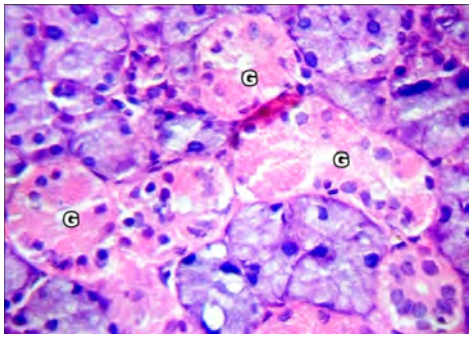


(Haematoxylin and Eosin x 400)

Fig.16 : photomicrograph of the submandibular gland 21 days after extra-oral duct de-ligation showing shortening of the basal striations and convolution of many striated ducts (S).

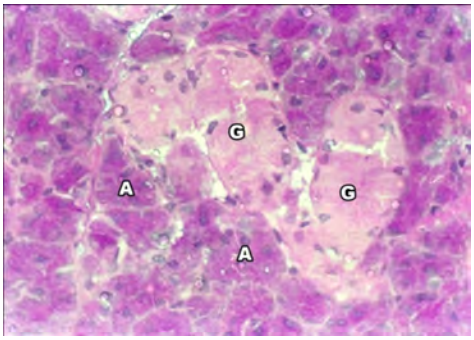


(Haematoxylin and Eosin x 100)



(Haematoxylin and Eosin x 400)

Fig.17, 18 : Photomicrograph of the submandibular gland 35 days after extra-oral duct de-ligation showing increased number, size of GCT (G) with full recovery of the secretory granules, the nuclei dislocated basally and the tubule architecture seemed the same as that of the normal adult gland.



(AB/PAS x 400)

Fig 19 : Photomicrograph of the sub-mandibular gland of control rat showing strong magenta colour in the acini (A) and weak magenta colour in GCT (G).

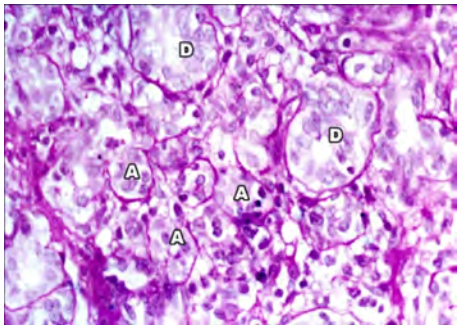


Fig 20 : Photomicrograph of the sub-mandibular gland 7 days after extra-oral duct ligation showing no secretion of any kind in the acini with interstitial fibrosis and thickening of the basement membrane of both acini (A) and ducts (D).

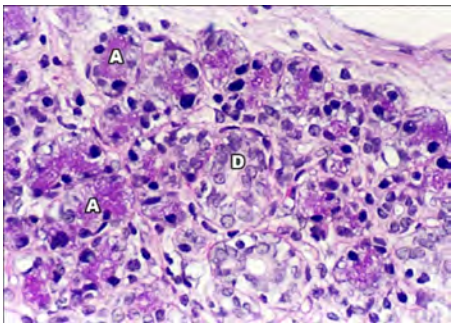
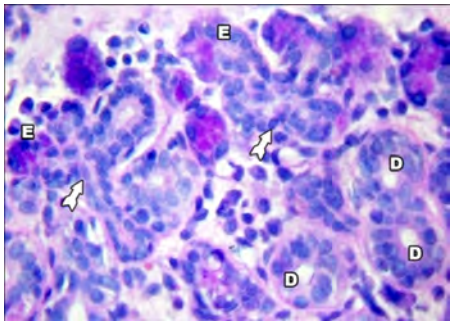
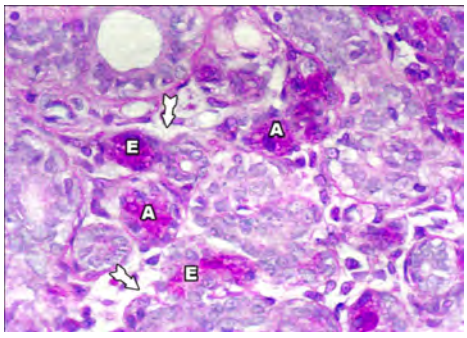


Fig 21 : Photomicrograph of the submandibular gland 1 day after extra-oral duct de-ligation showing secretory granules in acini (A) at the periphery of the lobule but not in the duct like structure (D).



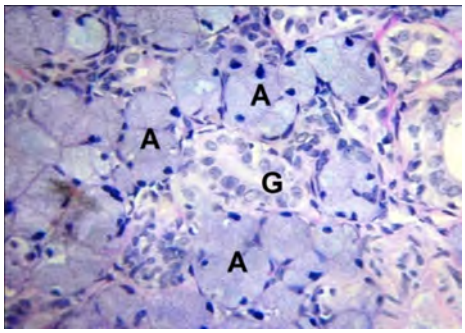
(AB/PAS x 400)

Fig. 23 : Photomicrograph of the submandibular gland 7 days after extra-oral duct de-ligation showing secretory granules in the epithelial bulgings (E) arising from branched structures (forked arrow) and no secretion in the duct like structures (D).



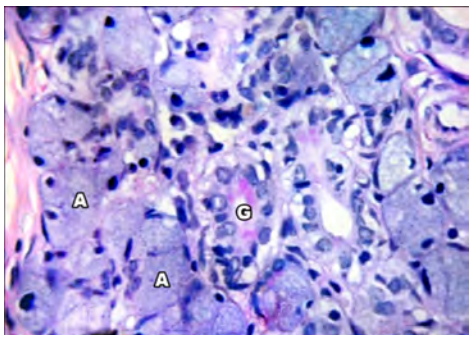
(AB/PAS x 400)

Fig. 22 : Photomicrograph of the submandibular gland 3 days after extra-oral duct de-ligation showing secretory granules in the epithelial bulgings (E) arising from duct like structures (D) as well as in the newly formed acini (A).



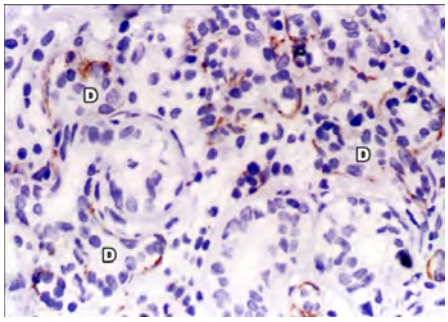
(AB/PAS x 400)

Fig. 24 : Photomicrograph of the submandibular gland 14 days after extra-oral duct de-ligation showing the acini (A) full of secretion but the GCT (G) had no secretion.



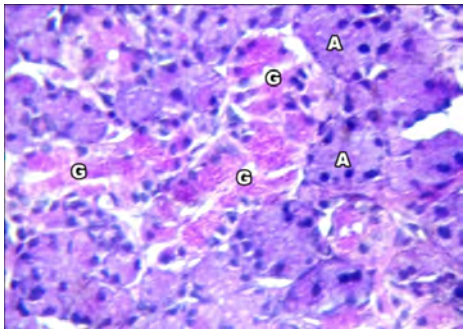
(AB/PAS x 400)

Fig. 25 : Photomicrograph of the submandibular gland 21 days after extra-oral duct de-ligation showing the acini (A) full of secretion and the GCT (G) had little secretion.



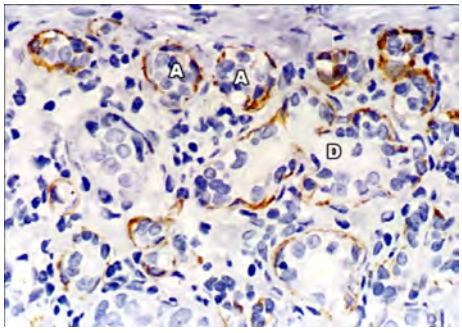
(alpha smooth actin x 400)

Fig. 27 : Photomicrograph of the submandibular gland after 7 days extra-oral duct ligation showing duct like structures (D) were surrounded by myoepithelial cells with disappearance of most of acini.



(AB/PAS x 400)

Fig. 26 : Photomicrograph of the submandibular gland 35 days after extra-oral duct de-ligation showing both the acini (A) and the GCT (G) were full of secretion.



(alpha smooth actin x 400)

Fig. 28 : Photomicrograph of the submandibular gland 1 day after extra-oral duct de-ligation showing myoepithelial cells around shrunken acini (A) at the periphery of the lobule and around the dilated duct (D).

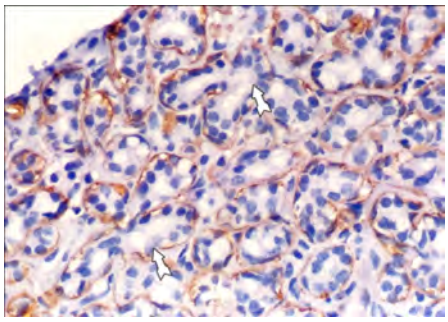


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Fig. 29 : Photomicrograph of the submandibular gland 3 days after extra-oral duct de-ligation showing myoepithelial cells around the branched (forked arrow) structure at the periphery of the lobule.

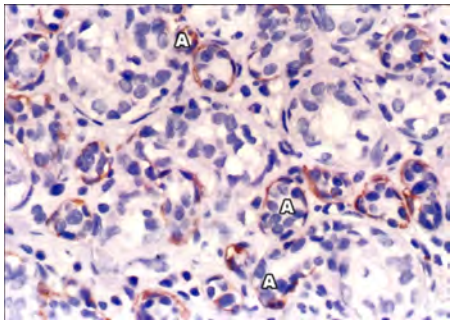

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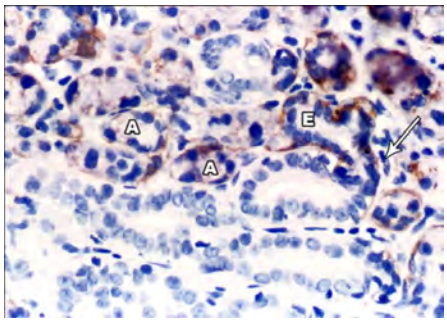
Fig. 31 : photomicrograph of the submandibular gland 7 days after extra-oral duct de-ligation showing myoepithelial cells around the growing acini (A).



(alpha smooth actin x 400)

Fig. 30 : Photomicrograph of the submandibular gland 3 days after extra-oral duct de-ligation showing myoepithelial cells around the epithelial bulging (E) from branched structure and the junction (arrow) between them.

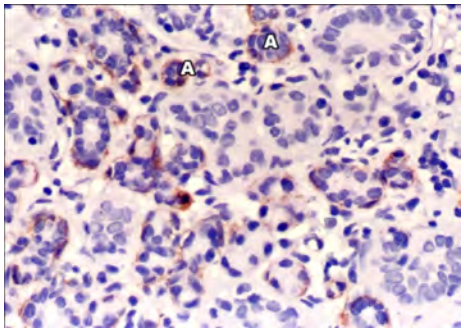


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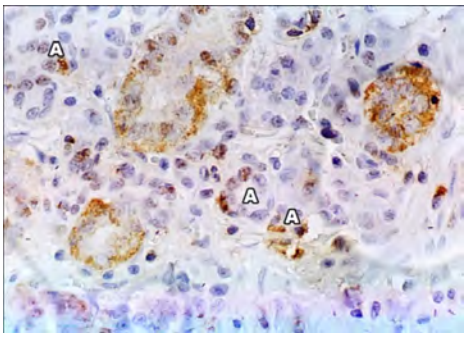
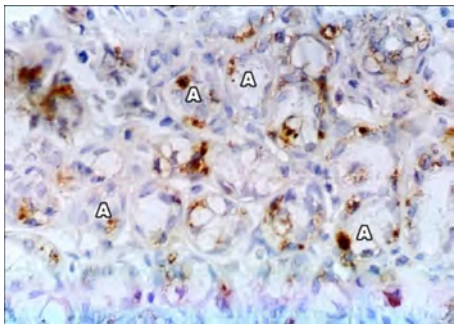
Fig 32 : Photomicrograph of the submandibular gland 7 days after extra-oral duct de-ligation showing myoepithelial cells around immature acini (A).



(Ki-67 x400)

Fig. 37 : Photomicrograph of the submandibular gland 3 days after extra-oral duct de-ligation showing several Ki 67-positive cells in immature acini (A) at the middle of the lobule.



(Ki-67 x400)

Fig. 38 : Photomicrograph of the submandibular gland 7 days after extra-oral duct de-ligation showing Ki 67-positive cells in the acini (A).

Table (2): Mean number of the branched structures after deligation of the submandibular duct.

Time of lig./delig.	Number	Mean	Standard Deviation
2 weeks lig.	3	1.56	0.351
1 day delig.	3	4.93	1.006
3 days delig.	3	12.33*	2.516
7 days delig.	3	17.66*	2.081
14 days delig.	3	0.90	0.300
21 days delig.	3	0.00	0.000
35 days delig.	3	0.00	0.000

p (the probability) ≤ 0.05 significant

Table (3): The Ki 67 labelling index for acinar cells and duct cells after deligation of the submandibular duct.

	Time of deligation	Number	Mean	Standard Deviation
Ki 67 labelling index of acini	control	3	0.00	0.000
	day 1	3	4.33*	0.416
	day 3	3	15.70*	0.435
	day 7	3	14.00*	0.916
	day 14	3	4.60*	0.529
	day 21	3	0.96	0.251
	day 35	3	0.00	0.000
Ki 67 labelling index of ducts	control	3	0.66	0.208
	day 1	3	6.60*	0.360
	day 3	3	4.26*	0.305
	day 7	3	2.23*	0.321
	day 14	3	0.86	0.152
	day 21	3	0.66	0.251
	day 35	3	0.63	0.208

Discussion

In this study the duct ligation-deligation technique was used to investigate the regeneration of secretory tissue in the rat submandibular gland. After removal of the obstruction the glands showed recovery in weight together with recovery of the parenchyma. All these features support the idea that deligation induces submandibular gland regeneration as previously described[14,15,18]. Following duct ligation previous studies have suggested that residual shrunken acini persist during atrophy[9,10,11,18,23,24]. These acini are thought to be the first cell population to actively proliferate (day 1), followed later by differentiation and proliferation of newly formed acinar cells (day 3). In 3 day deligated submandibular gland, the presence of acinar-

ductal branched structures was reported which were significantly more frequent in the regenerated gland than in the atrophic. Due to their similarity with the structures found in the submandibular gland at birth (i.e. during postnatal development), we referred to them as developmental like branched structures and we proposed them as a source of newly differentiated acinar cells. In the later regeneration time point (7 days), we have identified normal-looking acinar cells developing directly from these developmental-like branched structures. This data further supports the previously suggested correlation between the formation of new secretory tissue during the regeneration and the early stage of postnatal glandular development[9,10,11,18,23,24]. Furthermore, the ending acini appeared to undergo active mitosis. They appeared morphologically more differentiated in the 7 days than in the 3 days regenerated gland as reported by Cotroneo et al., 2008. These regenerating acinar cells also showed apparent increase of AB/PAS staining seen at this time point (day 7). Further proof that the terminal acinar cells were differentiating into mature acini was the presence of surrounding myoepithelial cells. During glandular development myoepithelial cell differentiation starts in the late embryonic stage and carries on postnatally in parallel with the maturation of acinar cell differentiation[2,25]. Eventually in the adult submandibular gland they remain localized around the mature acini and the intercalated duct[26,27]. In our study the 3 and 7 days regenerated glands had myoepithelial cells around both the bulgings arising from the branched structures and the bulging-duct junction, then they were seen around the mature acini and intercalated ducts in the 14 days regenerating gland, reflecting the arrangement of the normal gland. Myoepithelial cells covering newly formed acini have been previously described during submandibular regeneration[19,20,28], supporting the suggestion that these cells may play a role in supporting acini differentiation.

In the present study, the proliferative activity of acinar cells

was high during the regeneration of the submandibular glands. This shows that acinar cell proliferation is significant for the glandular tissue regeneration. Two types of acinar cell proliferation are considered in this study. One is the proliferation of residual acinar cells, and as newly formed acinar cells appeaered after day 3 of regeneration, at least the Ki-67 positive acinar cells at day 1 were residual ones. This indicates that residual acinar cells start proliferation immediately after removal of the ligation and that they contribute to the early stage of regeneration.

The other type considered is the proliferation of newly formed acinar cells where several Ki 67-positive cells in the wall of duct acinar branched structures were detected at day 3 of regeneration as well as several Ki 67-positive cells in immature acini at the middle of the lobule. This type of acinar cell proliferation has been reported in the regeneration of atrophic parotid glands without remaining acinar cells as reported by[29,30]. This also coincides with the ultrastructural study of [19].

The ki 67 labelling index for acinar cells was very high at 3 days when both types of acinar cells proliferate. The ki 67 labelling index for duct cells gradually declined from the maximum at day 1 after deligation. This agrees with the index in the regeneration of parotid glands after atophy under the same experimental conditions as reported by[31]. This suggests that active proliferation of duct cells is unnecessary in regeneration of salivary glands after atrophy. The probaple reason for this is that duct cells proliferate and are residual during atrophy. This coincides with[16,20].

Ductal cell proliferation also occurs during ligation and rapidly decreases in early stages of regeneration which in agreement with Takahashi et al., 2004a, 2004b. This suggests that the duct cells, from which the new acinar cells originate, were pluripotent or at least undifferentiated cells. Whether these undifferentiated duct cells derive from de-differentiation of the ductal cells during the atrophy[9,10,11,31,32] or from a pre-existent population of pluripotent intercalated duct cells[33-35] is still unclear.

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REGENERATION OF SUBMANDIBULAR GLAND FOLLOWING LIGATION OF ITS DUCT RETRACES THE POSTNATAL PATHWAY OF CYTODIFFERENTIATION

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PROTECTIVE EFFECTS OF VITAMINS C AND E ON CISPLATIN - INDUCED RENAL DAMAGE IN ADULT ALBINO RATS (A Light and Electron Microscopic Study)

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Abstract

Background: *Cisplatin is an effective chemotherapeutic agent used in the treatment of a wide variety of solid tumors. The major side effects limiting its clinical use is the nephrotoxicity.*

The aim of this work: *was to study the possible protective effects of vitamins C and E on Cisplatin-induced nephrotoxicity in adult albino rats.*

Materials and Methods: *Thirty adult male rats were divided into three equal groups: The control group, Cisplatin group and Cisplatin plus vitamins C and E group. In the control group, the rats were injected intraperitoneally with 2ml of normal saline /kg.B.W. once daily for 3 consecutive days. In Cisplatin group, the rats were injected intraperitoneally with Cisplatin at dose of 10mg /kg .B.W. once daily for 3 consecutive days. In Cisplatin plus vitamins group, the rats were injected intraperitoneally with Cisplatin as the second group, in addition to vitamins C and E at dose of 250mg/kg.B.W.each. The vitamins were administrated orally with a cannula one hour prior to Cisplatin injection. All animals were sacrificed 3 days after the last injection. The kidney specimens were prepared for light and electron microscopies.*

Results: *Cisplatin produced necrosis of the epithelial lining of most of the proximal convoluted tubules with subsequent dilatations of their lumens. Some of these tubules contained esinophilic material .The epithelial cells of some tubules contained many vacuoles. The Cisplatin induced focal condensation of the connective tissue and inflammatory cells in the interstitial spaces. Electron microscopic examination showed that Cisplatin produced reduction in the numbers of mitochon-*

dria and the microvilli with increase the numbers of lysosomes, vacuoles and vesicles. Cisplatin obliterated the pores in the glomerular endothelium and in between the foot processes of the podocytes. Administration of vitamins C and E during the Cisplatin injection reduced the pathological changes induced by Cisplatin. The severity of these changes in the tubules and the glomeruli were less than those in the Cisplatin group.

Conclusion: *The toxic effects of Cisplatin on the kidney was minimized by administration of combination of vitamins C and E .*

Keywords: *Cisplatin, nephrotoxicity, vitamins C and E, rat kidney.*

Introduction

Cisplatin is a platinum - containing antineoplastic agent. It is one of the most potent chemotherapeutic antitumor agents. It has been demonstrated against a variety of neoplasms, particularly for head and neck, testicular, ovarian, bladder and lung neoplasms[1,2,3]. High doses of Cisplatin produce hepatoxicity, otoxicity, neurotoxicity and nephrotoxicity[3,4,5,6,7,8].

Cisplatin - induced renal damage is associated with increased renal vascular resistance and histological damage to the proximal tubular cells[3,9]. Cisplatin - induced nephrotoxicity is closely associated with inhibition of the activity of antioxidant enzymes in renal tissues[8,10,11].

Vitamin C acts as a potent water - soluble antioxidant in biological fluids[12]. It may prevent oxidative damage to important biological macromolecules such DNA, lipids and proteins[13]. High doses of antioxidant vitamins C and E were demonstrated to be effective against Cisplatin - induced oxidative renal damage in rats[14,15]. However, few papers have reported the effects of vitamins C and E in Cisplatin treated rats. So, the present study has been performed to investigate the possible protective effects of vitamins C and E on Cisplatin nephrotoxicity in adult albino rats.

Materials and Methods

A- Chemicals:

1- Cisplatin (platinol) is produced by Orna Chemicals and Pharmaceutics. It is in the form of vials. Each vial contains 10mg/ 20ml of Cisplatin. The dose of Cisplatin used in this study

was 10 mg /kg. B. w.

2- Vitamin C (cevarol tablet) is produced by Memphis Chemical Company. Each tablet contains 500 mg ascorbic acid. One tablet was dissolved in 10ml distilled water. Each 1ml =50 mg. The dose of vitamin C used in this study was 250 mg/kg.B.W.

3- Vitamin E (E- viton capsule) is produced by Kahira Pharmaceutical Company. Each capsule contains 100mg vitamin E. The 5 capsules (500mg)were pinched using the tip of a sterile needle ,the contents were squeezed and dissolved in 10ml olive oil .Each 1ml =50mg of vitamin E. The dose of vitamin E used in this study was 250mg /kg. B.W.

B- Animals:

Thirty adult male albino rats were used in this study. Their ages ranged from 2.5-3 months old. Their weight ranged from 200-250gm. They were fed daily with tap water and pellet foods at room temperature.

C- Experimental design:

The animals were divided into three groups of ten rats each.

Group I (control group) were injected intraperitoneally with 2ml of normal saline/kg. B.W. once daily for 3 consecutive days.

Group II (Cisplatin group) were injected intraperitoneally with Cisplatin at a dose of 10mg /kg. B.W. once daily for 3 consecutive days.

Group III (Cisplatin plus vitamins group) were injected with Cisplatin as the second group, with oral administration of vitamins C and E at a dose of 250 mg/kg each. Vitamin C was dissolved in distilled water, while vitamin E was dissolved in olive oil. Vitamins C and E were administrated orally with a cannula one hour prior to Cisplatin injection.

All animals were sacrificed 3 days after the last injection using ether anesthesia.

D- Histopathological procedures :

The kidneys of all groups were excised and washed with saline to remove the blood. The kidney specimens were processed for light and electron microscopical exami-

nation. For light microscopical observation, the kidney sections were stained with Hematoxylin and Eosin, Masson´s trichrome and Periodic acid -Schiff (P.A.S.). The sections were photographed using a Camera connected with light microscope. For electron microscopical observation, ultrathin sections were collected on copper grids for double staining(uranyl acetate and lead citrate). Stained sections were finally observed under a transmission electron microscope and photographed.

Results

Control group:

By light microscopy, the histological structure of the renal cortex of the control group showed the normal structure of both glomeruli (renal corpuscles)and tubules. Each glomerulus appeared as a dense rounded structure which was surrounded by narrow space called renal space(Bowman´s space). The glomerulus consisted of tuft of capillaries which was covered by the Bowman´s capsule. The Bowman´s capsule consisted of an inner or visceral layer covering the glomerulus and an outer or parietal layer and the renal space in between. The visceral layer consisted of epithelial cells called the podocytes. These cells had large deeply stained nuclei .The parietal layer was composed of simple squamous epithelium resting on a thin basal lamina (Figs. 1, 2).

The tubules which were seen in the sections consisted mainly of the proximal convoluted tubules and some distal convoluted tubules. The proximal convoluted tubules had narrow lumens and were lined by a single layer of columnar cells with rounded, basal, vesicular nuclei. The distal convoluted tubules had wide lumen and were lined by low cubical cells (Figs.1,2).

P.A.S. positive reaction was evident in the basement membranes of the parietal layer of the Bowman´s capsule and the tubules. Also, the brushing border of the tubules showed positive P.A.S. reaction (Fig. 3).

By electron microscopy, the proximal convoluted tubules were lined by columnar cells which had rounded heterochromatic nuclei with prominent nucleoli. Its cytoplasm contained columns of

elongated mitochondria resting on the basement membrane. At high magnification, the wall of mitochondria had double membranes with translucent space in between. The lumens of the proximal convoluted tubules revealed profuse tall microvilli constituting the brush border seen by light microscopy (Figs 4, 5).

The glomerular filter consisted of three components (inward to outward): 1-Fenestrated capillaries endothelium. 2-Glomerular basement membrane. 3-secondary foot processes of the podocytes, separated by slit pores (Fig. 6).

Cisplatin group:

By light microscopy ,most of the proximal convoluted tubules showed necrosis of their epithelial lining which lead to dilations of their lumens. Some of these degenerated tubules contained esinophilic material which accumulated in their lumens. Some of these epithelial cells contained vacuoles and their brush borders were disrupted in some areas. Few tubules were still intact. The glomeruli appeared intact with intact basement membrane of its parietal layer. The interstitial space contained focal accumulation of connective tissue. In some sections there were focal accumulation of inflammatory cells in the interstitial space (Figs.7,8,9,10).

By electron microscopy ,the convoluted tubules showed increase in the numbers of lysosomes, vacuoles and vesicles. The contents of some lysosomes were homogenous,while the others were heterogeneous. The cytoplasm of these cells contained few numbers of mitochondria. Some nuclei showed basal indentation. The lumens of these tubules contained few microvilli (Figs.11,12). In the glomerular filter, there were absence of the fenestration in the capillary endothelium and obliteration of the slit pores between the secondary foot processes of the podocytes (Fig. 13).

Cisplatin plus vitamins (C & E) group:

By light microscopy, the glomeruli and some tubules were intact. Other tubules had varying degrees of changes in the form of 1- Some tubules were dilated with intact epithelial lining. 2-Other tu-

bules were not dilated, but some of their cells had vacuolations. 3-The lumen of one tubule contained esinophilic material. 4-The epithelial lining of few tubules were completely degenerated, but their basement membranes were intact (Figs. 14,16). Minimal connective tissue were seen in the sections of this group (Fig. 15).

By electron microscopy, the elongated basal columns of mitochondria were seen in the cells lining the tubules. Their nuclei were heterochromatic with prominent nucleoli (Fig. 17). The components of the glomerular filter consisted of three layers as in control group: fenestrated glomerular endothelium, glomerular basement membrane and foot processes of podocytes with slit pores in between (Fig.18).

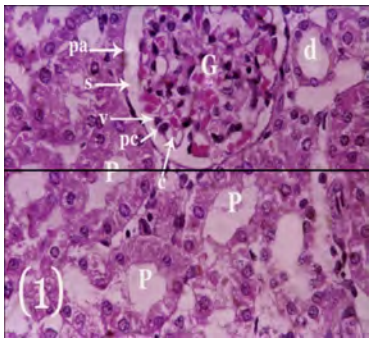

Fig. 1 : A light micrograph of adult control rat kidney showing :The glomerulus (G),the proximal convoluted tubules(P) and the distal convoluted (d).The glomerulus consists of capillary tuft (c),visceral layer of Bowman´s capsule(V) which separates from its parietal layer (pa)by renal space (s). Notice the podocytes (pc)with deeply stained nuclei in the visceral layer of Bowman´s capsule. The parietal layer was composed of simple squamous epithelium resting on the basement membrane. The proximal convoluted tubules consist of columnar cells with rounded basal vesicular nuclei. The distal convoluted tubules have low cuboidal cells with vesicular nuclei and wide lumen. (HX &E. X400)

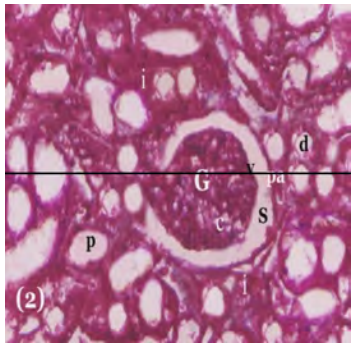

Fig. 2 : A light micrograph of adult control rat kidney showing : The glomerulus (G)consisting of tuft of capillaries (c) , visceral layer (v),renal space (s) and parietal layer (pa). The glomerulus is surrounded by cut sections of the proximal(p) and distal (d) convoluted tubules. The interstitial space (i) is rose in colour.

(Masson´s trichrome x 400)

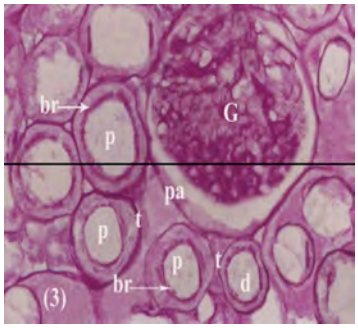

Fig. 3 : A light micrograph of adult control rat kidney showing : The glomerulus (G) surrounding by proximal(p) and distal (d) convoluted tubules . Notice a well defined basement membranes of the parietal layer of Bowman´s capsule (pa) and the convoluted tubules(t).Also, notice a well defined brush border(br) in the proximal convoluted tubules. (P.A.S. X400)

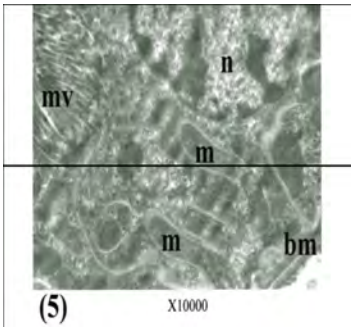

Fig. 5 : An electron micrograph of adult control rat kidney showing: A part of the proximal convoluted tubule containing heterochromatic nucleus (n) , elongated basal mitochondria(m) resting on the basement membrane(bm). Notice the wall of mitochondria has double membranes with translucent space. Also, notice the microvilli(mv) projecting into the lumen. (E.MX10,000)

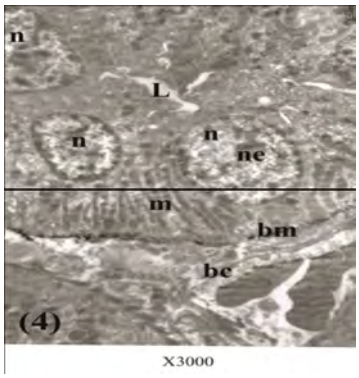

Fig. 4 : An electron micrograph of adult control rat kidney showing: A part of the proximal convoluted tubule lining with columnar cells. These columnar cells have rounded, heterochromatic nuclei (n) with nucleoli (ne) and columns of elongated mitochondria(m) resting on the basement membrane(bm). Notice a narrow lumen (L) of the proximal convoluted tubule .The interstitial space contains blood capillary(bc). (E. M. X3000)

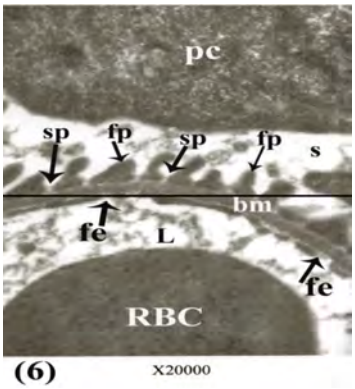

Fig. 6 : An electron micrograph of adult control rat kidney showing: The components of the glomerular filter consisting of fenestrated capillary endothelium (fe), glomerular basement membrane (bm) and secondary foot processes (fp) of podocytes(pc) which separated from each other by slit pores (sp) . Notice the lumen(L) of the glomerular capillary containing RBC. Also, notice the renal space (s). (E.M. X 20,000)

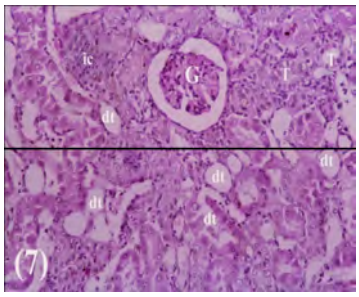

Fig. 7 : A light micrograph of adult rat kidney treated with Cisplatin showing : Most of the cells of tubules are degenerating (dt) with dilatations of their lumens. Notice the interstitial space containing focal accumulation of the inflammatory cells(ic). Also, notice intact glomerulus (G) and some tubules (T). (HX .&E. X 200)

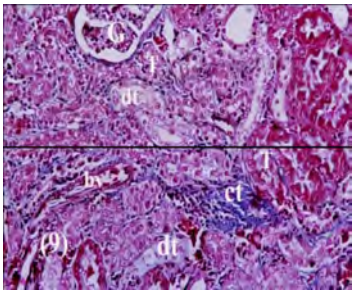

Fig. 9 : A light micrograph of adult rat kidney treated with Cisplatin showing : Focal accumulation of the connective tissue (ct) in the interstitial space. Some tubules(dt) are degenerating ,which other tubules (T) are intact . Notice the glomerulus(G) and blood vessel (bv). (Masson´s trichrome x 400)

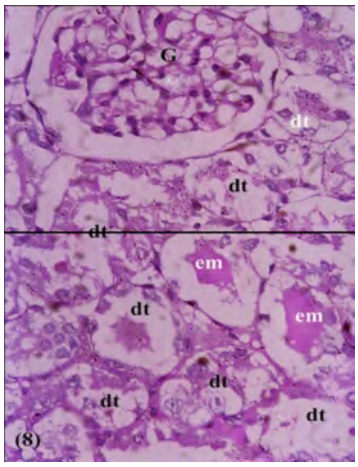

Fig. 8 : : A light micrograph of adult rat kidney treated with Cisplatin showing : Degeneration of the epithelial cells lining the tubules (dt). The lumens of some tubules are dilating with accumulation of esinophilic material (em). Notice the intact glomerulus (g) . (HX .&E. X 400)

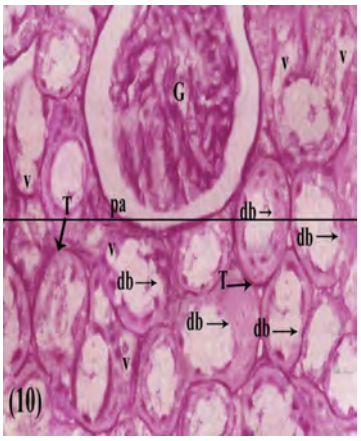

Fig. 10 : A light micrograph of adult rat kidney treated with Cisplatin showing :Partial disruption of the brush border (db) of the tubules. Some vacuoles(v) are seen in the epithelial cells lining the tubules . Notice intact basement membranes of the tubules(T)and the parietal layer (pa) of the glomerulus(G) . (P.A.S. X400)

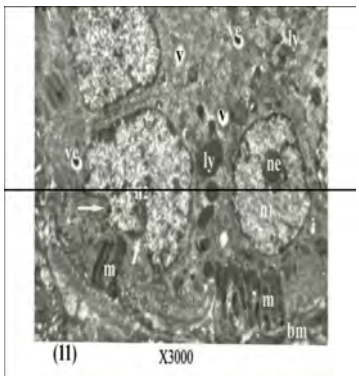

Fig. 11 : An electron micrograph of adult rat kidney treated with Cisplatin showing: Less number of basal mitochondria (m) resting on the basement membrane (bm). The cytoplasm contains different sizes of lysosomes (ly), vacuoles (v) and vesicles (ve) . Notice rounded nucleus (n1) with central nucleolus (ne), while other nucleus(n2) has basal indentation (arrow) .
(E.M. X 3000)

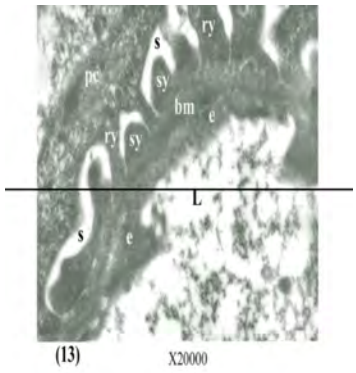

Fig. 13 : An electron micrograph of adult rat kidney treated with Cisplatin showing: Absence of the fenestration in the capillary endothelium (e) and slit pores in between the foot processes (primary"ry" , secondary "sy") of the podocytes (pc). Notice the lumen (L) of glomerular capillary , glomerular basement membrane (bm) and the renal space (s) .
(E.M. X 20,000)

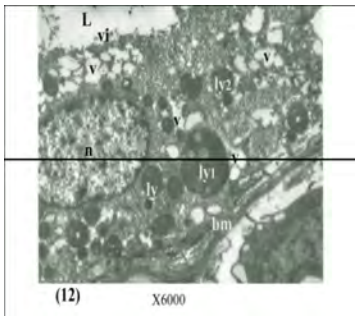

Fig. 12 : An electron micrograph of adult rat kidney treated with Cisplatin showing: The cells of the proximal convoluted tubules containing different sizes of lysosomes (ly). Some of these lysosomes are heterogeneous (ly1), while other lysosomes are homogeneous(ly2). Also, the cytoplasm of this cell contains groups of vacuoles (v) and oval intact nucleus (n) . Notice the lumen (L)of this tubule containing few microvilli(vi) . Also notice the basement membrane (bm) . (E.M. X 6000)

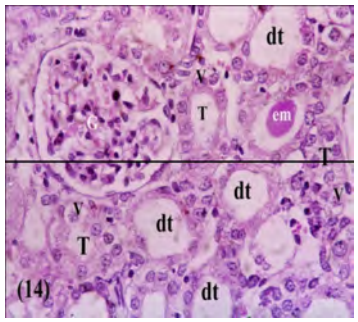

Fig. 14 : A light micrograph of adult rat kidney treated with Cisplatin plus vitamins C and E showing : Some tubules are dilating (dt) with intact epithelial lining . Other tubules (T)are not dilating but some of their cells having vacuolations (v) .Notice the lumen of one tubule has central esinophilic material (em) . Also, notice intact glomerulus(G) .
(HX.&E. X 400)

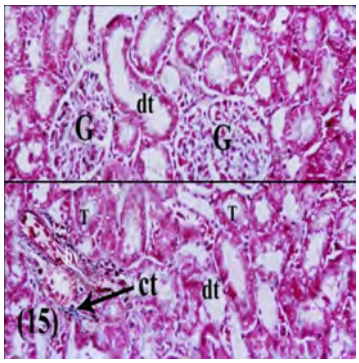

Fig. 15 : A light micrograph of adult rat kidney treated with Cisplatin plus vitamins C and E showing : Minimal amount of connective tissue (ct) in the interstitial space. Some tubules are dilating (dt), while other tubules (T) are intact. Notice intact glomeruli (G) and blood vessel (bv).

(Masson´s trichrome x 400)

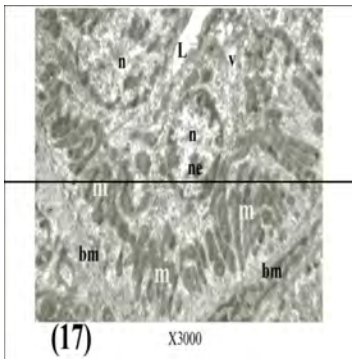

Fig. 17 : An electron micrograph of adult rat kidney treated with Cisplatin plus vitamins C and E showing : The epithelial cells of the proximal convoluted tubules containing elongated basal striations of the mitochondria (m), oval heterochromatic nuclei (n) with nucleoli (ne) and few vacuoles (v). Notice the lumen (L) and the basement membrane (bm) .

(E.M. X 3000)

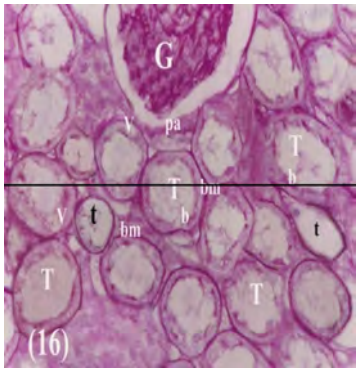

Fig. 16 : : A light micrograph of adult rat kidney treated with Cisplatin plus vitamins C and E showing : Some tubules (T) are dilating with some vacuoles(v)in their cells . Their brush border(b) and the basement membrane (bm) are intact . Few tubules (t) shows degeneration of their epithelial cells with intact basement membrane . Notice intact parietal layer (pa) of the glomeruli (G) .

(P.A.S. X400)

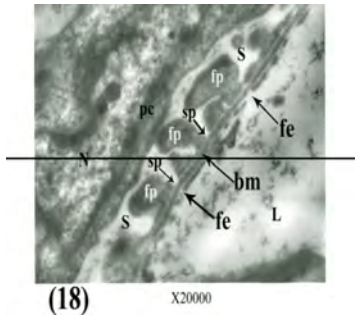

Fig. 18 : An electron micrograph of adult rat kidney treated with Cisplatin plus vitamins C and E showing : The intact glomerular filter which consisting of fenestrated capillary endothelium (fe), the glomerular basement membrane (bm) and foot processes (fp) of the podocyte (pc) which separated from each other by slit pores (sp) . Notice the capillary lumen(L) , renal space (s) and the nucleus of podocytes (N).

(E.M. X 20,000)

Discussion

The present study deals with the normal structure of the renal cortex of adult rat and the effects of Cisplatin on the renal cortex . Also, this study evaluates the protective effects of vitamins C and E on Cisplatin - induced nephrotoxicity in rats.

In the present study, the histopathological examination of renal sections demonstrated that Cisplatin produced necrosis of the epithelial cells of the proximal convoluted tubules with subsequent dilatations of their lumens. Some of these tubules contained esinophilic material (cast formation). The epithelial cells of some tubules contained many vacuoles. These results are similar to the results of [17,18,19,20,21,22]. Sheikh - Hamad et al. and Uehara et al. who reported that Cisplatin induced cell injury and necrosis in the rat kidney are predominantly localized in the S3 segment of the proximal convoluted tubules[17,18]. Sueishi et al. reported that Cisplatin produced vacuolations, necrosis and protein casts were observed in the proximal convoluted tubules on the fourth day after Cisplatin injection in rats[16]. Cisplatin is mainly excreted by the kidney and the kidney tissue content of this drug is higher than concentrations in other organs. As Cisplatin is retained in the kidney tissue for a long duration, it may readily cause nephrotoxicity[23].

In the present study , Cisplatin induced focal condensation of connective tissue and infiltration of the inflammatory cells in the interstitial space. These data are corroborated by previous studies reported by other investigators on Cisplatin - induced nephrotoxicity in rats[19,20,21,22,24,25,26,27,28]. Tarladacalisir et al. reported that Cisplatin induced focal mononuclear cell infiltration among some peritubular and Periglomerular areas [27]. Guinee et al. observed that these cells are lymphocytes[26]. Martinez et al. reported that the renal interstitial fibrosis is a major complication of Cisplatin treatment, due to the increased accumulation of extracellular matrix protein[28].

In the present study, electron microscopic examination of the

renal tubular cells showed reduction of the numbers of the mitochondria and the microvilli, while the numbers of lysosomes, vacuoles and vesicles were increased. These are early signs of degeneration of the tubular cells which are similar to the results of previous studies [21,27,29,30,31]. These previous studies explained the mechanisms of tubular cell damage: As the cell membrane represented the first organelle exposed to the heavy metal (platinum in Cisplatin), this metal could directly bind to brush border membrane and damage its integrity. This may increase the permeability of the membrane and cause the loss of microvilli. An interaction of heavy metals with the proximal convoluted tubular cell may lead to loss of mitochondria with a release of mitochondrial serin protease with subsequent increasing of the lysosomes, vacuoles and vesicles [21,27,29,30,32] .

In the present study, Cisplatin produced electron microscopic changes in the glomerular filter in the form of obliteration of the pores in the glomerular endothelium and obliteration of the slit pores in between the foot processes of the podocytes. These results run parallel with the reports documented by[3,16] who reported that Cisplatin -induced renal damage is associated with increase in blood urea nitrogen (BUN) and creatinine in serum with increase in the renal vascular resistance.

In the present study, administration of combination of vitamins C and E during Cisplatin injection reduced the pathological changes induced by Cisplatin. The severity of degenerative changes in the tubular cells and the vascular resistance in the glomerular filter were less than those in Cisplatin group. Vitamins C and E decreased the tubular necrosis, formation of cast in the tubular lumens, vacuolations and connective tissue formation. They protect the mitochondria and the glomerular filter from any pathological changes. These findings are similar the results of [14,15,27,32,33] who reported that the administration of vitamins C and E protect against Cisplatin -induced renal toxicity in animal studies. Vitamins C and E exhibit a protective effect against free radical-induced oxidative damage [34,35].

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PROTECTIVE EFFECTS OF VITAMINS C AND E ON CISPLATIN-INDUCED RENAL DAMAGE IN ADULT ALBINO RATS
(A Light and Electron Microscopic Study)

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LIGHT AND ELECTRON MICROSCOPIC STUDY OF THE BRONCHI OF GUINEA PIG AFTER EXPERIMENTALLY-INDUCED BRONCHIAL ASTHMA

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Abstract

Objective : *To study the histological and ultrastructural changes in the guinea pig bronchi after induction of bronchial asthma and its treatment.*

Materials and Methods : *Thirty adult guinea pigs were used in this study. They were classified into three equal groups:* ***Group A*** *as control group,* ***Group B:*** *asthmatic group and* ***Group C:*** *asthmatic group treated with corticosteroids.*

- *Specimens from bronchi were taken and fixed in 10% formalin; paraffin sections (5μm thick) prepared and stained with Mallory trichrome stain.*
- *Small fragments from bronchi and lung tissue were processed for examination with electron microscope.*

Results : *After induction of bronchial asthma, fibrous tissue was seen in sections stained with Mallory trichrome stain. Subepithelial collagen fibers deposition was noted to diminish after corticosteroid therapy. Transmission electron microscopic study in asthmatic group exhibited ciliary loss in most epithelial cells which appeared vacuolated with distorted tight junctions between cells. Increased sub epithelial collagen fibers deposition was also noted.*

Conclusion: *From the present study we concluded that bronchial asthma includes major hisopathologic changes in bronchi. Most of these changes were effectively treated with corticosteroid drug therapy.*

Introduction

Bronchial asthma is a chronic inflammatory disease of the airways, characterized by bronchial hyper-responsiveness and obstruction[21]. The prevalence of asthma in particular atopic asthma has markedly increased in recent years. Accumulating evidence suggests that environmental factors associated with allergic sensitization and exposure to microbial stimuli during infancy and early childhood, are associated with these changes in prevalence[10].

Periodic attacks of asthma may result in smooth muscle hyperplasia, mucous glands hypertrophy, subepithelial fibrosis, angiogenesis and change of extracellular matrix. These pathological findings are called airway remodeling to which great attention has been paid in recent years for its effects on asthma. Growth factors, cytokines, enzymes and inflammatory mediators all play an important role on airway remodeling[21].

The Aim of the Work

The present study represents a way to explore the pathology of asthma and also provides an idea about the base for its treatment in adult guinea pigs.

Materials and Methods

Thirty adult guinea pigs ranging in weight from 300-400 grams were used in this study and were classified into 3 equal groups:

Group A: animals served as control.

Group B: asthma was induced to animals by being sensitized (injected intraperitoneally with 1ml/350gm of 10% ovalbumin). Thereafter animals were challenged; exposed to nebulized solution of 1% ovalbumin in closed chamber for 10 minutes once weekly for 6 weeks[23].

Group C: animals after induction of bronchial asthma as in group B were given corticosteroid therapy; oral Prednisolone in a dose of 5mg/kg once daily for 6 weeks by means of gastric tubes[19].

Animals from each group were scarified at the end of the experiment, bronchi of each guinea pig was obtained and processed for

light and electron microscopic studies.

Tissue preparation:

At the assigned time of the experiment, guinea pigs from each group were obtained and anaesthetized with ketamine HCL (46 mg/kg body weight/pig).

Bronchi of each pig were dissected out, sliced and processed as follows:

I-Mallory Trichrome Stain[2]:

This technique was used for the differential demonstration of the connective tissue fibers and muscles by the following steps:

1- Bring formalin-fixed paraffin sections down to water.
2- Stain in 1% acid fuchsine for 2 minutes and wash in distilled water.
3- Put the sections in 2% phosphomolybdic acid solution for 10 minutes and wash in distilled water.
4- Immerse sections in a solution prepared by dissolving 2 gm. Orange G, 2 gm. Oxalic acid and 1 gm aniline blue in 100 ml distilled waterfor a period of 30 minutes and wash in distilled water.
5- Dehydrate in alcohols, clear in xylene and mount in Canada balsam.

II- Preparation of Ultrathin Sections for Transmission Electron Microscopy (T.E.M)[3]:

The animals were perfused through the heart apex with 200 ml saline followed by 300 ml 2.5% gluteraldehyde in 0.1 M cacodylate buffer (Ph 7.3).

- The lungs and extrapulmonary bronchi were excised and small pieces of about 1 mm3 were sliced from the bronchial tree.
- The specimens were immersed in the same fixative for 4-hours and postfixed in 1% osmium tetraoxide in 0.1 M Cacodylate buffer (PH 7.3) for 2 hours.
- The specimens were dehydrated in ascending grades of alcohol then passed in two changes of propylene oxide to be lastly embedded in Epon.
- Semithin sections (1 micron thick) were cut with glass knife and stained with toluidine blue to evaluate fixation quality and select sites for ul-

trathin sections.

- Ultrathin section of 60 nm thickness each, were cut with glass knife and stained with 2% uranyl acetate and lead citrate.
- Stained sections were examined with the transmission electron microscope.

Results

- Mallory trichrome stain:

- Control group:

Few elastic and collagen fibers in lamina propria and submucosa appeared blue in color (Fig. 1).

- Asthmatic group:

Thickened corium due to collagen fibers deposition was seen with increased fibrosis in lamina propria and submucosa (Fig. 2).

- Asthmatic group received corticosteroid therapy:

Few collagen fibers in lamina propria and submucosa than in asthmatic group were seen with decongestion of most blood vessels and capillaries (Fig. 3).

- Transmission electron microscopy (T.E.M.):

- Control group:

Examination of bronchial epithelium of guinea pig by T.E.M. revealed many cell types.The most common was ciliated cells. Its free border showed both cilia and microvilli.The cell membrane showed junctional complexes with adjacent cells. The cytoplasm had low electron density. The apical part of the cytoplasm showed numerous mitochondria and basal bodies. The 2nd cell type was the goblet cell. Its luminal border demonstrated few short microvilli. The cytoplasm was electron dense and packed with membrane bound secretory granules giving the goblet shape. The secretory granules were variable sized and of low to moderate electron density. Fusion was seen between the adjacent secretory granules (Fig. 4).

- Asthmatic group:

Bronchial epithelial ciliated cells appeared vacuolated with even loss of most cilia and distorted tight junction between them. Increased secretory granules in goblet cells is seen. Thickened basement membrane was clear by collagen fibers deposited under it (Fig. 5, 6).

- Asthmatic group received cor-

ticosteroid therapy:

Bronchial epithelial ciliated cells appeared less swollen with minimal vacuolization and cilia mostly preserved than in asthmatic group (Fig. 7).

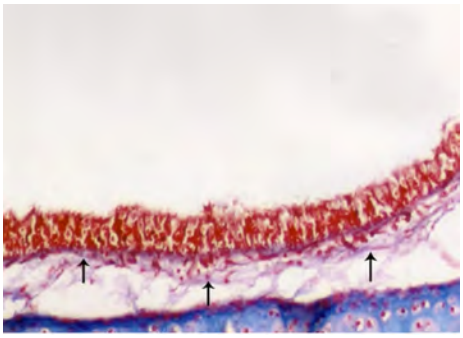

Fig (1): A photomicrograph of a paraffin section of control guinea pig extrapulmonary bronchus showing minimal collagen fibers in the corium and submucosa (arrows).
(Mallory trichrome X 250)

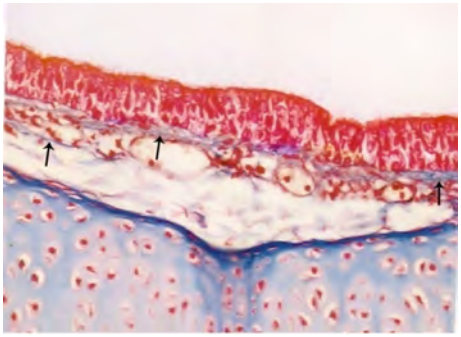

Fig (3): A photomicrograph of a paraffin section of asthmatic guinea pig extrapulmonary bronchus received corticosteroid therapy showing decreased collagen deposition (arrows).
(Mallory trichrome X400)

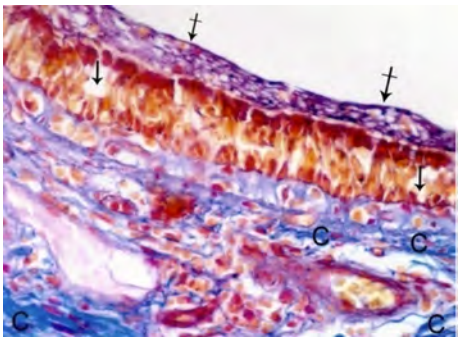

Fig (2): A photomicrograph of a paraffin section of asthmatic guinea pig extrapulmonary bronchus showing disrupted epithelial lining (arrows), increased collagen fibers deposition under it (C) and mucous film covering the disrupted epithelium (crossed arrows).
(Mallory trichrome X400)

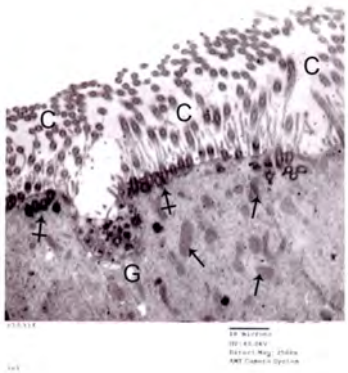

Fig (4): Transmission election micrograph of control guinea pig extrapulmonary bronchus lining epithelium. It shows ciliated cells with its free border having cilia and microvilli (C). The apical part shows numerous basal bodies (crossed arrows) and mitochondria (arrows). Note, solitary goblet cell (G).
(Uranyl acetate & Lead citrate X 2500)

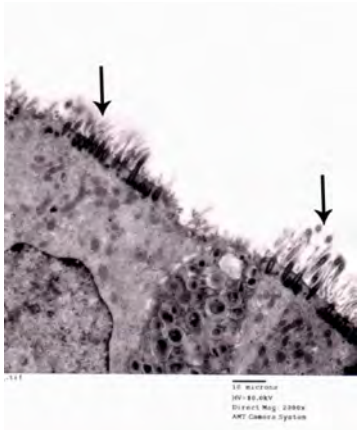

Fig (5): Transmission electron micrograph of asthmatic guinea pig extrapulmonary bronchus epithelial lining showing apparent decreased cilia (C) and increased secretory granules in goblet cells.
(Uranyl acetate & lead citrate X 2000)

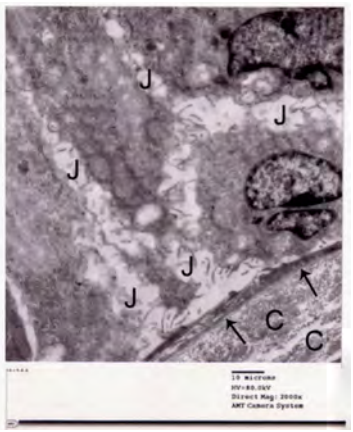

Fig (6): Transmission electron micrograph of asthmatic guinea pig extrapulmonary bronchus. It shows increased subepithelial collagen fibers deposition (C). Note, disrupted tight junction between epithelial cells (J) and thickened basement membrane under it (arrows).
(Uranyl acetate & lead citrate X 2000)

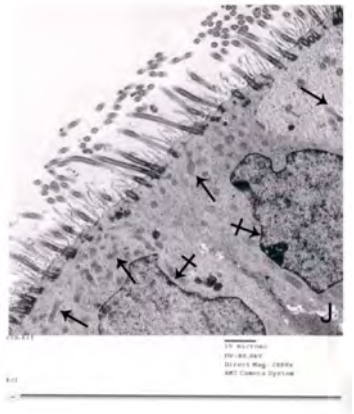

Fig (7): Transmission electron micrograph of asthmatic guinea pig extrapulmonary bronchus receiving corticosteroids therapy showing ciliated cells with mitochondria (arrows) and vesicular nuclei (crossed arrows). The cell junction shows some areas of disruption (J).
(Uranyl acetate & lead citrate X 2000)

Discussion

Bronchial asthma is a complex disorder in which major genetic and environmental factors interact to initiate the disease and modify its progression. While asthma is recognized as a disorder of the conducting airways characterized by inflammation, it is being increasingly apparent that alteration of the structural cells of the airways (airway remodeling) is also fundamental to disease chronicity and severity [7].

Corticosteroids are the cornerstone of asthma treatment. However, several tests have shown that this condition doesn't improve or even deteriorate further epithelial cells of the airways. Some studies have shown that these drugs induce apoptosis of airway epithelium and further denudation and therefore the detachment from the basement membrane and for this reason this effect is controversial [15].

[1] and [5] stated that the extra pulmonary bronchi are lined by ciliated, pseudo stratified columnar epithelium with numerous goblet cells and thick basal lamina. Under the epithelium lies the lamina propria which structure was clarified by [16] and [4] to be loose connective tissue rich in elastic fibers containing numerous bronchial sub mucosal glands. The bronchi as viewed by electron micrograph by [1] and [5] are lined by pseudostratified ciliated columnar epithelium with the ciliated cells having apical border through which the cilia project into the lumen with apical cytoplasm containing mitochondria. Also [16] proved the shape of goblet cells with their expanded apical region occupied by closely packed mucinogen granules of low electron density. These results agreed with the present study.

The present study clarifies the microscopic alteration in guinea pig bronchial tree after induction of bronchial asthma, and also the alteration introduced after treatment with corticosteroids.

After sensitization by ovalbumin, structural changes occurred in bronchi due to bronchial asthma developed such as loss of cilia and accumulation of inflammatory cells as nodules surrounding

bronchi [22].

[6] found that the gross pathology of asthma displays lamina reticularis thickening, mucosal edema, epithelial cell sloughing, ciliated cells disruption and mucus gland hypersecretion. [17] results included bronchial epithelium degeneration, fibrosis of the stroma, vascularization increased and lymphoid tissue hyperplasia in the bronchial wall and most of these descriptions were coincides with the present study. [24] postulated subepithelial fibrosis traced by Mallory trichrome stain to find its increase in asthmatic airways. Same methods were also used by present study.

By electron microscopic examination of asthmatic airway epithelium, [20] estimated that it is partially shed, ciliated cells appear swollen, vacuolated and there is often loss of cilia and [18] clarified goblet cells filled with secretory granules and epithelial cells with and without cilia. These results were all observed in present study.

[13] and [11] postulated that light and electron microscopic studies of bronchial tree in asthmatic cases revealed damage to the epithelium, retention of mucous in bronchial lumens. These outcomes were also seen in present study. [9] showed that in addition to adopting an activated phenotype, the barrier function of the epithelium is impaired through defective tight junction formation. This was also proved by the present study.

[15] evaluated the effects of treatment with corticosteroids on asthmatic airways reporting that it did not effectively inhibit mucosal regeneration, inflammation and mucus secretion. Similar data was reported by the present work. It also reported that some studies showed that corticosteroids cause apoptosis in the epithelium of the airways, the additional denudation and therefore its detachment from basement membrane, while in the present study corticosteroids reduced epithelial denudaion effectively.

[14] reported using electron microscopy improvement in the epithelium of the corticosteroids

treated cases. This improvement included regeneration of ciliated cells and restoration of damaged cilia, decreased lymphatic infiltration, reduced vascular area and decreased subepithelial collagen deposition. The present study concluded the same mentioned results.

Considering asthma as a disease of impaired function opens new opportunities for therapeutic intervention or prevention by agents that could increase the airway resistance to the inhaled environment rather suppressing the immune or inflammatory response [8].

The current bronchial asthma therapies lake the ability to completely prevent or reverse the remodeling of the airways, therefore indicating the need for new therapeutic strategies to counter this important aspect of asthma [12].

Summary and Conclusion

This work was undertaken to study the histological and ultrastructural changes in the guinea pig bronchial tree after induction of bronchial asthma and its treatment.

Thirty adult guinea pigs were used in this study. They were classified into three equal groups: Group A as control group, Group B: asthmatic group and Group C: asthmatic group treated with corticosteroids.

- Specimens from bronchi and lung tissue were taken and fixed in 10% formalin; paraffin sections (5μm thick) prepared and stained with Mallory trichrome stain.

- Small fragments from bronchi and lung tissue were processed for examination with electron microscope.

After induction of bronchial asthma, fibrous tissue was seen in sections stained with Mallory trichrome stain. Subepithelial collagen fibers deposition was noted to diminish after corticosteroid therapy more than in group treated with anti-inflammatory drug therapy. Transmission electron microscopic study in asthmatic group exhibited ciliary loss in most epithelial cells which appeared vacu-

olated with distorted tight junctions between cells. Increased sub epithelial collagen fibers deposition was also noted.

From the present study we concluded that bronchial asthma includes major hisopathologic changes in bronchial tree. Most of these changes were effectively treated with corticosteroid drug therapy.

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REPRINT

BENHA MEDICAL JOURNAL

LIGHT AND ELECTRON MICROSCOPIC STUDY OF THE BRONCHI OF GUINEA PIG AFTER EXPERIMENTALLY-INDUCED BRONCHIAL ASTHMA

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PROSTHETIC HEMIARTHROPLASTY FOR FRACTURES AND FRACTURE DISLOCATIONS OF THE PROXIMAL HUMERUS

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Abstract

Treatment of severe proximal humeral fractures is considered a challenge with regards to poor results after conservative treatment, the difficulties facing internal fixation and the vascular compromise associating this type of fracture. The aim of this study is to evaluate the results of hemiarthroplasty for treatment of type III and type IV fractures and fracture dislocations of the proximal humerus. The material of this prospective study included 26 patients with average age of 32-83 years. All cases were managed with hemiarthroplasty within a period ranged from 5-31 days. For final evaluation, each patient was assessed both functionally and radiologically. The modified Constant score in this study ranged from 11 to 93 points with a mean score of 61.7 ± 21.4 points. Shoulder hemiarthroplasty is indicated as a primary procedure in cases with severe proximal humeral fractures that cannot be reconstructed.

Introduction

Fractures and fracture-dislocations of the proximal humerus account for 4% to 5% of all fractures;[1,2] Displaced and severely comminuted fractures and fracture-dislocations of the proximal humerus present a serious problem in management and prognosis. Malunion, nonunion, avascular necrosis are common complications.[3] Many surgeons have preferred the conservative treatment of complex proximal humeral fractures in some patients.[4,5,6,7] Open reduction and internal fixation was indicated in severe angulations', rotational

deformity, displacement and loss of contact between the fragments, neurovascular compromise or irreducible fracture dislocation.[8,9,10] As proximal humeral fractures are more common in elderly patients with osteoporotic bone, there is an obvious risk of plate failure due to the reduced capacity of holding the screws.[2] Proximal humeral fractures that are at risk of avascular necrosis humeral head replacement should be considered.[11] The development of humeral head prosthesis and the availability of recent generations that can reproduce anatomy and mechanics of the shoulder joint has allowed for possible improvement of the functional outcome. The aim of this study is to evaluation and analysis of the results of shoulder hemi-arthroplasty in patients with type III and type IV fractures and fracture-dislocation.

Patients and Methods

A prospective study on 26 patients with severe proximal humeral fractures was carried out in Mansoura Emergency Hospital. There were 13 females (50%) and 13 males (50%). Their ages ranged from 35 years to 83 years with a mean of 56.6±12.5 years. The right side was affected in 14 patients (53.8%), and the left side was affected in 12 patients (46.2%). In this study, 50% of our cases were affected on their dominant side. All patients were examined clinically and radiologically by a true AP view and lateral scapular view. CT scan was done for all patients to clarify the diagnosis. Fractures were identified according to Neer classification.(8) Half of the cases in this study (13 patients) were IV part fracture, and 7 patients (27%) were III part fracture. 3 cases (11.5%) were III part fracture-dislocation. Three cases (11.5%) were VI part fracture-dislocation. Three types of prostheses were used: PEP prosthesis (Prosthèse d' èpaule) in 20 (76.9%), Baumer (MSP) in 4 (15.4%), and Global Fx in 2 (7.7%) cases. All of these prostheses are modular, with variable head diameters and short humeral stems.

Surgical technique:

General controlled hypotensive anesthesia was used in all patients. All patients were given 1gm third generation cephalosporins

on induction of anesthesia, and another dose during surgery. The patient was seated in a beach-chair position with the back elevated between 30-45°. A long deltopectoral approach was used in all patents.[12] Two No. 5 nonabsorbable sutures (Ethibond) were placed through the bone of the greater tuberosity. Another one sutures was placed through the lesser tuberosity in all cases. These sutures were used for retraction of the tuberosities and later on for fixation. The intramedullary canal was exposed. The arm was extended and adducted to deliver the shaft anteriorly. The canal was prepared with progressive hand-held sequential reamers and rasps starting with the smallest size reamer (8 PEP, 7 Baumer, and 6 Global Fx) until reaching the best fitting size reamer. Two drill holes (2.5 mm) were done about one finger breadth distal to the surgical neck fracture. One hole was medial to the bicipital groove and the other was lateral to it. A No. 5 Ethibond suture was inserted in these holes entering from one hole to the medulla and emerging from the other. Trial prosthesis was used to evaluate the position as regards height and retroversion. The humeral head was measured and the size which is anatomically equal to the removed head was considered. Retroversion was evaluated by the bicondylar axis as in Hempfing et al 2001[13] The trial prosthesis was removed and the prosthetic stem was inserted in the determined version and height. Bone cement was used in all cases to fix the humeral stem in the medulla. The tuberosities were mobilized utilizing the sutures previously inserted in each one. They were positioned on each side of the lateral fin of the prosthesis. The shoulder range of motion was evaluated before closure to assure stable tuberosity fixation and to determine the safe range of motion allowed during early rehabilitation.

Follow up:

All patients were followed for a period of at least 6 months. They were examined both clinically and radiologically on the first few days after operation, one month, three months, six months, one year, 18 months after operation

Final assessment:

Evaluation of results is formed by both functional and radiological assessment as well as by evaluation of complications. The functional outcome was assessed with the Constant-Murley scoring system (1985).[14] Radiological assessment of postoperative radiographs was performed including true AP view and lateral Y view.

Results

Overall functional results: By using the modified Constant score, the score in our series ranged from 11 to 93 points with a mean score of 61.7 ± 21.4 points. There were four patients (15.4%) with excellent results, 6 patients (23.1%) with good results, 7 patients (27%) fair results and 9 patients (34.6%) with poor results (Table 1).

Radiological Results: Concentric reduction was achieved in 19 (73.1%) cases. Six (23.1%) cases had superior migration (>5mm superior head displacement) and in 1 (3.8%) case there was evidence of inferior subluxation (>5 mm inferior head displacement). In initial radiographs, the greater tuberosity was considered in an anatomic position in the vertical plane 10 patients. Initial tuberosity malposition in the vertical plane was present in 16 (61.5%) cases. Tuberosity detachment and migration was detected in 8 (30.8%) cases. Tuberosity resorption was present in one of these cases. One case showed tuberosity nonunion. Stem loosening was detected in only two (7.7%) cases. The stem position was neutral in half of the cases. Nine (34.6%) cases were found in valgus (1-5°), and 4 (15.4%) were in varus position (2-4°).

Complications:

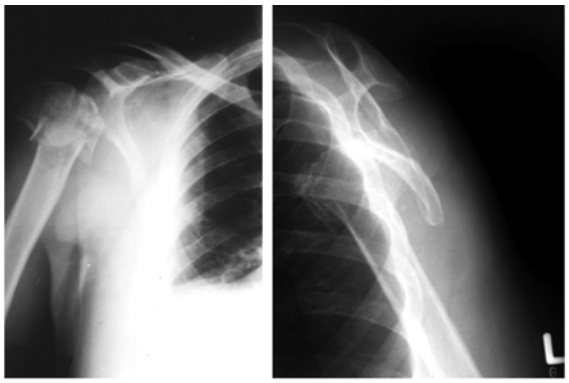
There were three patients with superficial infection that responded well to antibiotic treatment. The prosthesis was removed in one case due to persistent deep infection. Superior migration of the humerus was detected radiologically in 6 (23.1%) cases, inferior subluxation in one (3.8%) case.

Table 1 : Overall final results by the modified Constant Score.

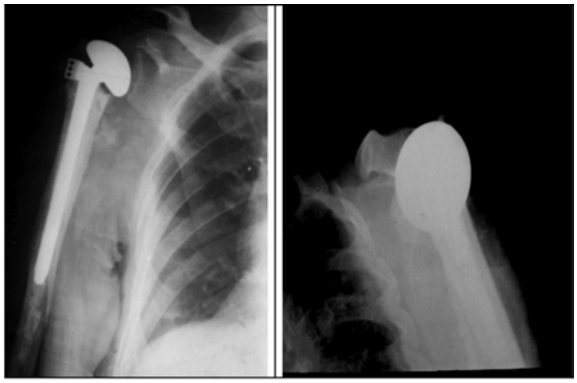
Results	Modified Constant score	
	No of patients	%
Excellent	4	15.3%
Good	6	23.1%
Fair	7	27%
Poor	9	34.6%
total	26	100%

Case presentation:

Case No 1

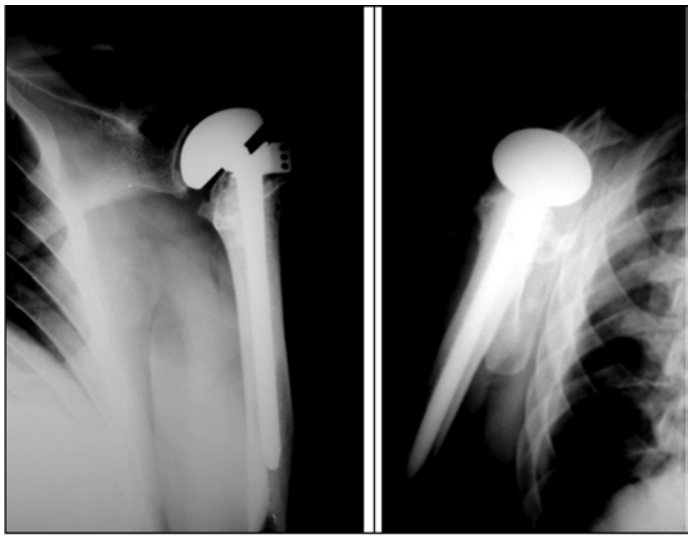


Preoperative radiograph of a IV part fracture of a male aged 48 years old.



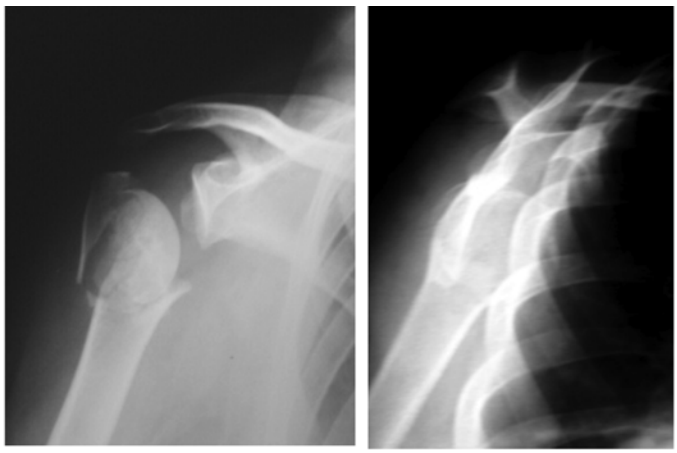
Immediate postoperative radiographs

Case No 1



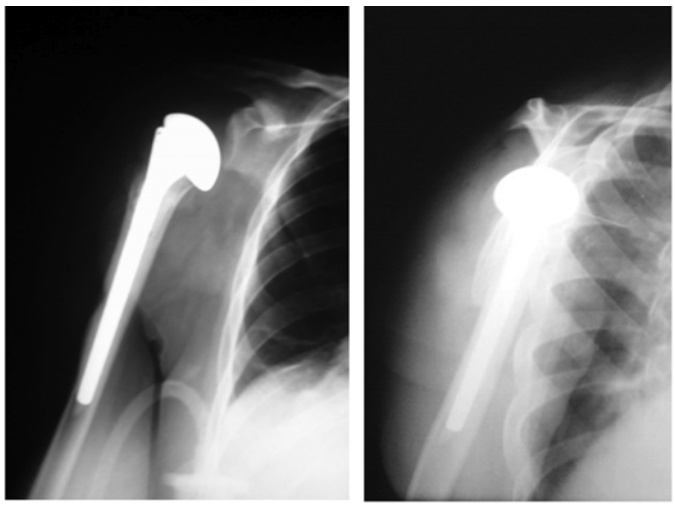
Final follow up obtained 18 months after operation. The patient was graded as excellent (91 points)

Case No 2

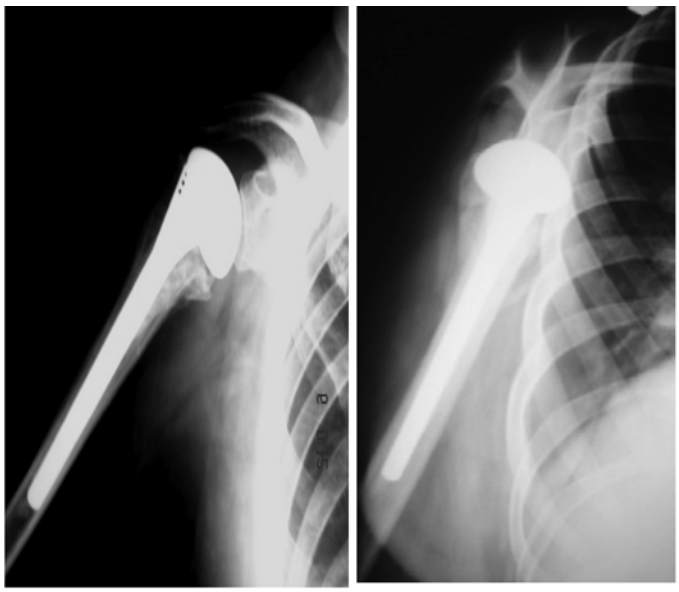


Preoperative radiograph of a IV part fracture of a female aged 53 years old.

Case No 2



Immediate postoperative radiographs



Final follow up obtained 10 months after operation. The patient was graded as poor (46 points)

Discussion

Complex fractures of the proximal humerus remain a therapeutic challenge for many reasons. These reasons include: First; the vascular compromise associating these fractures, second; internal fixation difficulties in osteoprotic bone in elderly patients with high incidence of failure,[15] third; difficulties in obtaining stable internal fixation due to significant comminution.[16] Although, primary hemiarthroplasty for proximal humeral fractures is recommended by many authors since Neer (1970),[9] Tanner and Cofield (1983),[17] Kay and Amstutz (1988),[18] Moeckel et al (1992),[19] Goldman et al (1995),[20] Mighell (2003)[21] and Krishnan et al (2005),[22] its outcome is still questionable. The indications of hemiarthroplasty of the proximal humerus in acute fractures include IV part fractures, fracture-dislocations, impression fractures involving more than 40% of the humeral head, split head fractures and III part fractures in selected cases.[23] This prospective study was carried out on 26 patients with severe proximal humeral fractures to evaluate the results of primary shoulder hemiarthroplasty. In our study, there were 38.4% excellent and good results, 27% fair results and 34.6% were reported as poor results. The modified Constant score ranged 11 to 93 points with a mean score of 61.7 ± 21.4 points. Many other studies have reported similar results. Kralenger et al (2004) in his study on 167 patients presented a mean overall Constant score of 55.37 points.[24] Robinson et al (2003) reported a median modified Constant score of 64 points after one year interval.[25] The most common type of fracture in this study was IV part fractures (50%). Cases associated with dislocation were 23%.In our study and in agreement with other authors, there was no statistically significant difference between the type of proximal humeral fracture with regard to active range of movement.[19,26,27] In this study, there was no statistically significant correlation between the presence of dislocation and results. This was similar to the results reported by both Prakash et al (2002) and Mighell et al (2003).[21,27] In this study, there

were 3patients (11.5%) superficial infection that responded well to antibiotic treatment. Amstutz et al (1988) reported superficial wound infection in one case (9%).[18] In this study, one case suffered from deep infection. Compito et al (1994) on his study on 28 cases with failed shoulder arthroplasty, there were deep wound infection in 16% of cases.[28] In this series, none of our cases were complicated with postoperative dislocation, unlike Boileau et al (2002) who reported such complication in one case.

Conclusion

Shoulder hemiarthroplasty is indicated as a primary procedure in cases with severe proximal humeral fractures that cannot be reconstructed. In elderly patients with osteoporotic bone, this indication can be extended to include all III and IV part fractures. Patient selection is essential for success of operation. Old sedentary patients who appear to be incooperative will not allow for a good rehabilitation program to be undertaken. Anatomical and secure tuberosity reconstruction is a cornerstone in success of shoulder arthroplasty in trauma patients both to restore good muscle function and to avoid detachment of the tuberosities.

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PROSTHETIC HEMIARTHROPLASTY FOR FRACTURES AND FRACTURE DISLOCATIONS OF THE PROXIMAL HUMERUS

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EVALUATION OF PREOPERATIVE CHEMOTHERAPY IN GASTRIC CARCINOMA

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Abstract

Background and Objectives : *The prognosis of locally advanced gastric carcinoma remains dismal even after radical surgery. To date, most therapeutic efforts are directed toward tailoring the extent of surgery and integrating it with the administration of pre- and/or postoperative treatment. Accordingly, our objective in this study is to determine whether chemotherapy before surgery improves the rate of curative resection and the outcomes of gastric cancer.*

Patients and Methods : *Twenty three patients with treatment naïve locally advanced gastric carcinoma, with no evidence of distant metastases, received 3 cycles of pre-operative chemotherapy protocol (cisplatin and flurouracil); clinical response to treatment was assessed. Surgery was scheduled to take place 3-4 weeks after completion of preoperative chemotherapy. Curability of resection was determined. The responding patients received another 3 cycles postoperatively. Paired t-test was used to test for significance of change in tumor diameter after chemotherapy. Survival was calculated by Kaplan-Meier method and differences were assessed by the Log-rank test.*

Results : *All patients received preoperative chemotherapy as scheduled; there was significant tumor shrinkage (from 8±1.86 cm to 5±2.4 cm, $p < 0.001$). Response to chemotherapy was assessed for all patients, 12 patients (52.2%) had partial response, nine patients (39.1%) had stationary disease and 2 patients (8.7%) showed disease progression. No patient reached clinical complete response. Curative resection (R0) rate was 83.3%. At the end of follow up, 11 out of 23 patients are*

alive with no evidence of disease. The 2-year actuarial overall survival (OS) and PFS after curative resection were 86% and 78%, respectively. At univariate analysis, curative resection (R0) found to be the only prognostic factor affecting survival (Log-rank test; p=0.015).

Conclusion: *Preoperative chemotherapy resulted in significant tumor shrinkage with improving patients' survival after actual curative resection. Accordingly, preoperative chemotherapy is a promising modality for gastric carcinoma patients who could benefit from a curative surgery.*

Key Words: *gastric carcinoma, preoperative chemotherapy, R0 resection.*

Introduction

Gastric cancer represents a challenging health problem around the world. It is the fourth most common cancer worldwide. Each year, approximately 700,000 people die of gastric cancer, representing about 10% of all cancer deaths. [1]

The mainstay of treatment in non-metastatic gastric carcinoma is radical surgery, but even with optimal surgical resection, the prognosis remains dismal with a substantial proportion of recurrence and death. Extended surgical resection did not improve the results in some series.[2,3] To date, most therapeutic efforts are directed toward an individualization of therapeutic protocols, tailoring the extent of surgery and integrating it with the administration of preoperative and/or postoperative treatment. [4]

The potential benefits of administering preoperative chemotherapy include increasing the likelihood of curative resection by down-staging the tumor, eliminating micrometastases, rapidly improving tumor-related symptoms, and determining whether the tumor is sensitive to the chemotherapy or not. [5]

The aim of the present study is to determine whether chemotherapy before surgery improves the outcomes of gastric cancer. The main end points were response rate to preoperative chemotherapy, rate of curative resection, overall survival and progression free survival.

Patients and Methods

From January 2009 to May 2011, 23 patients attended Clinical Oncology and Nuclear Medicine Department outpatient clinic, Surgical Gastroenterology Center and Oncology Center Mansoura University Hospital, diagnosed as locally advanced gastric carcinoma, with no evidence of distant metastases, with an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1, and had adequate liver and renal function tests and normal complete blood count. All patients were fit for surgery and gave informed consent.

As pre-treatment Evaluation, all patients were subjected to thorough medical history, physical examination (including anaethetic fitness and electrocardiography), complete blood count, liver and renal function tests, CA19-9 and CEA. Upper esophagogastic endoscopy was done to evaluate site and size of the lesion, assessment of surrounding mucosa and multiple biopsies were taken for histopathological examination. Computed tomography (CT) scan of the abdomen, pelvis and chest (if gastroesophageal junction involved), chest radiograph and bone survey or scan were performed to exclude bone metastases.

All 23 patients received 3 cycles of pre-operative chemotherapy protocol. Each 4-week cycle consisted of cisplatin and flurouracil. Cisplatin (100 mg/m^2) was given intravenously on day 1 over a period of 2 hours with adequate hydration, mannitol diuresis and anti-emetic coverage, and fluorouracil (800 mg/m^2) daily for 5 days by continuous intravenous infusion. Severity of adverse effects, defined according to the National Cancer Institute Common Toxicity Criteria (version 3), were assessed before each cycle.

Three weeks after the third cycle, response to treatment was assessed using CT scan for abdomen, pelvis and chest (if gastroesophageal junction involved). Criteria from the Response Evaluation Criteria in Solid Tumor (RECIST) were used, depending on one-dimensional measurements. [6]

Surgery was scheduled to take place three to four weeks after completion of preoperative chemotherapy.

The extent of resection was determined according to extent of the disease in each case. In radical total gastrectomy (8 patients), the whole stomach was removed, along with the greater and lesser omenta and any other organs involved by extension of the primary growth. The procedure for a radical subtotal distal gastrectomy (5 patients) was the same, but a small, variable gastric remnant was left intact. In both procedures, the resection lines had to be at least 3cm from the edge of the macroscopic tumor. For proximal tumors and tumors extended to the gastroesophageal junction (GEJ), proximal gastrectomy was done (3 patients) with frozen-section-guided resection of the distal esophagus. In D1 lymphadenectomy (6 patients), lymph nodes along the lesser and greater curvatures were included. While in D2 type, dissection extended to celiac group (10 patients). All resected specimens were pathologically examined according to a standard protocol that used the tumor- node- metastasis (TNM) classification system for staging gastric carcinoma.[7] The resection was judged curative if all macroscopic and microscopic disease seemed to have been removed (R0).

Three to six weeks after surgical resection, the responding patients received the same preoperative chemotherapy protocol to complete a total of six cycles.

After the end of therapy, all patients were followed up with physical examination and laboratory investigation (monthly for one year and every 3 months thereafter), CT scan and upper esophagogastric endoscopy every six months, and diet regimen for patients suffering from postgastrectomy feeding complications.

Statistical analysis :

Frequency, median, mean and standard deviation were used to describe data. Chi-square test was used to test for significant association between categorical variables and response. Paired t-test was used to test for significance of

change in tumor diameter after chemotherapy. Kendall's non parametric correlation was used to test for linear relationship between response and quantitative variables.

Life table, Kaplan-Meier test and Log-rank test were used to test for effect of different variables on patient survivals. P value was considered significant if less than 0.05. These tests were run on an IBM compatible personal computer using the Statistical Package for Social scientists (SPSS) for windows 17 (SPSS Inc., Chicago, IL, USA).

Results

Table (1) represents patients' characteristics. The mean age of patients was 47.2 ± 16.6 years (range 19 - 75 years), with a peak incidence of 39% between 40-59 years. There was a slight male predominance (52%). At initial presentation, 13 patients (56.5%) had ECOG performance status score 0, and 10 patients (43.5%) had score 1. The majority of patients suffered from upper abdominal pain (60.9%); followed by dysphagia (26.1%), vomiting (21.7%), dyspepsia (21.7%) and hematemsis (8.7%).

Upper endoscopic examination revealed body lesions in 10 patients (43.5%), antral lesions in 7 patients (30.4%), cardiac lesions in 4 patients (17.4%), and diffuse lesions in 2 patients (8.7%). Adenocarcinoma was the most common pathological type (10 patients); followed by signet ring carcinoma (9 patients), mucinous carcinoma (2 patients), and undifferentiated carcinoma (2 patients).

All patients received the preoperative chemotherapy as planned. Grade 1 and 2 nausea and vomiting were observed in 78.3% of patients, followed by grade 1 and 2 diarrhea and renal toxicity (13.3%). Grade 1 and 2 mucositis occurred in 2 patients (8.7%). As regard hematological toxicity, grade 1 and 2 anemia occurred in 74% of treated patients and grade 1 thrombocytopenia in one patient only (4.3%). The treatment was generally well tolerated and all observed toxicities were manageable (table 2). Symptomatic improvement, particularly pain and dysphagia, was reported in 16 out of

23 patients. Moreover, there was improvement in satiety which reflected on body weight and nutritional status.

The maximum diameter of gastric lesions based on pretreatment CT scanning was 8±1.86 cm. After preoperative chemotherapy the maximum diameter of gastric lesion was 5±2.4 cm, which was consistent with significant tumor shrinkage ($p < 0.001$) (table 3).

Response to chemotherapy was assessed for all patients, 12 patients (52.2%) had partial response, nine patients (39.1%) had stationary disease and 2 patients (8.7%) showed disease progression. No patient reached clinical complete response.

The relation of response to different patients' characteristics was evaluated (table 4), patients' gender and site of gastric lesion had statistically significant effect on response to preoperative chemotherapy in favor of male gender and lesion confined to the body of stomach ($p=0.03$ and 0.004, respectively); response was better in patients with PS score 0, but the effect was not statistically significant ($p=0.583$).

Kendall's non parametric correlation was used to test for relationship between response and patients' age and tumor pathology, there were no statistical significance ($p=0.952$, and 0.196 respectively).

Eighteen out of 23 patients were suggested clinically to be candidate for resection and submitted to surgery; of them, 8 patients (44.4%) underwent total gastrectomy, 5 patients (27.8%) underwent subtotal gastrectomy and 3 patients (16.6%) underwent proximal gastrectomy. Two patients (11%) had advanced disease because of direct infiltration of the surrounding organs, palliative resection and gastrojejunostomy was done for them. Regarding lymph node dissection, 10 patients (55.6%) had extended lymphadenectomy (D2) and 6 patients (33.3%) had limited lymphadenectomy (D1) (table 5). The other 5 patients not referred to surgery due to: patient request (2 patients), medical unfitness for sur-

gery (1 patient), and distant metastases (2 patients).

After final pathological examination, curative resection (R0) was confirmed in 15 patients (83.3%) and 1 patient found to have microscopic positive margin (R1) (table 5).

Postoperative hospital stay ranged from 6 to 10 days. The reported early postoperative complication was anastomotic leakage in 1 patient, who died after 1 week. No history of delayed wound healing or infections. Regarding late complications, there were biliary reflux and anastomotic benign stricture (1 patient), late dumping (1 patient), and adhesive intestinal obstruction (2 patients) (table 5).

Post operative pathological results are illustrated in table (5). According to depth of tumor invasion, 2 patients showed no histopathologic evidence of the primary tumor (ypT0), 8 patients had tumors with invasion up to the muscle layers (ypT2a). Subserosal invasion was detected in 6 patients (ypT2b). Two patients were staged as T4 (due to direct invasion of the surrounding organs). Four patients had pathological node negative diseases (one of them had evidence of pathologic complete response as infarction necrosis was detected). Twelve patients had pN1; there was no pathological N2 or N3 disease. Negative safety margins were documented in 15 patients (83.3%) and positive margin in one patient only (5.6%).

Although treatment was well tolerated in preoperative settings, some patients experienced more adverse effect of postoperative chemotherapy; details of toxicity of postoperative chemotherapy are shown in table (2).

At the end of follow up; 11 patients (47.8%) were alive with no evidence of disease (NED), 6 patients (26.1%) were alive with disease (LWD), 4 patients (17.4%) died with their disease (DWD) and 2 patients (8.7%) died from unrelated causes.

Overall median survival for the 23 patients was 15 months (range 6-29 months), the 2-year actuarial overall survival (OS) was 70% (fig

1), and the 2-year progression free survival (PFS) was 64% (fig 2).

At a median postoperative follow-up of 20 months (range 8-26 months); the 2-year actuarial overall survival (OS) and PFS were estimated after curative resection (15 patients), they were 86% and 78%, respectively. Two out of 15 patients (13.3%) proved to have local recurrence; and no patient developed distant metastases till the end of follow up.

At univariate analysis, age and gender of patients, performance status at presentation, tumor site, WHO pathologic type, tumor depth, pathological nodal status and response to preoperative chemotherapy did not show a significant impact on survival of patients submitted to surgery after preoperative chemotherapy. Curative resection (R0) found to be the only prognostic factor affecting survival (Log-rank test; p=0.015) (fig 3).

Table (1): Patients' characteristics.

Variable	Number (n=23)	%
Age (*year*)		
<40	8	35
40-59	9	39
≥60	6	26
Mean 47.2±16.6		
Range 19-75		
Gender		
Male	12	52
Female	11	48
ECOG PS		
0	13	56.5
1	10	43.5
Presenting symptoms*		
Pain	14	60.9
Dysphagia	6	26.1
Vomiting	5	21.7
Dyspepsia	5	21.7
Hematemsis	2	8.7
Site of tumor		
Body	10	43.5
Antrum	7	30.4
Cardia	4	17.4
Diffuse	2	8.7
Pretreatment tumor diameter (*cm*)		
6-8	14	61
>8-10	2	8.7
>10	7	30.4
Mean 8±1.86		
Pathology		
Adenocarcinoma	10	43.5
Signet ring cell	9	39.1
Undifferentiated	2	8.7
Mucinous	2	8.7

* More than one symptom may present per one patient

Table (*2*): Chemotherapy related toxicities.

Adverse effect		Preoperative (N=23)		Postoperative (N=15)	
		Grade 1&2 *No. (%)*	Grade 3&4 *No. (%)*	Grade 1&2 *No. (%)*	Grade 3&4 *No. (%)*
Non-hematological toxicity	Nausea &vomiting	18 (78.3)	0	11 (73.3)	2 (13.3)
	Diarrhea	3 (13)	0	5 (33.3)	0
	Mucositis	2 (8.7)	0	7 (46.7)	0
	Renal	3 (13)	0	3 (20)	0
Hematological Toxicity	Anemia	14 (74)	0	12 (80)	0
	Thrombocytopenia	1 (4.3)	0	2 (13.3)	2 (13.3)

Table (3): Evaluation of preoperative chemotherapy effect according to mean of maximum tumor diameter

Tumor maximum diameter	Mean (cm)	P value
Pre-chemotherapy	8.0 ±1.86	<0.001
Post- chemotherapy	5.19 ±2.43	

Table (4): Evaluation of clinical response to preoperative chemotherapy according to patients' characteristics

	PR		SD		PD		P value
Gender	**No.**	**%**	**No.**	**%**	**No.**	**%**	
Male	8	66.7	2	16.7	2	16.7	0.030
Female	4	36.4	7	63.6	0	0	
Site							0.004
Body	8	80	2	20	0	0	
Antrum	2	28.6	5	71.4	0	0	
Cardia	2	50	0	0	2	50	
Diffuse	0	0	2	100	0	0	
PS							0.583
0	8	61.5	4	30.8	1	7.7	
1	4	40	5	50	1	10	

Table (5): Evaluation of surgical treatment and pathology results

	Number (n=18)	%
Type of operation		
Total gastrectomy	8	44.4
Subtotal gastrectomy	5	27.8
Proximal gastrectomy	3	16.7
Palliative surgery	2	11.1
Extent of lymph node dissection		
Extended (D2)	10	55.6
Limited (D1)	6	33.3
No dissection (D0)	2	11.1
Extent of surgical resection		
Curative (R0)	15	83.3
Microscopic residual (R1)	1	5.6
Palliative surgery (R2)	2	11.1
Postoperative complications:		
Early:		
Anastomotic leakage	1	5.6
Delayed wound healing	0	0
Infection	0	0
Mortality	1	5.6
Late:		
Anastomotic stricture	1	5.6
Biliary reflux	1	5.6
Late dumping	1	5.6
Adhesive intestinal obstruction	2	11
Tumor stage		
T0	2	11.1
T2a	8	44.4
T2b	6	33.3
T4	2	11.1
Nodal stage		
Nx	2	11.1
N0	4	22.2
N1	12	66.7
yp TNM stage		
Ib	6	33.3
II	10	55.6
IIIa	2	11.1

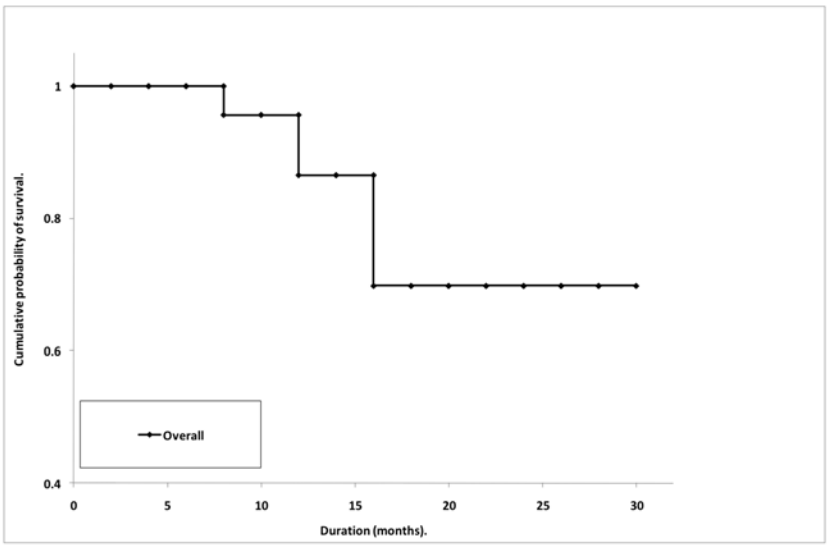

Fig (1): Kaplan-Meier estimation of over all survival of 23 patients with locally advanced gastric cancer submitted to preoperative chemotherapy

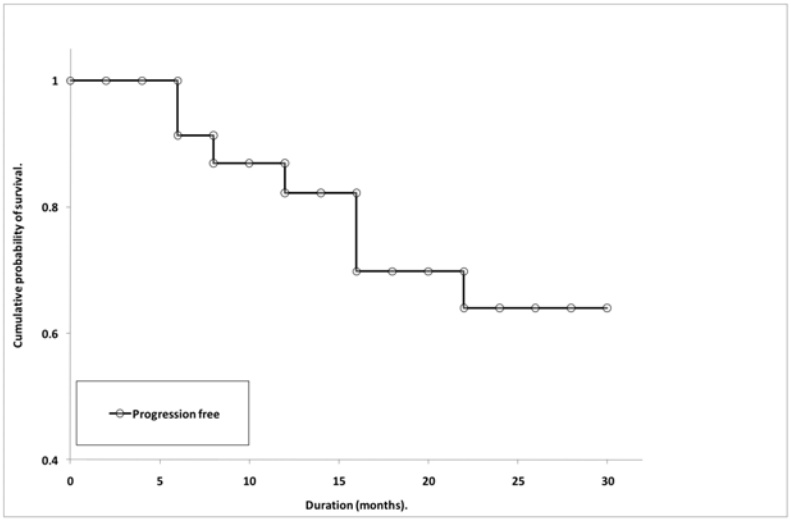

Fig (2): Kaplan-Meier estimation of progression free survival of 23 patients with locally advanced gastric cancer submitted to preoperative chemotherapy

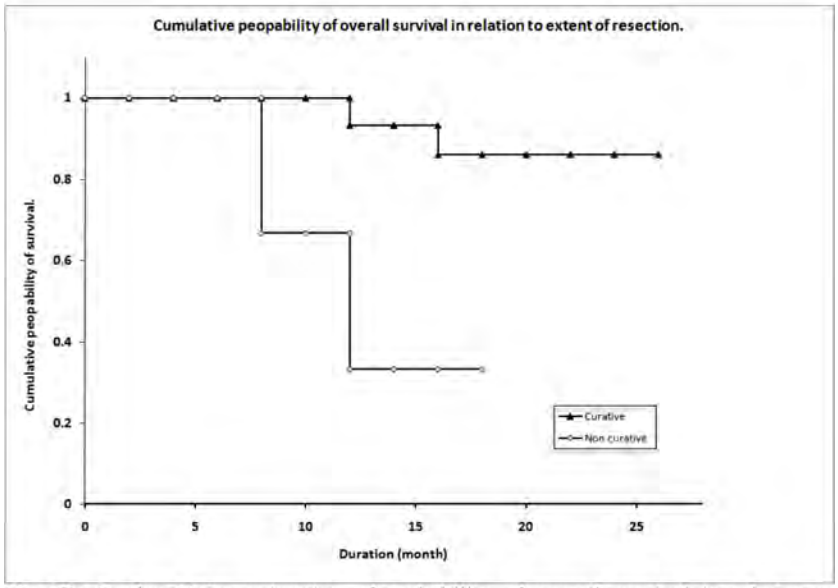

Fig (3): Kaplan-Meier estimation of probability of overall survival in relation to extent of resection

Discussion

Gastric cancer accounted for 14.5% of gastrointestinal cancers (286/1976 patients) and 1.3% of the total malignant cases (21,524 patients) attended to Clinical Oncology and Nuclear medicine Department, Mansoura University, in the last 10 years.

This study was conducted prospectively on 23 patients with treatment naïve potentially resectable, and locally advanced non-metastatic gastric carcinoma. All patients received three cycles of preoperative chemotherapy (cisplatin- 5 FU) then assessed for surgical resection.

Cisplatin-5-FU (CF) based regimen have been proposed as reference regimen in advanced gastric cancer for clinical use because they have been widely investigated in clinical studies and have demonstrated favorable survival outcomes. [8]

In this study, the mean age at presentation was 47.2 ± 16.6 year with the peak age incidence 39%

between 40-59 years. There was a slight male predominance (52%). Fifty two percent of our patients presented with ECOG performance status score 0. Abdominal pain was the most common presenting symptom (60.9%). Regarding the site of gastric lesions, the medium- distal third represented 73.9% of cases. The most common pathologic type was adenocarcinoma (43.5%).

In the present study, the gastric lesions were more advanced; the mean size of the tumors at presentation was 8±1.86 cm (range 6-12 cm). In Egyptian study conducted on surgically candidate patients, the mean size of the tumors was 5.5 cm (range 2-11 cm). [9] On contrary, in their study of perioperative chemotherapy in advanced gastric cancer, Cunningham et al.[10] found that the mean size of the tumors at presentation was 5 cm (range 3-7cm).

Preoperative chemotherapy was completed in all patients, with good tolerance; no history of treatment delay or dose reduction or treatment related deaths. Candidate patients for surgical resection (78.3%) were ready after 3-4 weeks from the last cycle. These findings differ from that reported in other series using the same or other chemotherapy protocols. In a study conducted by Ott et al [11], to evaluate PLF (cisplatin, Folinic acid, fluorouracil) in 49 patients with potentially resectable gastric cancer, chemotherapy was stopped during the first cycle in 7 patients (14%). One patient developed a severe pulmonary embolism, and in 2 patients a subclavian vein thrombosis occurred. One patient developed grade 3 diarrhea and refused further chemotherapy. Two other patients developed deterioration of their overall performance status. One patient demonstrated tumor progression during the first cycle. Persiani et al.[12] used three preoperative courses of either EEP (Etoposide, epirubicin, and Cisplatin) for 25 patients, or ECF (Epirubicin, Cisplatin, and 5-fluorouracil) for 9 patients with locally advanced gastric cancer. One patient died due to septic complications after completion of the first cycle of EEP chemotherapy. Grade 3-4 neutropenia occurred in 59%, and

administration of G-CSF was needed for 47%. Dose reduction was necessary in 29.4% of patients while treatment was delayed for 17.6% of patients, on account of grade 3-4 neutropenia and/or anemia.

Cunningham et al.[10] reported death rate of 1.7% within 60 days after commencing treatment with ECF, half of them died due to cardiac toxicity. Also, Boige et al.[13] reported toxicity-related death in one patient during preoperative treatment with cisplatin-fluorouracil.

In the present study, 16/23 patients (70%) experienced early control of abdominal pain, dysphagia, and so, diet tolerance and sense of well-being after one or two courses of treatment. The same observation was documented in 25/34 patients (73.5%) in Persiani et al. [12] series.

Clinical response rate to preoperative chemotherapy was estimated; PR was documented in 12 patients (52.2%), SD in 9 patients (39.1%), and PD in 2 patients (8.7%). Rougier et al.[14] found that 2 cycles of cisplatin-5FU in locally advanced gastric cancer resulted in 46.6% PR. Ott et al.[11] reported 26% clinical response rate (11/42) after 2 cycles of PLF in patients with locally advanced gastric cancer. On the other hand, Hartgrink et al. [3] reported a high rate of tumor progression (36%) after 4 cycles of FAMTX (fluorouracil, doxorubicin, and methotrexate), which was, at that time, the gold standard of treatment for adenocarcinoma of the stomach.

There was a significant reduction of tumor bulk in response to preoperative chemotherapy, as the maximum diameter of gastric lesions decreased from 8 ±1.86cm to 5 ±2.4 cm ($p < 0.0001$). Our results were comparable to Cunningham et al.[10] results, as the maximum diameter of the tumor was decreased from 5cm to 3cm after 3 cycles of ECF (epirubicin, cisplatin, and fluorouracil) ($p<0.0001$).

The possible increase in the actual R0 resection rate has been an important goal of the present study. The rate of curative resection reached 83.3% of patients

submitted to surgery which was in agreement with Schuhmacher et al.[15]; in their study R0 resection rate was 81.9% after two cycles of neoadjuvant chemotherapy (cisplatin- 5 FU) for 72 patients with locally advanced gastric cancer. Also, it was in agreement with D'Ugo et al.,[16]; in their phase II study of perioperative chemotherapy using different protocols (EEP or ECF) conducted on 34 patients with locally advanced non-metastatic gastric cancer, as R0 resection rate was 82%. Boige et al. [13] reported higher rate of curative resection (87%), after 2-3 cycles of (cisplatin- 5FU). On the other hand, the Dutch Gastric Cancer Group [3] reported a disappointing result after 4 cycles of FAMTX, as the curative resection was 56% only.

In this study, the extension of gastric resection depended upon the site of primary tumor. Among all patients submitted to surgery, limited lymph node dissection (D1) was done for 33.3%, while extended lymph node dissection (D2) was done for 55.6% of patients; while 2 patients (11.1%) had no lymph node dissection. The extent of lymphadenectomy was comparable to that occurred in MAGIC trial as the rate of D1 and D2 were 17.8% and 43.5% respectively, while 13.2% had nonresectional surgery. [10]

Median hospital stay was 8 days, which was the same as patients underwent upfront surgery as documented by our surgeons. In the western series the median hospital stay was 14 days.[10, 13] Postoperative mortality was reported in only one patient (5.6%) in the present study, which was similar to that reported in other series. In the MAGIC trial, 5.6% of patients died in the surgery-alone arm, and 5.9% died in the perioperative chemotherapy arm.[10] In the French trial, the corresponding numbers were 4% and 5%, respectively. [13]

In the present study, early surgical morbidity was recorded in one patient only (5.6%) in the form of anastomotic leakage, specific complication that may reflect chemotherapy effect was not observed. On the other hand, Persiani et al. [12] recorded 24% complication rate including anastomotic

leakage, pneumonia, abdominal abscess, and major wound infection. This may be contributed to the type of chemotherapy used in their study (EEP and ECF), as grade 3-4 neutropenia occurred in 59 % during preoperative settings. When compared to series used the same chemotherapy, Schuhmacher et al. [15] reported higher Postoperative complications after 2 cycles of (cisplatin, 5-FU) than in upfront surgery group (27.1% v 16.2%; P = .09).

Late postoperative complication rate in present study was 27.7%, which was comparable to that reported in MAGIC trial, in which complication rate was 29.1% after neoadjuvant chemotherapy and 28.9% after surgery alone; respectively. [10]

Regarding histo-pathologic reports, depth of tumor invasion was assessed: ypT0 was observed in 11.1%, ypT2a in 44.4% and ypT2b in 33.3%, denoting that in patients who did benefit from preoperative chemotherapy and curative resection there was less tumor invasion, T4 disease was found in 2 patients (11.1%). Over all the percentage of early T stage was higher in our study than that reported by Persiani et al.[12] as ypT0 was observed in 3%, ypT1 in 3%, ypT2a in 24.3%, ypT2b in 27.3%, ypT3 in 30.3%, and ypT4 in 12%.

As regard to pathological nodal staging, 22.2% had node negative disease, and 66.7% had pathological N1 disease (range 2-5 nodes), in Cunningham et al.[10] series, pN0 detected in 31%, pN1 in 53.3%, pN2 in 14% and pN3 in 4.7%.

In accordance with Cunningham et al.[10], and Boige et al.[13], our study illuminates that preperative chemotherapy is tolerated better than postoperative therapy. Postoperative chemotherapy was completed by our patients after curative resection. However, postoperative chemotherapy could be completed by only 42% and 50% in Cunningham et al.[10] and Boige et al.[13] series, respectively. This may be caused by slow postoperative recovery and the poor food intake after gastrectomy.

In Egypt gastric cancer usually

presents late in advanced stage. Before 1998, the 2- year overall survival rate after limited surgical resection was 30.1%[17]; after radical gastrectomy and systematic lymph node dissection, the estimated 2 year overall survival rate was 43.6%.[9] In this study, the estimated overall survival for the 23 patients was 70%. The survival rates at 12 and 24 months after curative resection are 93% and 86%, respectively. These results were comparable to the results reported by Persiani et al.[12], as 2-year overall survival rate for all patients was 67%; and the estimated survival rates after curative resection (27/33 patients) were 92% and 80% at 12 and 24 months, respectively.

Gastric cancer metastasizes very early and often represents a systemic disease, early preoperative treatment strategies are of special interest aiming at treatment of micrometastases early in the course of disease. In the present study, at the end of follow up (range 8-26 months) of curatively resected patients, 11 out of 15 patients (73.3%) had no evidence of disease, while locoregional recurrence was observed in 2 patients (13.3%); there was no evidence of distant metastases. At their last follow up (range 2-91 months), Persiani et al [12] had 19 out of 33 (57.6%) patients alive and 16 free of recurrence (48.5%); however, longer follow up and larger sample size are required to confirm our finding.

At univariate analysis, age and gender of patients, performance status at presentation, tumor site, WHO pathologic type, tumor depth, pathological nodal status and response to preoperative chemotherapy did not show a significant impact on survival of patients submitted to surgery after preoperative chemotherapy. Curative resection (R0) found to be the only prognostic factor affecting survival (p=0.015). This was in accordance with Persiani et al.[12], as they concluded that, R0 resection was the only independent variable in determining the probability of long-term survival in locally advanced gastric carcinoma (p=0.0002), while tumor depth, pathologic type, response to chemotherapy, tumor site, age of patients, did not show significant

impact on survival of patients submitted to preoperative chemotherapy. However, larger sample size and longer follow up period are required to confirm our results.

Conclusion

According to our results regarding the significant tumor shrinkage and improving patients' survival after actual curative resection, we can conclude that preoperative chemotherapy is a promising modality for gastric carcinoma patients who could benefit from a curative surgery. Additional studies on new regimens and well-designed powerful trials with accurate staging are highly encouraged in patients with locally advanced gastric cancer.

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EVALUATION OF PREOPERATIVE CHEMOTHERAPY IN GASTRIC CARCINOMA

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SEX DETERMINATION BY THE LENGTHS OF METACARPALS AND PHALANGES : X-RAY STUDY ON EGYPTIAN POPULATION

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Abstract

Measurements of hand bones length have been shown to be sexually dimorphic in many nationalities. The aim of this study is to assess the accuracy of sex determination from the lengths of all metacarpals and phalanges of right and left hands using X-ray radiographs and to develop a discriminant formula that can be used in Egyptians. One hundred Egyptians are included in the study (50 males and 50 females) in the period from December 2009 to January 2011 with mean age 31.60 ± 9.44. Each is subjected to X-ray radiographs on both hands. The results reveal that males have significantly greater mean values than females for all metacarpals and phalanges of both hands and the Egyptian population has greater measurements in comparison to the other ones (e.g. Turkish and European Americans). In addition there is no significant difference between the right and the left hands in either males or females. The correct classification reached an accuracy of 88%-94% by using both hands, while that for right hand only is 88% and 88%-90 % for the left hand only. Regarding the accuracy of each bone, the present results revealed that 1st DP & PP and 3rd and 4th MC in the right and left hands are the best bones that can be used in correct sex determination. It is concluded that the length of metacarpals and phalanges (especially the 1st DP & PP and 3rd and 4th MC) could be used for sex determination. The right hand could be used as the left hand in determination of sex. Also the X-ray radiographs are good non invasive and simple tool in the determination of sex from the hand bones. Furthermore the regression equation for both hands and each hand separately is specific to Egyptian population and should be used cautiously with other ones.

Keywords: *Sex Determination, Metacarpals, Phalanges, Egyptians.*

Introduction

Identification of living, dead or human remains is an imperative element of any medico - legal investigations. The determination of race, sex, age and stature remains the cornerstone in forensic medicine. Actual sexing of the remains is vital as it primarily narrows down the pool of possible victim matches [1].

Sex determination is one of the foremost criteria in establishing the identity of an individual. It is considered as one of the simplest tasks in forensic analysis when the external and internal genitalia can directly suggest the sex of an individual. But sex differentiation remains a complex one in case of intersex; bodies in advanced state of putrefaction; mutilated; fragmentary and skeletonized remains [2, 3].

Sex estimation is the classification of an individual as either male or female. To achieve an assignation of sex, anthropologists use biological traits that vary between both sexes[4]. Although there are only two biological sexes exist in humans, sex estimation of a human skeleton remains a challenge [5].

With the increasing frequency of mass disaster, either natural or in cases of wars; acts of terrorism; bombing and traffic accidents; it is not unlikely to find dismembered human remains and peripheral parts of the body[6,7]. While the most useful bones in this regard are cranial and pelvic ones, these are not always available[8]. For this purpose, when skeletal remains are incomplete or damaged, the sex determination must be attempted from other bones which show some degree of sexual dimorphism [9].

Anatomically the short tubular bones have some advantages over other bones in a forensic context. The shafts of long bones often stay intact, but their epiphyses are prone to damage because of the overlying fragile cancellous bone. However, the smaller long bones of the hands often remain complete [5].

The hands especially the metacarpal bones and feet have been addressed for sex estimation with

varying results in terms of accuracies [10]. In addition the phalanges were used for the same purpose, although their accuracy has been found to be higher than that of metacarpals [11].

The aim of this study is to assess the accuracy of sex determination from the lengths of all metacarpals and phalanges of right and left hands using X-ray radiographs and to develop a discriminant formula that can be used in Egyptians.

Subject & Methods

Subjects: This study was conducted on 100 Egyptian subjects (3800 bones) classified to 50 males (their ages range from 17 - 53 years) and 50 females (their ages range from 17 - 65 years) in the period from December 2009 to January 2011. They were randomly chosen from those attending to the Radiology Center in Mansoura University Hospital. The cases with skeletal immaturity, pathological lesions such as congenital and developmental dysplasia, metabolic bone diseases, recent trauma or surgery, as well as tumors, osteoarthritis and arthritis were excluded.

Methods: Separate X-ray radiographs were taken to the right and left hands of all subjects after obtaining informed consent. The subject was seated adjacent to the X - ray table with the forearm and hand flat and prone on the table with no lateral angulations at the wrist. The hand was centered on the cassette with fingers slightly apart from each other but flat. Images were obtained using a small focal point and a detail cassette. Exposures and distances were: 48 kV; 3.2 mA.s; 90 cm source to image distance. The length of all metacarpals and phalanges (proximal, middle and distal) of all fingers of the right and left hands were measured (in millimeters) on the X-ray films by one observer (First author). All measurements were made from the midpoint of the base to the midpoint of the tip of all metacarpals and phalanges[12]. The present study focused on one measurement (length) because the other measurements (the width of the base and head) of metacarpals and phalanges are not accurate on routine hand radiographs[10].

Statistical Analysis:

The statistical analysis of data was done by using SPSS (SPSS, Inc, Chicago, IL) program statistical package for social science version 16. To test the normality of data distribution K-S (Kolmogorov-Smirnov) test was done only significant data revealed to be nonparametric.

N.B: all tested data revealed to be parametric.

All results were expressed as mean ± standard deviation (SD), minimum and maximum values. The analysis of the data was done to test statistical significant difference between groups. For quantitative data student t-test was used to compare between two groups. Multivariate logistic regression analysis was used in order to be able to predict sex based on values of predictor variables of the metacarpals and phalanges. To detect cutoff values with highest sensitivity and specificity for each bone the Roc (Receiver Operating Characteristics) curve was done.

N.B: p is significant if < or = 0.05 at confidence interval 95%.

Results

Descriptive analyses by means of age of the subjects are summarized in table (1). The mean ± SD age for male subjects is 31.8600 ± 8.8755 years (ranges from 17 - 53 years) while mean ± SD age for female subjects is 31.3469 ± 10.0717 years (ranges from 17 - 65 years).

The summary statistics and comparison for all measurements (minimum, maximum, mean ± SD) of the right and the left hand bones (in millimeters) between both sexes are presented in table (2) & figure (1). All measurements are significantly higher in males than females regarding all metacarpals and phalanges of both right and left hands.

A comparison between the right and the left hand bones in males and females by using student t - test is represented in table (3). There is no significant difference between the right and the left hand in either males or females except for the first metacarpal bone in males.

Multivariate logistic regression

analysis with the use of most predictable measurements of both hand bones to determine sex is demonstrated in table (4).

Furthermore multivariate logistic regression analysis with the use of most predictable measurements of the right hand and the left hand bones separately to determine sex is demonstrated in table (6).

So for determination of sex (z), the following equation is used where z = 0 for males and z = 1 for females, was regressed on the bone measurements (Lf PP1, Lf MC4, Rt PP2, Rt PP3 and Rt PP4) to produce equations with the generalized formula. z = Constant (53.584) + B (-1.746) X (PP1) + -0.640 X MC4 + 1.559 X PP2 + -1.310 X PP3 + 0.815 X PP4.

If z > 0.5, the bone is male, and, if z < 0.5, the bone is female.

As a result 94 % of the males (47/50) and 88 % of the females (44/50) are correctly classified by using both hands as shown in table (5). While 88 % of both males and females (44/50) are correctly classified by using the right hand only and 90% of males (45/50) and 88% of females (44/50) are correctly classified by using the left hand only (Table 7).

In addition, the cut off values (in mm) and accuracy percentage for sex determination for each bone in the right and left hand is illustrated in table (8).

Table (1): Descriptive Statistics of the Studied Subjects (n = 100) by Means of Age.

	Minimum	**Maximum**	**Mean ± SD**
Males (n = 50)	17	53	31.86 ± 8.87
Females (n = 50)	17	65	31.34 ± 10.07
Total (n = 100)	17	65	31.60 ± 9.44

Table (2): Summary Statistics (minimum, maximum, mean ± SD) and comparison for all measurements of the right and the left hand bones (in millimeters) between both sexes (n = 100).

Bone Rt			Fingers									
			1st		2nd		3rd		4th		5th	
			Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
DP	Range	Male	18.00	28.00	15.00	21.00	16.00	23.00	16.00	29.00	14.00	22.00
	Mean ± SD		23.1400 ± 1.818		17.9200 ± 1.588		19.2600 ± 1.816		20.0408 ± 2.198		17.8000 ± 1.525	
	Range	Female	17.00	24.00	14.00	27.00	15.00	28.00	15.00	23.00	12.00	19.00
	Mean ± SD		20.3200 ± 1.463		16.4400 ± 1.928		17.2600 ± 2.028		17.7800 ± 1.681		15.7200 ± 1.429	
		t	8.544		4.188		5.194		5.755		7.037	
		P	**0.000***		**0.000***		**0.000***		**0.000***		**0.000***	
MP	Range	Male	None		20.00	30.00	25.00	36.00	25.00	33.00	17.00	25.00
	Mean + SD				24.2800 ± 2.147		29.8200 ± 2.335		28.5800 ± 1.990		20.6200 ± 1.736	
	Range	Female	None		19.00	27.00	21.00	38.00	22.00	38.00	15.00	29.00
	Mean + SD				22.6400 ± 1.711		27.2000 ± 2.718		26.5800 ± 2.515		18.5200 ± 2.366	
		t	None		4.222		5.169		4.408		5.058	
		P			**0.000***		**0.000***		**0.000***		**0.000***	
PP	Range	Male	29.00	38.00	35.00	47.00	41.00	52.00	38.00	49.00	31.00	40.00
	Mean + SD		32.9600 ± 2.249		41.5800 ± 3.037		46.7200 ± 2.777		43.7000 ± 2.719		34.9000 ± 2.261	
	Range	Female	25.00	33.00	35.00	52.00	37.00	48.00	37.00	44.00	28.00	36.00
	Mean + SD		28.9800 ± 2.065		38.9200 ± 2.975		42.7600 ± 2.599		40.4400 ± 2.139		32.1200 ± 1.975	
		t	9.216		4.424		7.360		6.661		6.547	
		P	**0.000***		**0.000***		**0.000***		**0.000***		**0.000***	
MC	Range	Male	43.00	55.00	63.00	80.00	60.00	77.00	54.00	69.00	49.00	63.00
	Mean + SD		48.4400 ± 2.749		71.5800 ± 3.933		68.6200 ± 3.724		60.6600 ± 3.354		56.7200 ± 3.356	
	Range	Female	39.00	51.00	58.00	72.00	55.00	70.00	49.00	61.00	45.00	59.00
	Mean +SD		44.6400 ± 2.974		65.3400 ± 3.432		62.2800 ± 3.476		54.4400 ± 3.117		51.5600 ± 3.169	
		t	6.634		8.452		8.799		9.604		7.903	
		p	**0.000***		**0.000***		**0.000***		**0.000***		**0.000***	

Bone Lf			Fingers									
			1st		2nd		3rd		4th		5th	
			Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
DP	Range	Male	14.00	27.00	15.00	22.00	16.00	23.00	16.00	23.00	14.00	21.00
	Mean +SD		22.6800 ± 2.103		18.0800 ± 1.588		19.2600 ± 1.712		19.8200 ± 1.746		17.8000 ± 1.577	
	Range	Female	16.00	23.00	14.00	21.00	15.00	22.00	16.00	22.00	14.00	19.00
	Mean +SD		20.2800 ± 1.485		16.3400 ± 1.437		17.3800 ± 1.523		17.9000 ± 1.373		15.7600 ± 1.221	
		T	6.590		5.743		5.800		6.111		7.229	
		P	**0.000***		**0.000***		**0.000***		**0.000***		**0.000***	
MP	Range	Male	None		20.00	29.00	25.00	35.00	25.00	33.00	17.00	25.00
	Mean +SD				24.2400 ± 2.015		29.8000 ± 2.267		28.5600 ± 1.960		20.5800 ± 1.830	
	Range	Female	None		20.00	27.00	21.00	31.00	23.00	37.00	15.00	22.00
	Mean +SD				22.6200 ± 1.664		27.1200 ± 2.086		26.4400 ± 2.331		18.6600 ± 1.745	
		T	None		4.382		6.150		4.921		5.368	
		P			**0.000***		**0.000***		**0.000***		**0.000***	
PP	Range	Male	29.00	38.00	36.00	48.00	41.00	52.00	39.00	50.00	30.00	40.00
	Mean +SD		33.0000 ± 2.185		41.7800 ± 2.674		46.9800 ± 2.795		44.0200 ± 2.721		34.9400 ± 2.235	
	Range	Female	25.00	33.00	35.00	44.00	38.00	48.00	37.00	45.00	28.00	37.00
	Mean +SD		28.9800 ± 1.932		38.7200 ± 2.157		42.9400 ± 2.606		40.3400 ± 2.036		31.9600 ± 2.194	
		T	9.744		6.296		7.474		7.655		6.727	
		P	**0.000***		**0.000***		**0.000***		**0.000***		**0.000***	
MC	Range	Male	43.00	55.00	64.00	80.00	61.00	77.00	53.00	69.00	50.00	64.00
	Mean +SD		49.0200 ± 2.867		71.9800 ± 3.809		68.7600 ± 3.728		60.8800 ± 3.526		56.4600 ± 3.221	
	Range	Female	39.00	51.00	57.00	72.00	56.00	70.00	50.00	60.00	44.00	57.00
	Mean ± SD		44.6000 ± 2.725		65.3000 ± 3.189		62.1800 ± 3.293		54.6800 ± 2.758		51.3600 ± 3.042	
		T	7.900		9.507		9.353		9.792		8.139	
		P	**0.000***		**0.000***		**0.000***		**0.000***		**0.000***	

DP = distal phalanges, MP = middle phalanges, PP = proximal phalanges, MC = metacarpal bone. $p < 0.05$ is significant.

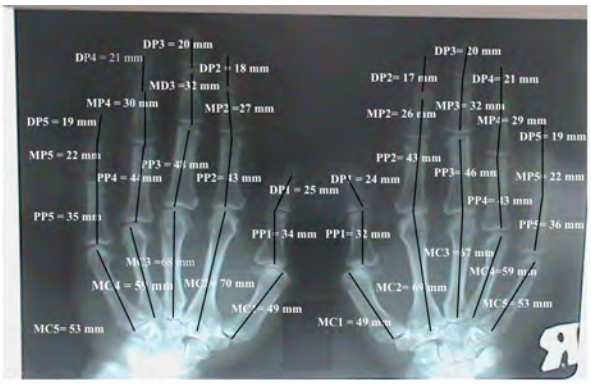

Figure (1): A Radiograph demonstrates the measurements of all metacarpals and phalanges of the right and the left hands (MC= metacarpal; PP= proximal phalanges; MP = middle phalanges and DP= distal phalanges).

Table (3): Comparison between the right and the left hand bones in males and females by using student t- test

Bone Rt & Lf		Fingers									
		1st		2nd		3rd		4th		5th	
		M	F	M	F	M	F	M	F	M	F
DP	t	1.949	0.350	1.307	0.440	0.000	0.496	0.984	1.030	0.000	0.270
	P	0.057	0.728	0.197	0.662	1.000	0.622	0.330	0.308	1.000	0.789
MP	t	None	None	0.221	0.111	0.141	0.270	0.163	1.124	0.531	0.655
	p	None	None	0.826	0.912	0.888	0.789	0.871	0.267	0.598	0.516
PP	t	0.275	0.000	1.492	0.714	1.695	1.243	2.030	0.798	0.286	1.091
	p	0.785	1.000	0.142	0.478	0.096	0.220	0.048	0.429	0.776	0.281
MC	t	3.584	0.230	1.750	0.207	0.765	0.538	1.244	1.014	1.500	0.889
	p	0.001*	0.819	0.086	0.837	0.448	0.593	0.219	0.315	0.140	0.378

M= male; F= female; Rt= right; Lf= left; DP= distal phalanges; MP= middle phalanges; PP= proximal phalanges; MC= metacarpal. $p < 0.05$ is significant*.

Table (4): Multivariate logistic regression analysis with the use of most predictable measurements of both hand bones to determine sex.

	Variables	B	SE	Wald	Df	Significance	Exp (B)
Step 1	Lf PP1	-1.107	0.227	23.874	1	0.000	0.331
	Constant	34.216	6.991	23.955	1	0.000	7.240
Step 2	Lf PP1	-0.751	0.242	9.604	1	0.002	0.472
	Lf MC4	-0.308	0.121	6.509	1	0.011	0.735
	Constant	40.926	7.958	26.450	1	0.000	5.939
Step 3	Lf PP1	-1.454	0.420	11.965	1	0.001	0.234
	Lf MC4	-.536	0.171	9.824	1	0.002	0.585
	Rt PP2	0.859	0.319	7.240	1	0.007	2.360
	Constant	41.821	9.637	18.833	1	0.000	1.455
Step 4	Lf PP1	-1.644	0.490	11.241	1	0.001	0.193
	Lf MC4	-0.496	0.189	6.869	1	0.009	0.609
	Rt PP2	1.596	0.519	9.461	1	0.002	4.931
	Rt PP3	-0.855	0.39	4.805	1	0.028	0.425
	Constant	54.452	13.954	15.227	1	0.000	4.449
Step 5	Lf PP1	-1.746	0.514	11.534	1	0.001	0.174
	Lf MC4	-0.640	0.231	7.690	1	0.006	0.527
	Rt PP2	1.559	0.519	9.028	1	0.003	4.752
	Rt PP3	-1.310	0.476	7.577	1	0.006	0.270
	Rt PP4	0.815	0.425	3.669	1	0.055	2.259
	Constant	53.584	13.645	15.422	1	0.000	1.868

Rt= right; Lf= left; PP= proximal phalanges; MC= metacarpal; 1 = first finger; 2 = second finger; 3 = third finger; 4 = fourth finger; SE = standard error; Wald: χ^2; df = degree of freedom; p = significance; Exp = exponential.

Table (5): The Correct classification of sex by using the multivariate logistic regression of both hands.

Sex	Original	Predicted group		Accuracy %	
		Male	Female	Correct	Incorrect
Males	50	47	3	94 %	6 %
Females	50	6	44	88 %	12 %

Overall predictive value is 91 %.

Table (6): Multivariate logistic regression analysis with the use of most predictable measurements of the right hand and left hand bones separately to determine sex.

	Variables of Rt hand	B	SE	Wald	df	Significance	Exp (B)
Step 1	4th MC	-0.554	0.105	28.073	1	0.000	0.575
	Constant	31.914	6.032	27.990	1	0.000	7.247
Step 2	1st PP	-0.574	0.213	7.295	1	0.007	0.563
	4th MC	-0.338	0.125	7.341	1	0.007	0.713
	Constant	37.233	7.335	25.769	1	0.000	1.479
Step 3	1st PP	-.890	.277	10.333	1	.001	.411
	2nd PP	.458	.221	4.282	1	.039	1.581
	4th MC	-.437	.146	9.031	1	.003	.646
	Constant	34.499	7.553	20.862	1	.000	9.612
Step 4	1st DP	-.627-	.280	5.008	1	.025	.534
	1st PP	-.933-	.311	8.986	1	.003	.394
	2nd PP	.648	.264	6.020	1	.014	1.911
	4th MC	-.324-	.150	4.670	1	.031	.723
	Constant	35.330	8.078	19.129	1	.000	2.206E15
Step 5	1st DP	- 0.634	0.311	4.143	1	0.042	0.531
	1st PP	-1.047	0.337	9.668	1	0.002	0.351
	2nd PP	1.351	0.469	8.281	1	0.004	3.860
	3rd PP	- 0.814	0.385	4.468	1	0.035	0.443
	4th MC	-.258	0.163	2.500	1	0.114	0.773
	Constant	43.602	10.392	17.603	1	.000	8.634
	Variables of Lf hand	**B**	**SE**	**Wald**	**df**	**Significance**	**Exp (B)**
Step 1	4th MC	-0.561	0.105	28.798	1	0.000	0.571
	Constant	32.297	6.008	28.894	1	0.000	1.062
Step 2	1st PP	-0.749	0.243	9.500	1	0.002	0.473
	4th MC	-0.315	0.121	6.767	1	0.009	0.730
	Constant	41.238	8.011	26.500	1	0.000	8.115
Step 3	1st PP	-1.189	0.342	12.076	1	0.001	0.305
	2nd PP	0.680	0.302	5.090	1	0.024	1.975
	4th MC	-0.511	0.167	9.412	1	0.002	0.600
	Constant	39.032	8.493	21.123	1	0.000	8.941

Rt= right; Lf= left; DP = distal phalanges; PP= proximal phalanges; MC= metacarpal; 1 = first finger; 2 = second finger; 3 = third finger; 4 = fourth finger; SE = standard error; Wald: χ^2; df = degree of freedom; p = significance; Exp = exponential.

Table (7): The Correct classification of sex by using the multivariate logistic regression of the right hand and the left hand separately.

Sex	Original	Predicted group		Accuracy % of right hand	
		Male	Female	Correct	Incorrect
Males	50	44	6	88 %	12 %
Females	50	6	44	88 %	12 %
Sex	Original	Predicted group		Accuracy % of left hand	
Males	50	45	5	90 %	10 %
Females	50	6	44	88 %	12 %

Overall predictive value is 88 % for the right hand and 89 % for the left hand.

Table (8): Cut off values (in mm) and accuracy percentage for sex determination for each bone (n = 100).

Bone	Cut off values	Accuracy %	Total %
Rt 1st DP	Male < 21.50 < Famale	Female: 85.7 % Male: 80 %	**82.8 %**
Rt 2nd DP	Male < 17.50 < Famale	Female: 63.3 % Male: 82 %	**72.6 %**
Rt 3rd DP	Male < 17.50 < Famale	Female: 81.6 % Male: 70 %	**75.8 %**
Rt 4th DP	Male < 18.50 < Famale	Female: 77.6 % Male: 74 %	**75.8 %**
Rt 5th DP	Male < 16.50 < Famale	Female: 85.7 % Male: 76 %	**80.8 %**
Rt 2nd MP	Male < 22.50 < Famale	Female: 79.6 % Male: 54 %	**66.8 %**
Rt 3rd MP	Male < 28.50 < Famale	Female: 71.4 % Male: 76 %	**73.7 %**
Rt 4th MP	Male < 27.50 < Famale	Female: 69.4 % Male: 70 %	**69.7 %**
Rt 5th MP	Male < 18.50 < Famale	Female: 91.8 % Male: 58 %	**74.9 %**
Rt 1st PP	Male < 30.50 < Famale	Female: 87.8 % Male: 80 %	**83.9 %**
Rt 2nd PP	Male < 41.50 < Famale	Female: 59.2 % Male: 86 %	**72.6 %**
Rt 3rd PP	Male < 44.50 < Famale	Female: 81.6 % Male: 74 %	**77.8 %**
Rt 4th PP	Male < 41.50 < Famale	Female: 77.6 % Male: 62 %	**69.8 %**
Rt 5th PP	Male < 32.50 < Famale	Female: 81.6 % Male: 64 %	**72.8 %**
Rt 1st MC	Male < 46.50 < Famale	Female: 71.4 % Male: 72 %	**71.7 %**
Rt 2nd MC	Male < 68.50 < Famale	Female: 77.6 % Male: 76 %	**76.8 %**
Rt 3rd MC	Male < 65.50 < Famale	Female: 77. 6 % Male: 86 %	**81.8 %**
Rt 4th MC	Male < 57.50 < Famale	Female: 83.7 % Male: 80 %	**81.8 %**
Rt 5th MC	Male < 52.50 < Famale	Female: 89.8 % Male: 72 %	**80.9 %**

Rt= right; DP= distal phalanges; MP= middle phalanges; PP= proximal phalanges; MC= metacarpal.

Table (8): Cutoff values (in mm) and accuracy percentage for sex determination for each bone (n = 100).

Bone	Cutoff values	Accuracy %	Total %
Rt 1st DP	Male < 21.50 < Famale	Female: 85.7 % Male: 80 %	**82.8 %**
Rt 2nd DP	Male < 17.50 < Famale	Female: 63.3 % Male: 82 %	**72.6 %**
Rt 3rd DP	Male < 17.50 < Famale	Female: 81.6 % Male: 70 %	**75.8 %**
Rt 4th DP	Male < 18.50 < Famale	Female: 77.6 % Male: 74 %	**75.8 %**
Rt 5th DP	Male < 16.50 < Famale	Female: 85.7 % Male: 76 %	**80.8 %**
Rt 2nd MP	Male < 22.50 < Famale	Female: 79.6 % Male: 54 %	**66.8 %**
Rt 3rd MP	Male < 28.50 < Famale	Female: 71.4 % Male: 76 %	**73.7 %**
Rt 4th MP	Male < 27.50 < Famale	Female: 69.4 % Male: 70 %	**69.7 %**
Rt 5th MP	Male < 18.50 < Famale	Female: 91.8 % Male: 58 %	**74.9 %**
Rt 1st PP	Male < 30.50 < Famale	Female: 87.8 % Male: 80 %	**83.9 %**
Rt 2nd PP	Male < 41.50 < Famale	Female: 59.2 % Male: 86 %	**72.6 %**
Rt 3rd PP	Male < 44.50 < Famale	Female: 81.6 % Male: 74 %	**77.8 %**
Rt 4th PP	Male < 41.50 < Famale	Female: 77.6 % Male: 62 %	**69.8 %**
Rt 5th PP	Male < 32.50 < Famale	Female: 81.6 % Male: 64 %	**72.8 %**
Rt 1st MC	Male < 46.50 < Famale	Female: 71.4 % Male: 72 %	**71.7 %**
Rt 2nd MC	Male < 68.50 < Famale	Female: 77.6 % Male: 76 %	**76.8 %**
Rt 3rd MC	Male < 65.50 < Famale	Female: 77. 6 % Male: 86 %	**81.8 %**
Rt 4th MC	Male < 57.50 < Famale	Female: 83.7 % Male: 80 %	**81.8 %**
Rt 5th MC	Male < 52.50 < Famale	Female: 89.8 % Male: 72 %	**80.9 %**

Rt= right; DP = distal phalanges; MP = middle phalanges; PP = proximal phalanges; MC = metacarpal; 1 = first finger; 2 = second finger; 3 = third finger; 4 = fourth finger; 5 = fifth finger.

Table (8): Continued……..

Bone	Cutoff values	Accuracy %	Total %
Lf 1st DP	Male < 21.50 < Famale	Female: 80 % Male: 84 %	**82 %**
Lf 2nd DP	Male < 21.50 < Famale	Female: 84 % Male: 66 %	**75 %**
Lf 3rd DP	Male < 21.50 < Famale	Female: 86 % Male: 62 %	**74 %**
Lf 4th DP	Male < 21.50 < Famale	Female: 78 % Male: 70 %	**74 %**
Lf 5th DP	Male < 21.50 < Famale	Female: 80 % Male: 76 %	**78 %**
Lf 2nd MP	Male < 21.50 < Famale	Female: 66 % Male: 76 %	**71 %**
Lf 3rd MP	Male < 21.50 < Famale	Female: 72 % Male: 74 %	**73 %**
Lf 4th MP	Male < 21.50 < Famale	Female: 70 % Male: 74 %	**72 %**
Lf 5th MP	Male < 21.50 < Famale	Female: 72 % Male: 74 %	**73 %**
Lf 1st PP	Male < 21.50 < Famale	Female: 88 % Male: 82 %	**85 %**
Lf 2nd PP	Male < 21.50 < Famale	Female: 78 % Male: 68 %	**73 %**
Lf 3rd PP	Male < 21.50 < Famale	Female: 84 % Male: 70 %	**77 %**
Lf 4th PP	Male < 21.50 < Famale	Female: 74 % Male: 82 %	**78 %**
Lf 5th PP	Male < 21.50 < Famale	Female: 68 % Male: 76 %	**72 %**
Lf 1st MC	Male < 21.50 < Famale	Female: 76 % Male: 76 %	**76 %**
Lf 2nd MC	Male < 21.50 < Famale	Female: 82 % Male: 82 %	**82 %**
Lf 3rd MC	Male < 21.50 < Famale	Female: 82 % Male: 88 %	**85 %**
Lf 4th MC	Male < 21.50 < Famale	Female: 82 % Male: 86 %	**84 %**
Lf 5th MC	Male < 21.50 < Famale	Female: 88 % Male: 70 %	**79 %**

Lf = left; DP= distal phalanges; MP= middle phalanges; PP = proximal phalanges; MC = metacarpal; 1 = first finger; 2 = second finger; 3 = third finger; 4 = fourth finger; 5 = fifth finger.

DISSCUSION

Many studies have been attempted to determine sex by using different body features such as foot shape, foot print ratio, foot and shoe dimensions, the femoral head, the patella, long bones of the arm and the teeth. Very few studies were done for the determination of sex from foot and hand dimensions (Ozden et al., 2005[13]; Agnihotri et al., 2006[14]; Moudgil et al.,2008[15]).

The aim of this study is to assess the accuracy of sex determi-

nation from the lengths of all metacarpals and phalanges of right and left hands using X - Ray radiographs and to develop a discriminant formula that can be used in Egyptians.

The present study revealed that the majority of measurements was slightly larger (decimal fraction) in the right hand than the left hands in females, while in males the majority of measurements were slightly larger (decimal fraction) in the left hand than the right hand; but with no significant difference in the measurements between right and left hands in both sexes except for the first metacarpal bone in males. This could be explained by daily activities which could affect the growth of bones.

This result is in contrast to that of Manolis et al. (2009)[16] in Athens population, who stated that the right metacarpals were generally larger than those of the left hand in both sexes with exceptions in some measurements but these differences were not statistically significant. The present results were on contrary to that of McFadden and Bracht (2009)[17] in European and African American populations, who stated that the right hand was slightly longer than the corresponding bones in the left hand, but the differences were small and there were numerous exceptions.

Moreover this is inconsistent with the studies of Alicioglu et al. (2009)[18] in Turkish population and Eshak et al. (2011)[2] in Egyptian population which reside on the left hand only because of the fact that the majority of populations are right handed and therefore will be less influenced by activity. So the present result proves that either the right or left hands could be used for sex determination. This reverses the findings of Smith (1996)[8] and Case and Rose (2007)[10] who stated that the accuracy rates for the left hand was higher than the right hand.

Moreover the present study revealed that males presented with significantly greater mean values than females ($p<0.05$) for the length of metacarpals and phalanges of all fingers of both hands. The ordering of metacarpals by

the length from longest to shortest was 2 > 3 > 4 > 5 > 1; the ordering for proximal phalanges by the length was 3 > 4 > 2 > 5 > 1; the ordering of middle phalanges by the length was 3 > 4 > 2 > 5 and lastly the ordering of distal phalanges by the length was 1 > 4 > 3 > 2 > 5. This could be explained by mechanical response of the bone owing to the greater muscular demand of males.

As regard the metacarpal bones, the present study is in agreement with the studies of Manolis et al. (2009)[16] in Athens and McFadden and Bracht (2009)[17] in USA which stated that male metacarpals were higher than those of females. Also McFadden and Bracht (2009)[17] mentioned that the ordering of metacarpals by the length from longest to shortest was 2 > 3 > 4 > 5 > 1.

These findings were on contrary to Alicioglu et al. (2009)[18] study on Turkish, who mentioned that male measurements were greater than females except for distal phalanges. Furthermore Eshak et al. (2011)[2] study on Egyptians by using computed tomography; stated that males presented with significantly greater mean values than females for the lengths of the distal phalanges of all fingers, 1st and 3rd proximal phalanges and all metacarpals. Neither the middle nor the 2nd, 4th or 5th proximal phalanges showed significant differences between males and females.

Comparing between different populations, the present study stated that the lengths of all metacarpals and phalanges of the left hands for both Egyptian males and females were greater than the measurements taken by Alicioglu et al. (2009)[18]; on a sample of Turkish population.

Also the lengths of all metacarpals of both the right and left hands of both Egyptian sexes were greater than the measurements taken by Manolis et al., (2009)[16]; McFadden and Bracht (2009)[17] on a sample of Athens and European American populations respectively.

While the measurements of all metacarpals of both right and left

hands of both Egyptian sexes in this study were smaller than the measurements taken by McFadden and Bracht (2009)[17] on a sample of African American population.

These differences could be the result of genetic factors, environmental factors affecting growth and development (nutrition, physical activity, pathological).

A study done on Egyptian population by Eshak et al. (2011)[2] by using CT scan; revealed that the measurements of all metacarpals and phalanges of the left hand of both sexes were slightly smaller than the measurements of the present study. This small difference could be explained by the different methods used for the measurements and the difference in their accuracy and may be due to the different sample size. Also they stated that the length of distal and middle phalanges for both Egyptian sexes were more than the measurements taken by Alicioglu et al. (2009)[18] in Turkish population, while those of proximal phalanges and metacarpals were less than them.

Furthermore Habib and Kamal in (2010)[19] studied the length of phalanges of the right and left hands in both Egyptian sexes with the exclusion of thumb fingers because of their flexibility as compared to other fingers which are straight. The measurements of the distal phalanges of both right and left hands in both sexes were greater than those of the present study; while the middle and proximal phalanges of both right and left hands of both sexes were smaller than those of the present study. This could be explained by different size sample and the difference in the method of measurements as they measured the phalanges as the distance between two phalange ridges by help of sliding caliper from the palmer side.

In addition to the previous findings, the present study indicated that five bones of both hands (left 1st proximal phalanges, left 4th metacarpal and right 2nd; 3rd and 4th proximal phalanges) were useful bones for sexing of Egyptian population and showed sexual dimorphism with accuracy 91% (94% for males and 88% for

females). This is explained by differences in body size between both sexes and the fact that men and women do different things to different degrees. The regression equation should be applied cautiously for different population and time periods.

While by using the right hand only; five bones also (1st proximal and distal phalanges & 2nd : 3rd proximal phalanges & 4th metacarpal) were useful bones for sexing of Egyptian population and showed sexual dimorphism with accuracy 88%. On the other side, by using the left hand only; 3 bones (1st & 2nd proximal phalanges & 4th metacarpal) were useful bones for sexing of Egyptian population and showed sexual dimorphism with accuracy 89% (90% for males & 88% for females).

This is in contrast to Alicioglu et al. (2009)[18] who obtained 72.7% accuracy for males (16/22) and 90.7% for females (39/43) by using the left hand only.

The misclassification in sex determination could be explained by wrong classified cases (i.e. males of reduced dimensions or females with very strong musculature).

Regarding the accuracy of each bone, the present results revealed that 1st DP & PP and 3rd and 4th MC are the best bones that can be used in correct sex determination.

This is in accordance to Manolis et al. (2009)[16] in Athens population who proved that 3rd & 4th MC had the highest percentage of correct prediction. In addition Stojanowski (1999)[20] in New Mexico revealed that the 4th MC had the highest accurate estimate.

On the other hand, Eshak et al. (2011)[2] in Egyptian population proved that the 2nd and 5th MC and 1st and 3rd PP had the highest accuracy. Furthermore, Falsetti (1995)[21] in European and African American stated that 2nd, 4th and 5th MC could provide method for sex assessment. Moreover Shreuer and Elkington (1993)[22] in British White population found that 1st MC had the highest degree of accuracy in identifying sex. In consistent with the present result Barrio et al. (2006)[11] in

Spanish population found that the 2nd, 4th and 5th MC had the highest accuracy.

These differences could be explained by different populations, different sample size and different methods used in the study.

From the previous results it is concluded that the length of metacarpals and phalanges (especially the 1st DP & PP and 3rd and 4th MC) could be used for sex determination. The right hand could be used as the left hand in determination of sex. Also the X-ray radio graphs are good non invasive and simple tool in the determination of sex from the hand bones. Furthermore the regression equation for both hands and each hand separately is specific to Egyptian population and should be used cautiously with other ones.

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SEX DETERMINATION BY THE LENGTHS OF METACARPALS AND PHALANGES : X-RAY STUDY ON EGYPTIAN POPULATION

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EMERGENCY CESAREAN SECTION IN MANSOURA UNIVERSITY HOSPITAL : RATES AND INDICATIONS

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Abstract

Objective: *To determine the frequency and the reasons for cesarean section in Mansoura University Hospital Emergency Unit. Study design: Retrospective descriptive study. Patients and methods: Data on cesarean section rates and reasons were collected from the medical records of 21887 women admitted in Mansoura University Hospital Emergency Unit for delivery in the period between December 2005 and December 2010. Results: Overall 29.2% of women had a cesarean section. The most common indication for cesarean section was previous cesarean section (45.4%) followed by breech presentation (7.94%), failure to progress (7.44%) and cephalopelvic disproportion (6.45%). Conclusion: The high rates of cesarean deliveries was mainly attributed to previous cesarean section and careful supervised vaginal delivery after cesarean section has to be encouraged. Breech presentation was the second common cause and routine practice of external cephalic version has to be implemented.*

Key word: *Cesarean section, rates, indications.*

Introduction

Rise in cesarean section (CS) rate during the last three decades, has been the cause of alarm. Actual CS rates in developing countries are largely unknown because of lack of reliable data.

The recommended upper limit rate of CS was 15% as advocated by the World Health Organization[1]. CS in developing countries is associated with significant increase in maternal morbidity[2-3]. Very low rate (less than 1%) has

been associated with higher maternal and infant mortality[2-4].

CS is not without hazards. Immediate risks include complications of anesthesia[5] and intraoperative complications like uterocervical, bladder laceration, blood loss and the need for hysterectomy in 12- 15% were reported[6]. Post operative complications; pelvic infection, sepsis, deep venous thrombosis, urinary infection and post partum hysterectomy for adherent placenta, uterine atony, uterine rupture and extension of uterine scar were recorded[7-8]. Also, respiratory distress of the neonate[9]. Late consequences of CS were reported including; placenta previa, adherent placenta and abruption[10-11]. Moreover, increased incidence of ectopic pregnancy[12-13]. Kennare et al[14], reported increased risks for malpresentation, placenta previa antepartum hemorrhage, placenta accreta prolonged labor, emergency cesarean and uterine rupture, preterm birth, low birth weight, small for gestational age, stillbirth, and unexplained stillbirth with cesarean delivery.

Interventions aimed at reducing maternal and perinatal morbidity associated with CS have included auditing of the rates, indications and associated health outcome[15-16].

Aim: To assess information on the rates and the indications for CS in Mansoura University Hospital Emergency Unit.

Subjects and Methods

Data were collected on cesarean section rates and indications from the medical records of 21887 women delivered in Mansoura University Hospital Emergency Unit, from December 2005 to December 2010. Approval was taken from the ethics committee of Mansoura Faculty of Medicine.

Statistical analysis : Was performed by using SPSS statistical package for social science program version "10" 1999.

Results

Out of 21887 women delivered at MUH (between 2005 and 2010) 6448 women had CS giving a rate of 29.2% (table 1).

The most common indication for CS was previous CS (45.5%) followed by breech presentation 7.94%, failure to progress 7.44%, cephalopelvic disproportion 6.45. While, acute fetal distress and severe PET represented 4.7% for each (table 2).

The main indication for repeated CS were unfavorable cevix in 34.97% and previous two or more CS in 28.97% (table 3).

Table (1): The number of deliveries by CS versus vaginal delivery.

	N	%
Total deliveries	22118	100%
Total vaginal	15670	70.8%
Total CS	6448	29.2%

Table (2): Indications of CS .

Indication	No	%
Repeat CS	2928/6448	45.4%
CPD	416	6.45%
Failure to progress	480	7.44%
PROM Unfav.cx	400	6.20%
Severe PET Unfav.cx	304	4.71%
Severe PET & IUGR	144	2.23%
Antepartum eclampsia	64	0.99%
Face & severe PET	16	0.24%
Breech	512	7.94%
Twin & 1st non vertex	256	3.97%
Twin & medical dse.	48	0.74%
Twin & prolapsed cord	32	0.49%
Triplet	16	0.25%
Acute fetal distress	304	4.7%
Placenta previa	160	2.48%
Accidental hge	128	1.99%
Other indications	240	3.72%

Table (3): Indications of CS after prior CS.

Indication	No	%
Previous CS & unfav. CX	1024	34.97
Previous cs & tender scar	224	7.65
Previous cs & breech	176	6.01
Previous cs & CPD	112	3.83
Previous cs & medical dis.	176	6.01
Previous CS & fetal distress	16	0.55
Previous cs & antepartum Hge	64	2.19
Previous cs & PROM	288	9.84
Previous 2 CS	608	20.77
Previous more than 2 CS	240	8.20

Discussion

In the present study the overall CS rates was 29.2% being higher than the WHO recommended rates[17]. This may be attributed to the fact that MUH is a referral centre, meaning high presentation of women with complications. Still this rate is higher than the nationally representative data available[18]. Also more than that reported by Khawaja et al[19], who reported a rate of 22% in Egypt which is much higher than the rate in the Arab countries where CS rate between 5-15% were reported[20].

The main indication of CS in the present study was previous CS, followed by malpresentation and cephalopelvic disproportion. The National Collaborating Centre for Women's and Children Health together with the Royal College of Obstetricans and Gynaecologists (NCCWCH/RCOG)[21] guidelines listed malpresentation, cephalopelvic disproportion and acute fetal distress as main indication for CS while previous CS is not a recommendation of NCCWCH/RCOG guidelines.

In the present study, the high incidence of this indication could be due to women or providers choosing this option after previous complicated birth common in developing countries[22]. Also, may

be attributed to limited Knowledge and training of health professionals causing limited implementation of recommendation of VBAC. VBAC is still a controversial issue and the danger of uterine rupture in women with previous CS is a threat to most pregnant women and their attending obstetricians. The National Study of Vaginal Birth after Cesarean in Birth Centers[23], reported 87% success rate of VBAC with 0.2% rates of uterine rupture and fetal/ neonatal death. Landon et al[24] reported that uterine rupture and delivery related perinatal deaths among planned VBAC are extremely low being 14/ 10,000 and 4 / 10,000 respectively. VBAC was found to be extremely safe[25]. Refaie and Abd El Aziz[25] on performing VBAC on 240 pregnant women found 90% success rate without any complications.

Many risks were reported with repeated CS including injuries to the bladder, bowel or ureters, placenta accrete, hysterectomy, admission to intensive care units, blood transfusion and long term hospital stay, dense adhesion. All these risks significantly increased with increasing number of repeat CS[26-27-28]. Also, Kamath et al [29], reported higher rates of respiratory morbidity and NICU admission and long hospital stay for neonates born after elective repeat CS in comparison with VBAC.

VBAC is a good option provided the details of the previous CS are available, with close monitoring during labor and the ability to proceed to an emergency CS if needed[30-31]. Trial of labor after previous CS is safe and appropriate for most women with previous cesarean delivery, including some women with two previous CS and twin pregnancy according to less restrictive guidelines issued by the American College of Obstetricians and Gynecologists 2010[32]. Also the National Institutes of Health conference has focused attention on VBAC[33]. The National Institute of Child Health and Human Development Maternal-Fetal Medicine Units reported that an increasing number of prior VBACs is associated with a greater probability of VBAC success, as well as a lower risk of uterine rupture and

perinatal complications[34].

Reducing the number of primary cesareans deals with the problem where it originates[35]. The Society of Obstetricians and Gynaecologists of Canada continues to raise concerns that the rising cesarean delivery rate reflects the use of the procedure in cases in which it is not medically indicated[36]. In the present study the cephalopelvic disproportion and failure to progress collectively constitute 13.89% for the indication of primary CS. This is in accordance to Hanley et al[37] who found that the most common indication for primary CS was dystocia and concluded that the variation in the rate of primary CS reflects differences in practitioners' approaches to medical decision-making.

In the present study, breech presentation represent 7.94% of primary CS and 6.1% of repeated CS and routine practice of external cephalic version has to be implemented, even in women with previous CS[32]. Also, seeking a second opinion from a senior obstetrician before performing cesarean section is recommended to reduce the incidence of cesarean section. Unless measures are instituted to reverse the rapidly rising cesarean rate, catastrophic complications from placenta accreta and percreta associated with multiple repeat cesareans soon may be a greater problem than uterine rupture.

Conclusion

bstetricians should carefully evaluate the indication of primary CS and the policy of "Once CS always CS" should not be followed. The concept of proper VBAC has to be implemented together with routine practice of external cephalic version. The debate regarding the appropriateness of CS will continue any discussion of risks and benefits must include risks of repeated CS.

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BENHA MEDICAL JOURNAL

EMERGENCY CESAREAN SECTION IN MANSOURA UNIVERSITY HOSPITAL : RATES AND INDICATIONS

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T3 VERSUS T4 SYMPATHICOTOMY FOR TREATMENT OF PRIMARY PALMAR HYPERHIDROSIS : A PROSPECTIVE RANDOMIZED STUDY

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Abstract

Objective: *This prospective study aimed to compare the efficacy of video-assisted thoracoscopic sympathicotomy at the T3 or T4 level in the treatment of palmar hyperhidrosis.*

Summary Background Data: *treatment of hyperhidrosis varied widely from conservative methods to a more selective surgical management. These variations originated from the complicated nature of postoperative complication like postoperative over dry hands and compensatory hyperhidrosis. So, most surgeons moved towards a more selective level of sympathicotomy to obtain satisfactory postoperative results.*

Methods: *Patients were operated on for palmar hyperhidrosis from February 2008 to September 2010, a total of 136 consecutive patients with PH were enrolled in this study. There were 85 males and 51 females, with mean age of 32.3 ± 11.5 years. The patients categorized randomly into two groups, group 1 or T3 sympathicotomy (65 patients); group 2 or T4 sympaticotomy (71 patients).*

Results: *The rate of dryness and compensatory hyperhidrosis (CH) was significantly lower in the T4 sympathicotomy group than the T3 group (P<.0001). Satisfaction rate, recurrence, and improvement of plantar sweating were of no statistical significance in either group.*

Conclusions: *Although both sympathicotomies were effective, safe,*

and minimally invasive methods for the treatment of palmar hyperhidrosis, T4 appeared to be a more optimal technique with less CH.

Introduction

Palmar hyperhidrosis (PH) is a benign sympathetic disorder that affects daily activities, and may causes social withdrawal and depression [1]. The incidence of PH is about 1%, men and women showed the same reported incidence in most surgical series[2,3].

Although Video-assisted thoracoscopic sympathetic surgery is being the most accepted and safest treatment of primary palmar hyperhidrosis (PH)[4], there are many other treatment options to be preferred for some patients [5]. A new classification of sympathetic disorder was introduced by Lin and Telaranta [6] based on sympathetic distribution of sympathetic innervations. According to the classification, surgical Procedures must take in consideration a more selective approach for treatment of PH in which T3 or/and T4 sympathicotomy as an interruption of the interganglion trunk will result in a higher success rate with significant decreases of major side effects[7,8]. Compensatory hyperhidrosis (CH) is the most common and serious side effect that occurs in 30-70% of patients after T2 or T2-3 sympathectomy[9]. Other complications include gustatory sweating, phantom sweating, a transient increase in upper limb sweating, facial anhydrosis, ptosis, meiosis, over dry hands and recurrence of hyperhidrosis but all presented with low incidence. Some patients still present with certain degrees of CH or over dry hands after T3 or/and T4 sympathicotomy [10].

The aim of this study is to compare the two methods for the treatment of PH, in which the sympathetic chain was transected in one segment, on the level of either the third or the fourth ribbed, defined as T3 sympathicotomy or T4 sympathicotomy, respectively. Evaluation of the efficacy, side effects, and patients' satisfaction rate to these two types of surgical therapy were analysed.

Materials and Methods

Patient's selection :

From February 2008 to September 2010, a total of 136 con-

secutive patients with PH were enrolled in this study. There were 85 males and 51 females, with mean age of 32.3 ± 11.5 years (range, 15- 38 years). The severity of palmar sweating, the sweating in other parts of the body, other concomitant symptoms and past medical history were recorded. All patients were subjected to careful history taking, clinical examination, and laboratory tests. Chest X-ray radiography, ECG and routine blood examination were performed before surgery. All patients had severe palmar hyperhidrosis that was refractory to conservative treatment and some preferred surgical therapy for permanent results. Patients with pleural adhesion, bleeding diathesis, local infection, and patients with certain anatomic anomalies were excluded from analysis.

Informed consent was obtained from all patients to be included in the study, after explanation of the nature of the disease and possible treatment. The study was approved by the local ethical committee.

Randomization was achieved through a computer - generated schedule, and the results were sealed into envelopes. The envelopes were drawn and opened by a nurse not otherwise engaged in the study in the operating room. The patients were then randomized into two groups: Group 1 underwent T3 sympathicotomy; Group 2 underwent T4 sympathicotomy .

Surgical techniques :

All surgical procedures were performed using the same surgical technique under general anaesthesia with a single-lumen endotracheal tube. Preanesthetic medication was indicated; short-duration benzodiazepines administered soon before the procedure. Anesthetic induction with propofol (1 to 2 $mg.kg^{-1}$), fentanyl (1 to 2$\mu g.kg^{-1}$) and atracurium (20 to 40 mg) to facilitate tracheal intubation, followed by maintenance with 1 MAC isoflurane and 100% oxygen.

Patients were placed in the supine position with their arms abducted. The procedures were performed with the patient in the semi-Fowler position and the side

to be operated on was slightly tilted down to shift the lung toward the diaphragm and mediastinum. The endotracheal tube was disconnected for a moment for deflation of the lung to avoid damage to lung parenchyma. Afterward, the first small stab incision to guide the 0° thoracoscope was made at level of the nipple at the anterior axillary line and pneumothorax was achieved by puncture with a Verres needle. A 5-mm blunt tip trocar was introduced after deflation of the lung with insufflation of 200 mL of CO2. The second small stab wound was made at the midaxillary line at the level of the second to third intercostal space for insertion of an electrocautery device through a 5-mm trocar. The sympathetic chain was identified at the level of the crossing of the third or fourth costal heads after dissection of the parietal pleura and completely divided about 1 cm wide at the upper margin of the rib. With assistance of anaesthesia team we reinflate the lung totally in sequence with removal of the trocars. The same procedure was performed on the opposite side and ablation of the sympathetic chain overlying the rib was performed bilaterally.

Adequate monitoring decreases potential post-anesthetic accidents by identifying abnormalities before they become severe or irreversible injuries. As in any surgical procedure, routine monitoring during thoracoscopic sympathectomy includes ECG, pulse oximetry, noninvasive blood pressure and capnography. Pulse oximetry is needed because there are always ventilatory changes attributed to periods of apnea or to intrapleural carbon dioxide inflation. At the end of surgery, a postoperative chest x-ray was routinely taken to rule out pneumothorax or hemothorax. Postoperative pain is common in general it is characterized as retro-sternal or in the upper chest region close to shoulders and related to incisions and its exact mechanism is not well explained. It is believed that when intrapleural carbon dioxide inflation is used, mediastinum shift results in pleural stretch and pain pathways activation. NSAIDs, may be used for post thoracoscopy pain, being an effective and safe alternative to opioids and decreas-

ing their postoperative need.

Patients were usually discharged on second postoperative day. Patients were evaluated according the results of sweating, compensatory hyperhidrosis, and degree of satisfaction, complications, and recurrence. A total of 65 patients who underwent bilateral thoracoscopic T3 sympathicotomy composed the T3 group. The T4 group included 71 patients who underwent T4 sympathicotomy. The results of the intervention were evaluated by patients as follows: "dry" when the patient was not aware of sweat on the palms, "nearly dry" when the patient had marked improvement whereby minimal sweat sometimes occurred under stressful conditions and "wet when the patient had limited improvement and was very aware of sweating. Compensatory sweating in other regions of the body was graded as "mild" when it was tolerable but sometimes interfered with daily activities, "moderate" when it was barely tolerable and frequently interfered with daily activities and "severe" when it was intolerable and always interfered with daily activities.

The degree of satisfaction for each patient was evaluated as very satisfied, satisfied and partially satisfied. Patient satisfaction was evaluated using an analogous scale ranging from 1 to 10 with 1 indicating no satisfaction at all and 10, the maximum possible satisfaction. We further divided the analogous scale in 4 parts: Not satisfied (1), partially satisfied (2-4), satisfied (5-7), very satisfied (8-10).

Recurrence was considered when patients felt severe discomfort similar to that before surgery in spite of improved symptoms of sweating.

Statistical analysis of comparisons between the 2 groups was performed by Student unpaired t test or chi-square test using SPSS 13.0 (SPSS, Inc, Chicago, IL). Analyzed data were shown as mean standard deviations and all P values 0.05 were considered statistically significant.

Results

Of 136 patients, the mean duration of palmar sweating was 9.6 ± 2.4 years (P=0.3). The mean fol-

low-up period was 20.9 ± 5.2 months. All operations were successfully performed using a video-assisted thoracoscope without severe morbidity and mortality. No life-threatening event or death occurred and no procedures were converted to thoracotomy. Other complications, such as Horner syndrome, hemothorax, intercostal neuralgia, pulmonary edema, and atelectasis, were not observed. One patient (0.7%) had subcutaneous emphysema which treated conservatively but no residual pneumothorax on follow-up chest x-ray was observed.

However, pneumothorax resulting from an injured lung in 1 patient (0.7%) required chest tube placement. Postoperative brachial paresthesia was encountered in 5 patients (3.7%); no postoperative rebound sweating, phantom sweating or a sensation of sweating that never actually occurred probably caused by residual sympathetic activity had reported. Postoperative gustatory sweating was not found in any patients in either T3 or T4 group.

All patients were satisfied with complete alleviation of their sweating in the immediate postoperative period. There were no significant differences between the 2 groups in terms of age, sex, duration of sweating, (Table 1). Duration of follow-up was not significantly different in both groups (Table 2). Most patients (73.8%) in the T3 group felt dryness in contrast to the mildly wet feeling (63.4%) in the T4 group.

Accompanying axillary sweating was improved in all patients in the T4 group. In terms of CH, the frequency of none to severe compensatory sweating was significantly different between the 2 groups, and the incidence of mild to severe compensatory sweating was higher in the T3 group than T4 (75 % vs. 22.5 %)with p =0.001. No case of severe CH was observed in the T4 group. The most frequent areas involved were chest, back, abdomen and groins. As regard the results of satisfaction, T4 group shows a great significance in the very satisfied category in comparison to T3 group (P= <0.001), (Table 2).

Some patients have over dry hands. The incidence of this side effect was significantly lower in

group T4 (0 out of 71) than in group T3 7.7% (6 out of 61).

As for recurrence (unilateral or bilateral) rate according to the level of sympathicotomy, the T3 group was superior to T4 (1.5 % vs. 2.8%) with P= 0.51.

Table (1): General condition of the 136 patients in the two groups.

	T3	T4	Total/average
Number	65	71	136
Age (years)	32.9±12.4	31.7 ±10.5	p=0.54
Gender (M/F)	37/28	41/30	
Duration of symptoms (years)	9.2 ± 2.2	9.8 ± 2.6	p=0.15

Table (2): Surgical outcomes in the two groups.

	T3	T4	P value
Follow-up period (mean ±SD) (mo)	20.5 ± 3.4	21.6 ± 6.4	P=0.37
Treatment success			
Dry hands	73.8%(48/65)	36.6%(26/71)	<0.001
Nearly dry hands	26.2%(17/65)	63.4%(45/71)	<0.001
Wet hands	0 (0/65)	0 (0/71)	
Side effects			
Compensatory hyperhidrosis			
None	24.6%(16/65)	77.5%(55/71)	
Mild	55.4%(36/65)	15.5%(11/71)	P=<0.001
Moderate	13.8%(9/65)	5.6%(4/71)	
Severe	6.2%(4/65)	1.4%(1/71)	
Over dry hand	7.7% (6/65)	(0/71)	
Satisfactory rate			
Very satisfied patients	26.2%(17/65)	60.6%(43/71)	<0.001
Satisfied patients	67.6% (44/65)	36.6%(26/71)	<0.001
Partially satisfied patients	6.2%(4/65)	2.8%(2/71)	0.34
Recurrence	1.5%(1/65)	2.8%(2/71)	0.51
Reduction in planter	17%(11/65)	24%(17/71)	0.31
Sweating			

Fisher's exact test was used for there are expected cell frequencies less than five.

Discussion

Primary palmar hyperhidrosis is a somatic disorder characterized by excessive perspiration in the hand. The aetiology is still unknown, but the condition usually associated with basal sympathetic hyperactivity and mostly leads to hyper functioning of the sudoriparous glands, which are frequently triggered by emotion [5]. There is a clear consensus now that video-assisted thoracoscopic sympathectomy is being established as a safe and effective treatment with a success rate greater than 95% in most series[11]. But T2 sympathetic ganglion resection as postulated by de Campos et al.[12] have a great relation to the severity and outcome in CH beside some other factors like obesity and prolonged history of primary complain. So, the technique was recently modified with a trend to minimize the extent of surgery to be a more selective and to ovoid the post-operative complications, namely the most serious and distressing one, compensatory hyperhidrois. Gray[13], reported an anatomical study in which he concluded that the third and fourth segments of sympathetic trunk were considered as the main lesion responsible for palmr hyperhidrosis and the one must to be in our consideration during surgery.

CH remains the most common and distressing complication post-sympathicotomy and many efforts have been made to ovoid its happening. Chou et al.[2] suggested that the underlying mechanism of CH may be due to a reflex response in sweating centre in hypothalamus but the exact mechanism beyond this phenomenon remain unclear.

Lin's hypothesis seemed to be correct until now[14]. According to the theory; changes in sympathetic tone and disturbance of the reflex arc in the hypothalamus due to the procedures of the upper thoracic sympathetic system are responsible for the postoperative result. Blocking of the lumbar sympathetic system for plantar sweating and peripheral block on palmar sweating by iontophoresis and botulinum toxin injection have no effect on CH, and these facts support disordering of the reflex feedback in sympathetic tone after surgery. The degree of

preservation of sympathetic tone and variation of sympathetic innervation are supposed to have individually different influences on palmar sweating and CH.

The reported incidence of mild to moderate CH was estimated to be as high as 89% of patients undergo sympathectomy. However, the sever form of CH is less tolerable, difficult to manage and more distressing to most patients if compared by the preoperative condition of these suffer patients. So optimum treatment of CH remains a problem to be managed as conventional treatment don't satisfy most patients [15,16].

Various surgical methods have been attempted to reduce the occurrence rates of compensatory sweating. Many authors felt that the frequency and severity of compensatory sweating were correlated to both the level and extent of resection. The more sympathetic segments excised, especially those including T2, the greater the incidence of severe compensatory symptoms. Dewey and et al. (9) limited the extent of resections for hyperhidrosis to a single level and found it can reduce the incidence of severe compensatory symptoms.

In the present study, mild to moderate CH accounts for 69% in T3 group and 21% in T4 group ($p<0.001$). So T4 sympathicotomy reduces the rate of CH and suggested to be more acceptable. In comparison, Niemeyer et al.[10] found a rate 8.5% of CH in T4 group while Yang et al.[16] (70.5% in T3 group and 44.7 in T4 group). Selective sympathicotomy is one method of controlling and avoiding CH with successful treatment of hyperhidrosis.

The frequency of CH was reduced in severity with less interruption of sympathetic trunk in group T4 compared to T3 (table 2), this results reflected on the degree of patients satisfaction. T4 patient showed mild witness in 63.4% and the patients who was in the very satisfied category was 60.6%, while T3 group showed mild witness in 26.2% with a degree of very satisfaction was 26.2% (p value was < 0.001 in each category). These mild hands witness was compensated and favoured by

most patients undergo T4 sympatheicotomy. However there is no significant between the two group in term of overall satisfaction p= 0.34.

The recurrence rate in T4 group was occurring in 2 patients (2.8%), whereas one recurrence found in T3 group (1.5%). in a study by Kim et al.[4] on 119 patients, the recurrence (unilateral or bilateral) rate according to the level of sympathicotomy, the T3 group was superior to T4 (1.8% vs 3.2%)

Over dry hands is one of the side effects of thoracic sympathetic surgery. In a study by Liu et al. (5), the over dry hands was very rare in T 4 group (1.4%) but common in group T3 (12.9%). in our study over dry hand was detected in (7.7%) in T3 group while we did not observed any patient in T4 group complaining these side effect. So T4 sympathicotomy, though theoretically means the least denervation of the hands, relieves the over-sweating condition of the hands, and gains the highest 'very satisfied' rate of the patients.

We conclude that palmar hyperhidrosis is well treated by video-assisted T3 or T4 sympathicotomy, and that it is a safe and effective procedure. T4 sympathicotomy results in less dryness and CH than T3 sympathicotomy at long-term follow-up. Slight moisture in the hands after surgery appears to be better tolerated than dryness. In this regard, T4 sympathicotomy can be a more useful and viable treatment for reduction of palmar sweating than T3. However T4 sympathicotomy showed small percentage of recurrence. Further study is needed to elucidate the physiologic and anatomical bases that influence factors in palmar dryness with CH.

Disclosures Drs. Ahmad Negm, Waleed Askar, Ashraf Abass, Amro Elhadidi, Salwa Hayes, and Ramadan Ellithy have no conflicts of interest or financial ties to disclose.

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T3 VERSUS T4 SYMPATHICOTOMY FOR TREATMENT OF PRIMARY PALMAR HYPERHIDROSIS : A PROSPECTIVE RANDOMIZED STUDY

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IMMUNOHISTOCHEMICAL EVALUATION OF ESTROGEN AND PROGESTERONE RECEPTORS EXPRESSION IN ADULT CHOLESTEATOMA TISSUE : AN OBSERVATIONAL STUDY

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Abstract

Objectives: *To investigate the frequency of estrogen receptor (ER) and progesterone receptor (PR) expression in cholesteatoma tissue as a trial to explore their role in pathogenesis of cholesteatoma.*

Patients & Methods: *The study included 40 patients with mean age of 39.1±6.8 years. There were 25 males and 15 female patients diagnosed clinically and by Computerized Tomography (CT) to have cholesteatoma, radicle mastoidectomy was done and surgical cholesteatoma specimens were taken to be examined histopathologically and used for determination of receptor positivity defined as nuclear staining in more than 10% of cells regardless of staining intensity. Four snips of normal skin specimens were obtained from external auditory canal as controls.*

Results: *29 specimens (72.5%) were receptor positive; 16 specimens (55.2%) were PR positive, while 13 specimens (44.8%) were ER positive. Twelve specimens showed marked receptor expression, 11 specimens showed moderate receptor expression and 6 showed weak receptor expression, all of control specimens were negative for ER and PR.*

Conclusion: *Female sex hormones expression rate was significant in cholesteatoma tissue, irrespective of patients' gender and clinical staging of cholesteatoma. These observations could open a way for evaluation of outcome of medical preoperative treatment of cholesteatoma*

using anti-estrogen and/or anti-progesterone drugs especially drugs with competitive receptor inhibition mechanism of action.

Keywords: *Cholesteatoma, Adult patients, Female sex hormones, Receptor expression, Immunohistochemistry.*

Introduction

Pathogenesis of acquired cholesteatoma is still a matter of debate and became no longer just whether it is cholesteatomatous epithelium in normal tissue merely in wrong place or pathologic tissue with qualitative changes. Multiple studies tried to explore the pathogenesis of acquired cholesteatoma. Local infection leads to a disturbance of self-cleaning mechanisms, with cell debris and keratinocytes accumulate inside the retraction pocket, and this is followed by an immigration of immune cells. Thus the resultant is an imbalance and a vicious circle of epithelial proliferation, keratinocyte differentiation and maturation, prolonged apoptosis, and disturbance of self-cleaning mechanisms. The inflammatory stimulus will induce an epithelial proliferation along with expression of lytic enzymes and cytokines. Moreover, bacteria inside the retraction pocket produce some antigens which will activate different lytic enzymes and cytokines especially interleukin-8 and tumor necrosis factor-alpha, mediators of bony destruction, leading to activation and maturing of osteoclasts with the consequence of degradation of extracellular bone matrix and hyperproliferation, bone erosion and finally progression of the disease [1,2,3].

Multiple hormone receptors were evaluated in non-endocrinal tumors and receptors of variant hormones were detected in benign and locally malignant lesions elsewhere in the body. Meningiomas, the most common intracranial extra-axial neoplasm, are commonly benign but have the ability of adjacent bony structures invasion with a recurrence rate of 12%[4]; however, Hirota et al. [5] reported that over half of meningiomas may be regulated by gonadotrophine-gonadotrophine receptor expression in an autocrine fashion.

Female steroid hormones regulate growth, differentiation, and function of diverse target tissues,

both inside and outside the reproductive system. Most of the actions of steroid hormones appear to be exerted via specific receptors on the target cells that function as ligand-actived transcription factors, regulating the synthesis of specific RNA and proteins. The human estrogen and progesterone receptors have been identified in normal target cells and their neoplastic counterparts. Evidence suggested that some dermatomes are related with female sex hormones including dermatomes arising during pregnancy, dermatomes improving or aggravating during pregnancy as psoriasis or systemic lupus erythmatosus and dermatomes showing female predominance as erythema nodosum [6,7,8].

The current study aimed to investigate the frequency of estrogen and progesterone receptor expression in cholesteatoma tissue as a trial to explore their role in pathogenesis of cholesteatoma.

Patients & Methods

The current study was conducted at Departments of Otorhinolaryngology and Pathology, Faculty of Medicine, Benha University hospital since Aug 2006 till Aug 2010. After approval of the study protocol by the Local Ethical Committee and obtaining fully informed patients' consent, patients with retraction pocket, attic perforation, persistent scanty bad odour discharge were collected and 40 patients (25 males and 15 females) with definite clinical and CT dignosis to have cholesteatoma were enrolled in this stydy, these patients aged 28-52 years with mean age of 39.1 $\pm$ 6.8.

Patients underwent radical mastoidectomy under general inhalational anesthesia. Cholesteatoma tissue specimens were taken and prepared for examination, Four snip of normal skin specimens were obtained from the external auditory canal as controls.

The aim of this study was To investigate the frequency of estrogen receptor (ER) and progesterone receptor (PR) expression in cholesteatoma tissue as a trial to explore their role in pathogenesis of cholesteatoma.

Immunohistochemical studies:

Surgical cholesteatoma specimens were fixed in 10% neutrally buffered formalin for 24 to 48 hours and embedded in paraffin. A representative block from each case containing an adequate tissue was selected. The DAKO ENVISION+kit (Dako Cytomation, Glostrup, Denmark) was used in conjunction with the DAKO Autostainer (DakoCytomation) according to instructions supplied by the manufacturer. Briefly, slides were deparaffinized in xylene and rehydrated in a graded series of ethanol/water rinses, then antigen retrieval was performed by heating slides in a water-bath to 95-99ÆC in Target RetrievalSolution High pH (DakoCytomation) for 40 min. After cooling for 20 min, the sections were treated with 3% hydrogen peroxide for 5 min followed by primary antibody for 30 min at room temperature.

The monoclonal mouse anti-human ER· (clone 1D5 DakoCytomation) and PgR (clone PgR636; DakoCytomation) antibodies were used at 1:50 and 1:800 dilutions, respectively. Visualization using the LSAB2 System was accomplished using a biotinylated link antibody, peroxidase-streptavidin and 3,3'-diaminobenzidine tetrachloride (1 mg/mL) containing 0.1% hydrogen peroxidase (30% w/v). Non-immune serum instead of the primary antibody was used for negative controls.

Assessment of immunohistochemistry :

Estrogen receptor or progesterone receptor positivity was defined as nuclear staining in more than 10% of cells regardless of staining intensity (9). For normal skin, the presence of nuclear-stained cells was considered as positive regardless of the number or staining intensity and the expression pattern was categorized into three groups: weak, moderate or marked according to number and intensity of staining of cells.

Results

The study included 40 patients with mean age of 39.1±6.8; range: 28-52 years. There were 25 males (62.5%) and 15 females (37.5%) patients. All of the control specimens were negative for ER (Fig. 1) and PR, (Fig. 2). Twenty-nine

specimens (72.5%) were receptor positive and 11 specimens (27.5%) were receptor negative, (Table 1). 16 specimens were PR positive, while 13 specimens were ER positive, (Table 2, Fig. 3).

12 specimens (41.4%) showed marked nuclear expression of receptor protein; 7 specimens showed marked nuclear expression of PR protein (Table 3, Fig. 4), while 5 specimens showed marked nuclear expression of ER protein (Table 3, Fig. 8). Eleven specimens (37.9%) showed moderate nuclear expression of receptor protein; 5 specimens showed moderate nuclear expression of PR protein (Table 3, Fig. 5, 6), while 6 specimens showed moderate nuclear expression of ER protein (Table 3, Fig. 9). Six specimens (20.7%) showed weak nuclear expression of receptor protein; 4 specimens showed weak nuclear expression of PR protein (Table 3, Fig. 7), while 2 specimens showed weak nuclear expression of ER protein, (Table 3, Fig. 10).

Table (1): Patients' distribution according to receptor positivity and gender.

	Receptor positive	Receptor negative	Total
Males	17 (42.5%)	8 (20%)	25 (62.5%)
Females	12 (30%)	3 (7.5%)	15 (37.5%)
Total	29 (72.5%)	11 (27.5%)	40 (100%)

Data are presented as numbers; percentages are in parenthesis

Table (2): Specimens' distribution according to receptor type.

	Estrogen receptor	Progesterone receptor
Positive	13	16
Negative	27	24

Data are presented as numbers;

Table (3): Specimens' distribution according to receptor type and intensity of staining .

	Estrogen receptor	Progesterone receptor	Total
Marked	5 (38.5%)	7 (43.8%)	12 (41.4%)
Moderate	6 (46.2%)	5 (31.2%)	11 (37.9%)
Weak	2 (15.3%)	4 (25%)	6 (20.7%)
Total	13 (100%)	16 (100%)	29 (100%)

Data are presented as numbers; percentages are in parenthesis

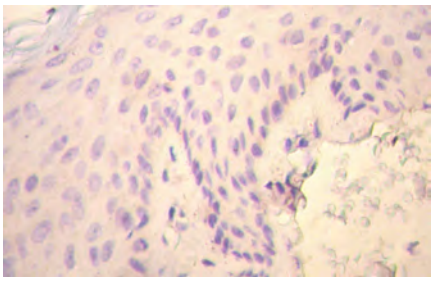

Fig. 1 : shows a specimen of a skin of the external auditory canal (Control Specimen) showing negative nuclear expression of ER protein (streptavidin-Biotin x200).

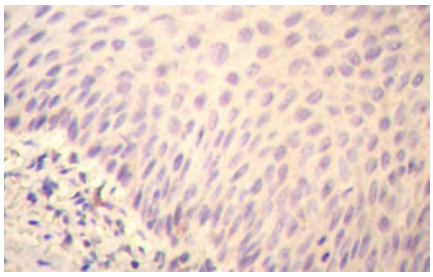

Fig. 2 : shows a specimen of a skin of the external auditory canal (Control Specimen) showing negative nuclear expression of PR protein (streptavidin-Biotin x200)

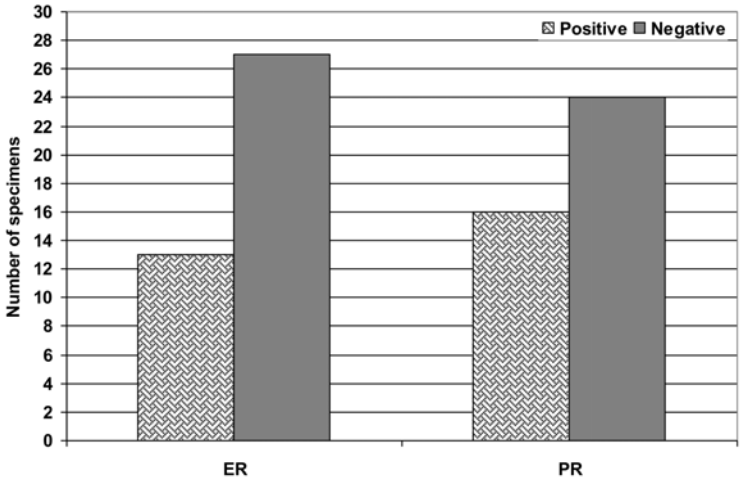

Fig. (3): Specimens' distribution according to receptor type and its positivity

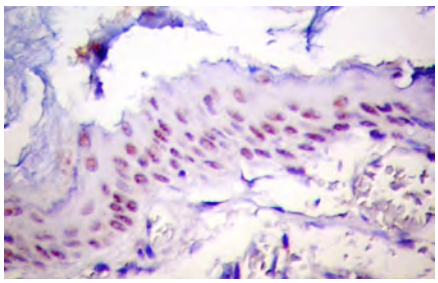

Fig. 4 : shows a specimen of a case of cholesteatoma obtained from female patient showing marked nuclear expression of PR protein (streptavidin-Biotin x200).

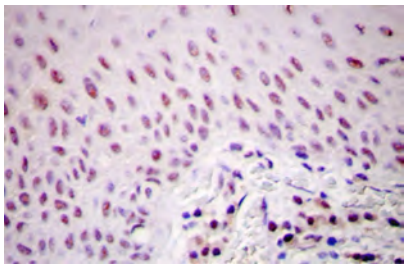

Fig. 5 : shows a specimen of a case of cholesteatoma obtained from male patient showing moderate nuclear expression of PR protein (streptavidin-Biotin x200).

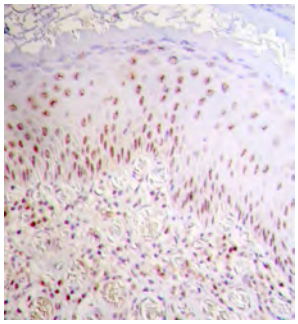

Fig. 6 : shows a specimen of a case of cholesteatoma obtained from male patient showing moderate nuclear expression of PR protein (streptavidin-Biotin x100).

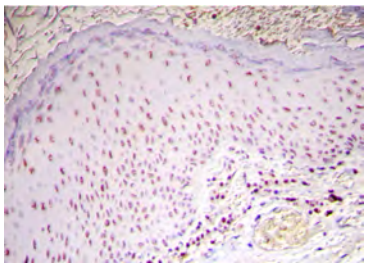

Fig. 7 : shows a specimen of a case of cholesteatoma obtained from female patient showing weak nuclear expression of PR protein (streptavidin-Biotin x100).

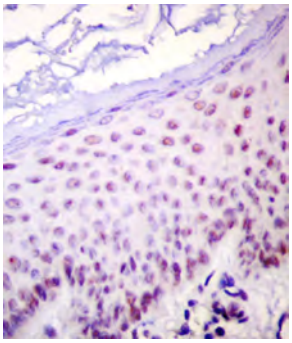

Fig. 8 : shows a specimen of a case of cholesteatoma obtained from female patient showing marked nuclear expression of ER protein (streptavidin-Biotin x200)

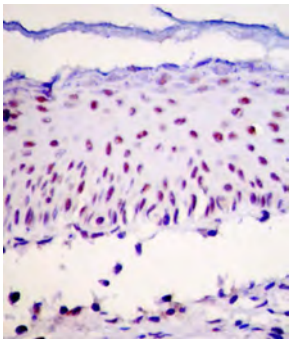

Fig. 9 : shows a specimen of a case of cholesteatoma obtained from female patient showing moderate nuclear expression of ER protein (streptavidin-Biotin x200).

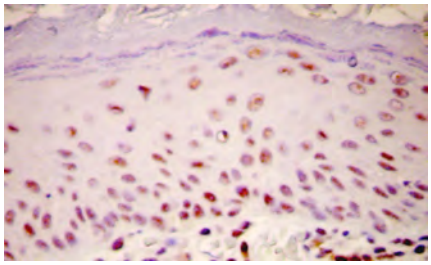

Fig. 10 : shows a specimen of a case of cholesteatoma obtained from male patient showing weak nuclear expression of ER protein (streptavidin-Biotin x200).

Discussion

The middle ear cholesteatoma is one of the most fascinating and complex topics in otology. Multiple factors contribute to the development and frequency of cholesteatomas as geography, genetics, sex, age, the environment, the social and economic status, health, incorrect use of antibiotics, and others. However, males predominated slightly (64.7%) compared to females (35.3%) among adults and children [10].

A role for estrogen in skin function may appear particularly relevant with aging and during wound healing; estrogen are known to affect wound healing by influencing different skin cellular components, including fibroblasts, vascular endothelial cells, and keratinocytes as well as infiltrating inflammatory cells [11,12].

Estrogen increases the mitotic rate in the epidermis of man and stimulates the synthesis, maturation, and turn over of collagen. Estrogen appears to increase the vascularization of the skin and the activity of pigment cells, while suppressing sebaceous gland activity. Progesterone has been shown to have anti-inflammatory and immunosuppressive properties, increases keratinocyte proliferation and blocks the action of 5-alpha-reductase. Steroid sex hormones have been shown to have a profound influence on the function of inflammatory cells including macrophages and on the secretion and activation of a range of plasma-borne inflammatory mediators. Polymorphisms in estrogen receptor genes have separately been shown to affect the incidence of a range of inflammatory disorders. Sex steroids themselves have been shown to be protective in certain conditions; harmful in others [13,14].

The obtained results of the current study reported significantly higher frequency of estrogen and progesterone receptors in cholesteatoma tissue compared to normal external auditory canal skin, irrespective of the patients' gender. A finding indicating an abnormal presentation of these hormones in a tissue which is not the main target of these hormones thereby indicating a role for these hormones in pathogenesis, pro-

gression or aggressiveness of cholesteatoma.

These findings go in hand with multiple studies detected feminine sex hormones in non-target tissues; Lukits et al.[15] reported estrogen and progesterone receptor expressions in head and neck cancer (HNC): 50.7% and 49.3% respectively and ER-alpha expression was predominant over ER-beta in both of oral cavity as well as laryngeal/hypopharyngeal cancers and concluded that ER and PR expressions are frequent in HNC and may affect the prognosis of the disease, at least in case of laryngeal/hypopharyngeal cancers. Bianchini et al.[16] reported specific estrogen and progesterone receptors in 53.3% and 73.3%, respectively of tissue specimens of laryngeal carcinoma.

Patel et al. [17] reported ER and PR expression in vestibular schwannomas and concluded that this might have implications for development of a vestibular schwannomas-specific drug delivery system using anti-hormone pathway for re-establishing cell density dependent growth inhibition. Cafer et al.[18] found that progesterone receptor is expressed in all acoustic neuroma samples and recommended further studies to find out about the inhibitory effect of antiprogesterone treatment on acoustic neuroma growth, which may be important particularly in elderly people or high-risk patients. Walton et al.[19] detected differential production of ER-beta splice variants in cancer prostate tissue and concluded that ER may play important roles in the genesis of prostate cancer.

Stratification of the obtained results according to patients' gender; ER expression was mostly found in specimens obtained from female patients, while PR expression was mostly found in specimens obtained from male patients. Such observation refuted the impact of patients' gender on receptor expression and the dependence of cholesteatoma pathogenesis is on expression of female sex hormone receptors irrespective of type of receptor. However, the preponderance of progesterone receptor in males supported that previously reported by Agras et al.[20] who detected progeste-

rone receptor protein at gestational day 12 in the urethral plate and mesenchyma and at later stages staining intensity increased with a greater progesterone receptor signal, especially in the urethra and in utero ethinyl estradiol led to 8.2, 9.7 and 5.2-fold increases in progesterone receptor mRNA in females and in males with and without hypospadias.

The obtained results indicated significant female sex hormones expression rate in cholesteatoma tissue, irrespective of patients' gender and clinical staging of cholesteatoma. These observations could open a way for evaluation of outcome of medical preoperative treatment of cholesteatoma using anti-estrogen and/or anti-progesterone drugs especially drugs with competitive receptor inhibition mechanism of action.

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IMMUNOHISTOCHEMICAL EVALUATION OF ESTROGEN AND PROGESTERONE RECEPTORS EXPRESSION IN ADULT CHOLESTEATOMA TISSUE : AN OBSERVATIONAL STUDY

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EFFECT OF FLUORIDE ON THE THYROID FOLLICLES OF YOUNG MALE ALBINO RATS
(HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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Abstract

Numerous reports on the effect of fluoride on the thyroid gland have been published but the results were highly controversial. The present study was done on twenty young (3 weeks old) male albino rats. They were divided into control and sodium fluoride treated groups (10 mg/L in drinking water). The rats were sacrificed after four and eight weeks. A significant decrease in serum T3 and T4 level was found. Morphological and histological examination of the thyroid gland revealed a significant increase in the follicular diameter, increased amount of colloid, a significant decrease in the follicular epithelial height, follicular hyperplasia and degeneration in both thyroid follicles and follicular cells. Ultra-structurally, follicular cell microvilli were lost, the mitochondria were swollen with loss of cristae, and the rER was dilated and irregular. Immunohistochemical examination of C-cells revealed a significant increase in the area percentage and density of reaction. It is concluded that fluoride has a deleterious effect on the structure and function of thyroid gland in young-aged. Therefore, parents must be carefully advised to avoid giving or exposing their children to fluoridated substances.

Introduction

The thyroid follicles are the structural and functional units of the thyroid gland. The follicles vary greatly in shape as well as in size, but are generally spheroidal,

measuring from 0.05 to 0.5 mm in diameter[1]. In rat and guinea pig, follicles at the periphery of the gland are larger than those more centrally situated[2].

A follicle consists of a layer of simple epithelium enclosing a cavity (the follicular cavity) containing a gel-like material, referred to as colloid. Each follicle is surrounded by an extremely thin basal lamina, which is not usually resolved with the light microscope but gives strong PAS-positive reaction [2&3].

The thyroid follicle has a double endocrine component; follicular cells, the most abundant cells in the gland responsible for secreting T3 and T4 hormones and C-cells, or parafollicular cells, which are very scarce and produce calcitonin, a hypocalcemic and hypophosphatemic hormone. Nevertheless, co-localization of follicular and C-cells in the thyroid gland is not accidental. C-cells are probably also involved in the intrathyroidal regulation of follicular cells[4&5]. This hypothesis is supported by different features, such as their characteristic 'parafollicular' position, their predominance in the central region of the thyroid lobe - the so-called C-cell region[6] and their implication in the secretion of many different regulatory peptides[7].

Exposure to fluoride components is widely spread, it includes not just water we drink but the air we breathe and the food we eat. Water fluoridation is a major source of ingested fluoride that was proven to alter enzymes used by living organisms to carry out a multitude of essential processes[8].

Exposure to fluoride is commonly complicated with hazards on different body organs; neurodegeneration[9], secondary hyperparathyroidism[10], osteomalacia, osteosclerosis, osteoporosis and osteosarcoma[11,12&13], marked lung congestion[14] and renal impairment[15].

Fluoride is an element of the halogen series; accordingly, it may have antagonistic properties toward iodine, another halogen.

Unfortunately, literature investigating the effect of fluoride on

the thyroid gland are very controversial[16]. Elevated T3 was reported with low fluoride doses [17]. Elevated T3 and T4 were found at the beginning of the experiment followed by decreased hormonal levels[18]. On the other hand, a decrease in the concentration of T3 and T4 hormones, producing hypothyroidism was documented[19]. In addition, normal thyroid function was lowered with low doses of fluoride (2.3 ppm fluoride in drinking water)[20]. However, fluoride in low doses (recommended for caries prevention) did not affect the thyroid function[16].

Thyroid hormones are essential for children metabolism and growth. Nevertheless, children are the most susceptible population group to fluoride toxicity as they may swallow toothpaste during teeth brushing; they are more exposed to fluoridated water in swimming pools, and may ingest fluoride in water, juice or prepared food.

Therefore, the aim of the present work is to assess the effect of low fluoride dose on thyroid function (T_3 & T_4) and to study the fluoride induced structural changes (light, electron microscopic and immunohistochemical) in the thyroid of young male rats.

Material and Method

Twenty young, three week-old, male albino rats were used in the present study. The animals were obtained from Mansoura Urology and Nephrology center, were housed in metal cages with meshes and had free access to standard diet. All cages were kept at room temperature.

The rats were divided into two groups:-

1- Control group (C group): consisted of 10 rats. They had free access to ordinary tap water.
2- Experimental group (E group): consisted of 10 rats. They received tap water containing sodium fluoride in a concentration of 10mg/L[21].

Four and eight weeks after the beginning of the experiment, five rats from each of the control and experimental groups were anaesthetized by ether inhalation. Blood samples were taken from the

heart of each rat, centrifuged and the serum was transported (surrounded by dry ice) to the Clinical Pathology Lab for assessment of the serum concentration of T3 and T4. The thyroid glands were dissected out (supported by the tracheas). In each sample the trachea was divided into two halves; each had one lobe of the gland. The right lobe was used for light microscopic study and the left one was used for electron microscopic examination.

*** Radioimmunoassay**

Radioimmunoassay was done for the assessment of total serum T3 and T4 in rat blood [22].

32 Light Microscopic (LM) Study

For LM study, the right lobes of the thyroid glands were fixed immediately in Bouin's fluid for 24 hours, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, impregnated and embedded in paraffin. Serial sections, 5 micrometer (μm) in thickness, were obtained, mounted on slides in groups of 10 sections each. The slides of the central third of the thyroid lobe were used and stained with:-

- Haematoxylin and eosin stain (Hx & E) [23].
- Immunohistochemical stain[24] for localization of calcitonin containing cells (C-cells) by labeled avidin-biotin method (LAB). After deparaffination and rehydration, sections were treated with 3% hydrogen peroxide for inhibition of endogenous peroxidase, washed in phosphate-buffered saline (PBS), sequentially incubated with a blocking reagent for 15 min, followed by specific rabbit antiserum anti-calcitonin overnight at 4^{o}C (Cat # RB-071-Al, Ready to use, Neomarkers, Westinghouse, USA). Slides were further incubated in biotinylated antiserum to rabbit / mouse immunoglobulins for 30 min, and then streptavidin peroxidase complex for 30 min. washing in PBS (three changes, 3 min. each) was performed after each incubation. Peroxidase activity was developed using 3, 3-diaminobenzidine tetrahydrochloride as chromogen, and hydrogen peroxide as substrate. Sections were counterstained with haematoxylin, dehydrated, cleared, and mounted. For nega-

tive controls, incubation with the specific antibody was omitted.

*** Electron Microscopic (EM) Study**

For EM study, the left lobes of the thyroid glands of the fluoride-treated group (four and eight weeks after the beginning of the experiment) and their corresponding control were obtained, fixed in glutaraldehyde (2.5%) and post fixed in osmium tetroxide (1.0%).

Semithin sections (1um thickness) were prepared and stained with toulidine blue. Ultrathin sections (80 nm thickness) were cut and stained with uranyl acetate and lead citrate[25].

*** Morphometric study**

Leica Qwin computerized image analyzer was used for this study.

- Haematoxylin and eosin stained sections were used for the measurement of follicular diameter and follicular epithelial height.
- Immune stained sections were used for the measurement of area percent and the density of immune reaction in C-cells.

*** Statistical analysis**

Statistical analysis of the data obtained from the morphometric study was done using Student's-t test in order to test the significance of difference between the experimental groups and their corresponding control.

Results

Control group

*** Four weeks after the beginning of the experiment:**

Haematoxylin and eosin (Hx. and E.) stained sections of the thyroid gland of the young control rats revealed the C.T. capsule covering the gland. Thin C.T. septa were seen dividing the gland into incomplete lobules. The follicles were variable in size; the mean follicular diameter was 62.3380 ± 10.5043 µm (table 1). The central follicles had a smaller diameter than the peripheral ones. The follicular lumen was filled with an acidophilic colloid which occasionally revealed marginal vacuoles, especially in the peripheral follicles. The wall of the follicles was composed of a single layer of cells with average height 8.2520± 1.0998 µm (table 2). The central follicles were lined by cubical epi-

thelium with rounded nuclei, whereas the peripheral ones were delimited with flat or low cubical epithelium. Interfollicular C.T. as well as small clumps of interfollicular cells, that represent tangential sections through the wall of the follicles, were seen (fig.1).

C-cells were scattered among the follicular and interfollicular cells, mainly in the central part of the gland. They were few in number, large in size and rounded or oval in shape. Their cytoplasm appeared pale and their nuclei were rounded and vesicular (fig.1). However, their precise identification in Hx. and E stained section was difficult.

Immunohistochemical technique is an accurate method for detection of C-cells. C-cells were singly distributed within the thyroid follicles and among interfollicular cells. The percentage of their surface area was 0.7440 ± 0.3932 (table 5) and their density of reaction was 0.8230 ± 0.03917 (table 6).

Ultrathin section examination showed that the thyroid follicles were lined by cubical cells with almost rounded vesicular nuclei and prominent nucleoli. The apical part of the cells revealed slender microvilli that projected into the homogeneous luminal colloid. The basal lamina of the cells was surrounded by a network of blood capillaries. The cytoplasm exhibited numerous cisternae of rER. The mitochondria showed intact cristae and moderate electron dense matrix. Few lysosomes, which appeared as electron dense granules of variable sizes, were seen in the apical part of the follicular cells (Fig. 3).

C-cells were occasionally observed among follicular and interfollicular cells. They were ovoid in shape and their cytoplasm exhibited rounded granules of variable size and electron density. The cells were lying in contact with blood capillaries but they did not abut on the follicular lumen. They were separated from the lumen by narrow cytoplasmic extensions from the follicular cells (fig. 4).

Mast cells were frequently seen in the connective tissue between the thyroid follicles; their

cytoplasm was stuffed with rounded granules larger in size than those of C-cells and of variable electron density. The cell membrane revealed many cytoplasmic processes (fig. 4).

*** Eight weeks after the beginning of the experiment:**

Hx. and E. stained sections of the young control thyroid gland eight weeks after the beginning of the experiment (fig. 5) revealed a structure similar to that of the four week control group. However, an increase in follicular diameter (72.5150 ± 12.3183 µm) was recorded (table 1).

Immunohistochemical examination revealed an increase in the number of immunostained C-cells, the area percentage of C-cells was 2.8220 ± 1.5175 (table 5). The density of reaction of calcitonin immunostained C-cells was also increased to 0.8650 ± 0.06187 (table 6). The cells were distributed mainly in clusters (fig. 6).

Ultrathin section examination revealed no ultra-structure difference from the previous control group (figs. 7).

Sodium fluoride treated group (E group):-

*** After four weeks of fluoride treatment**

Hx. and E. stained sections of the thyroid gland after four weeks of fluoride treatment exhibited a non-significant increase in follicular diameter 66.4100 ± 10.4841 ?m as compared with 62.3380 ±10.5043 ?m in the corresponding control group (table 1). The follicular epithelium height was non-significantly decreased from 8.2520 ± 1.0998 µm in the control to 7.6630 ± 1.4487 µm (table 2). Follicular deformity, hyperplasia and degeneration (partial loss of follicular cell lining together with the presence of desquamated cells in the follicular lumen) were encountered. Both C-cells and interfollicular cell masses were frequently seen in section. Cells with small darkly stained pyknotic nuclei were observed among follicular cells (fig. 8).

Immunohistochemical examination revealed a significant increase in the number of immunostained C-cells. The area percentage of C-cells was 1.0430 ± 0.7980 as compared with 0.7440

± 0.3932 in the corresponding control (table 5). The density of reaction of calcitonin immunostained cells was also significantly increased to 0.9410 ± 0.008987 as compared with 0.8230 ± 0.03917 (table 6). The C-cells were distributed either singly or in small clusters (fig. 9).

Ultrathin section examination revealed follicular cells with partial loss of their apical microvilli. The cytoplasm exhibited dilated rER cisternae. Most of the mitochondria were swollen with loss of their cristae. The nuclei were euchromatic except some few condensed irregular nuclei (fig. 10).

*** After eight weeks of fluoride treatment**

Hx. and E. stained sections of the thyroid gland after eight weeks of fluoride treatment showed a non-significant increase in the follicular diameter, 75.5810 ± 8.6475 µm as compared to 72.5150 ±12.3183 µm in the corresponding control group (table 1). However, a significant decrease in the epithelial height was detected; 7.5360 ± 1.1696 µm compared with 10.4690 ± 1.6444 µm in the control (table 2).

Follicular hyperplasia, deformity and degeneration were more frequently encountered. Cells with small darkly stained pyknotic nuclei and acidophilic cytoplasm were detected among follicular and interfollicular cells (fig. 11).

Immunohistochemical examination revealed a significant increase in the number of immunostained C-cells, the area percentage was 4.4700 ± 1.3050 compared with 2.8220 ±1.5175 in the corresponding control (table 5). The density of reaction of calcitonin immunostained cells was also significantly increased to 1.3190 ± 0.06707 as compared with 0.8650 ± 0.06187 in the control (table 6). The C-cells were distributed mainly in larger clusters (fig. 12).

Ultrathin section examination revealed follicular hyperplasia. The apical part of follicular cells showed loss of microvilli. The cytoplasm exhibited more dilated rER cisternae. The mitochondria were swollen with loss of their cristae (figs. 13).

The C-cells were more frequently seen in section. Their cytoplasm contained abundant rounded granules of variable electron density, cisternae of rER and free ribosomes (figs. 14).

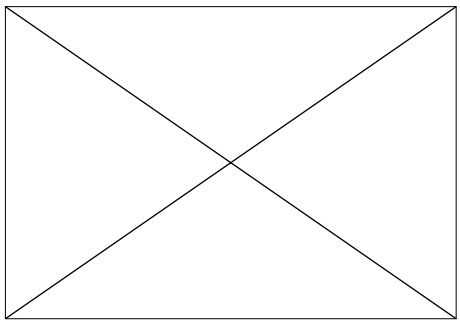


Fig. 1 : A section in the thyroid gland of a young control rat after four weeks from the beginning of the experiment showing the central follicles (c) lined by a single layer of cubical epithelium with large rounded nuclei, while the peripheral ones (P) are lined by low cubical or flattened epithelium. Small clusters of interfollicular cells are present in the C.T. between the follicles (I). Few scattered C cells are also seen (crossed arrow).
(Hx &E X 250)

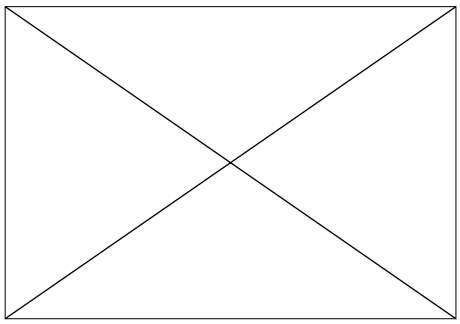


Fig. 2 : A section in the thyroid gland of a young control rat four weeks after the beginning of the experiment showing scattered follicular and interfollicular immunostained C-cells exhibiting moderate reaction for calcitonin. (Anti-calcitonin inmmunostaining X 250)

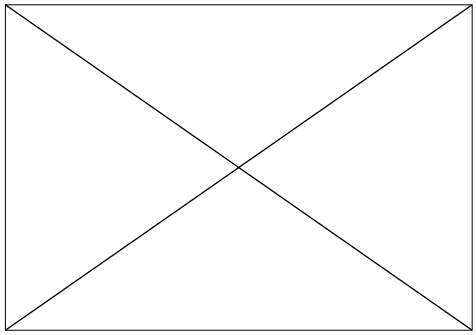


Fig. 3 : Electron photomicrograph of the thyroid gland of a young control rat four weeks after the beginning of the experiment showing part of a thyroid follicle. The follicular cells have vesicular nuclei (N) with prominent nucleoli. The apical surface exhibits numerous microvilli projecting into the colloid (arrows); the cytoplasm shows cisternae of rough endoplasmic reticulum (R) and mitochondria (M). Electron dense lysosomes are present mainly at the apical part of the follicular cells (L). A blood capillary (C) is seen under the basal lamina. (uranyl acetate and lead citrate X 5000)

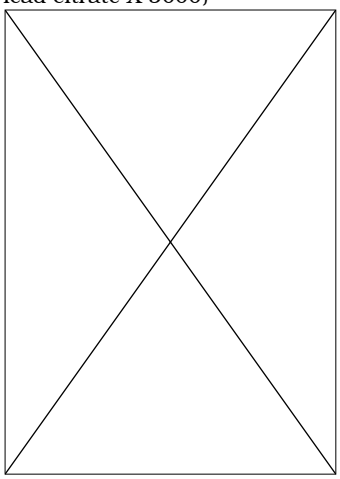


Fig. 4 : Electron photomicrograph of the thyroid gland of a young control rat four weeks after the beginning of the experiment showing an intrafollicular C-cells resting on a basement membrane. The cytoplasm contains rounded granules of variable electron density. (Uranyl acetate and lead citrate X 5000)

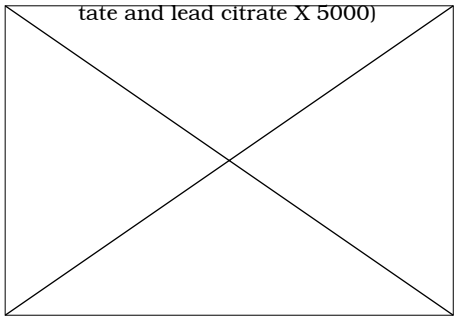


Fig. 5 : A section in the thyroid gland of a young rat after four weeks of fluoride treatment showing hyperplasia in the wall of some follicles (arrows), and increased interfollicular cell masses (I). Degenerated follicles (D) with desquamated cells in their lumen are also seen.

(Hx&E X 250)

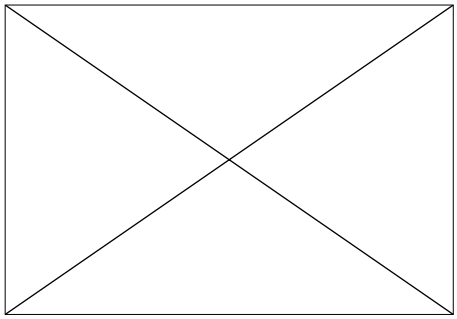


Fig. 6 : A section in the thyroid gland of a young rat after four weeks of fluoride treatment showing increased number and density of immunostained C-cells. The cells are distributed either singly (arrows) or in small groups (crossed arrows). Compare with the control (Fig. 2).

(Anti-calcitonin inmmunostaining X 250)

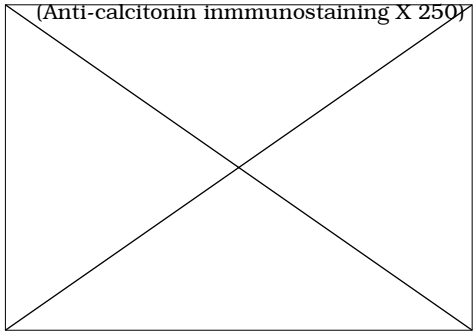


Fig. 7 : Electron photomicrograph of two adjacent thyroid follicles of a young rat after four weeks of fluoride treatment, showing a follicular cell with rounded vesicular nucleus (V) and two other cells with flat (F) or irregular (IR) nuclei. Partial loss of the projecting microvilli (arrows) and dilated rER cisternea (R) are seen in the follicular cells.
(Uranyl acetate and lead citrate X 5000)

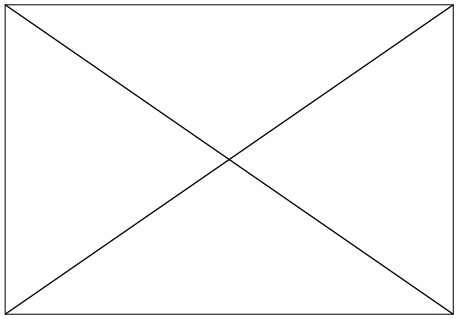


Fig. 9 : A section in the thyroid gland of a young control rat after eight weeks from the beginning of the experiment showing an increase in the size of the follicles and the inter follicular cell masses. C-cells are more frequently seen (arrows).
(Hx &E X 250)

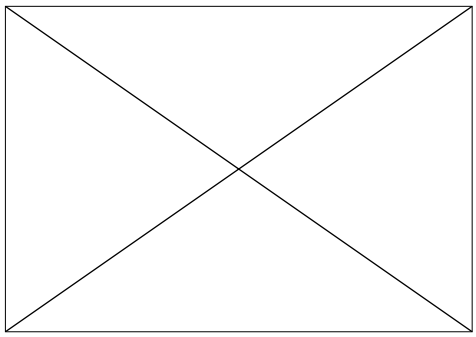


Fig. 8 : Electron photomicrograph of the thyroid gland of a young male rat after four weeks of fluoride treatment, showing parts of two thyroid follicles. The follicular cells show dilated rER (R), lysosomes (L), loss of apical microvilli (arrows) and flat nuclei (F). A degenerated follicular cell with small pyknotic nucleus is seen (P). An intra-follicular C–cell (crossed arrows) rich in rounded granules with variable electron density is also present
(Uranyl acetate and lead citrate X 5000)

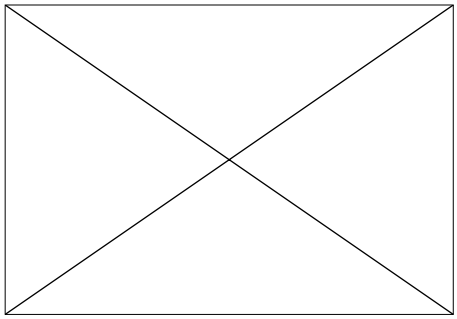


Fig. 10 : A section in the thyroid gland of a young control rat after eight weeks from the beginning of the experiment showing aggregations of immunostained C-cells with a moderate reaction product for calcitonin.
(Anti-calcitonin inmmunostaining X 250)

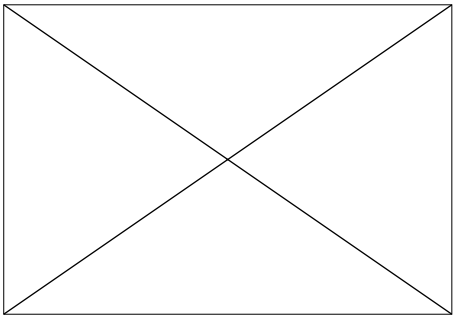


Fig. 11 : A section in the thyroid gland of a young rat after eight weeks of fluoride treatment showing hyperplasia in the wall of some follicles (arrows), follicular deformity (F) and degeneration (D). Necrotic cells characterized by darkly stained nuclei and acidophilic cytoplasm are seen among the follicular epithelial cells (crossed arrows).
(Hx &E X 250)

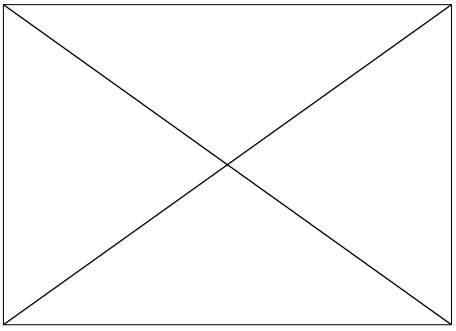


Fig.12 : A section in the thyroid gland of a young rat after eight weeks of fluoride treatment, showing large clusters of C-cells (arrows) with a strong immune reaction product for calcitonin.
Note: - The immunostained C-cells may form a collar around some of the follicles (crossed arrows).
(Anti-calcitonin inmmunostaining X 250)

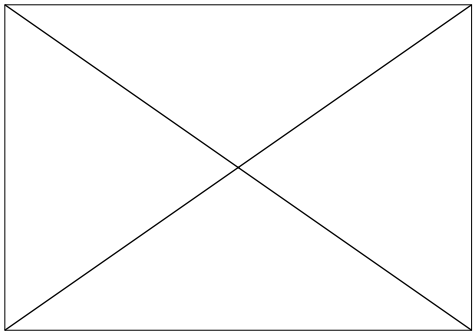


Fig. 13 : Electron photomicrograph of the thyroid gland of a young rat after eight weeks of fluoride treatment, showing part of a thyroid follicle with follicular hyperplasia (the wall is lined by three layers of cells). The rER cisternae are dilated in almost all follicular cells (R). The nuclei of the follicular cells appear hyperchromatic and show irregular nuclear membranes, compared with the control (fig.10).
(Uranyl acetate and lead citrate X 5000)

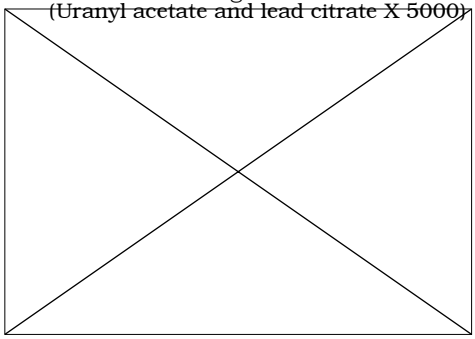


Fig. 14 : Electron photomicrograph of a thyroid follicle of a young rat after eight weeks of fluoride treatment showing showing part of a thyroid follicle containing two intrafollicular C-cells (crossed arrows) which rest on the basement membrane and do not reach to the luminal surface. The cytoplasm of C-cells contains numerous granules with variable electron density. The follicular cells appear degenerated with lost apical microvilli (arrows), many lysosomes (L) and dilated rER cisternae (R). One of the follicular cells is having a small pyknotic nucleus (P) and the other demonstrates an irregular hyperchromatic nucleus (IR).

Statistical Results

Table (1): The External Follicular Diameter (μm)

Duration of the experiment	Control group Mean	S.D	Experimental group Mean	S.D	P
4 weeks	62.3380	10.5043	66.4100	10.4841	0.15
8 weeks	72.5150	12.3183	75.5810	8.6475	0.33

- Number of animals in each group = 5 animals.
- Number of readings / animal = 10 central and 10 peripheral follicles.
- P: student test between experimental (E) group and control (C) group.
  The probability ($P \leq 0.05$ significant)

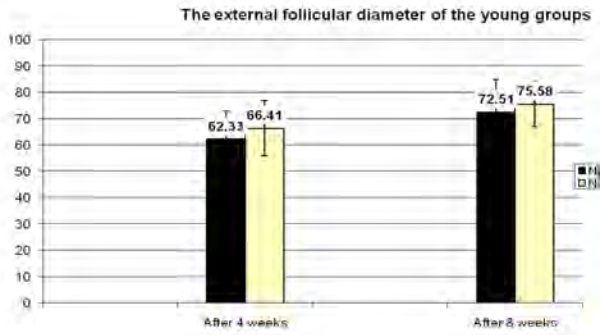


Table (2): The Follicular Epithelial Height (μm)

Duration of the experiment	Control group Mean	S.D	Experimental group Mean	S.D	P
4 weeks	8.2520	1.0998	7.6630	1.4487	0.61
8 weeks	10.4690	1.6444	7.5360	1.1696	<0.001

- Number of animals in each group = 5 animals
- 20 readings were obtained from each animal.
- P: student test between E group and C group.
  The probability ($P \leq 0.05$ significant)

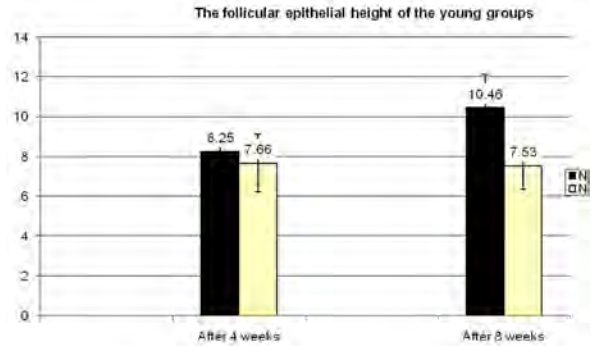


Table (3): Serum T_3 Level

Duration of the experiment	Control group		Experimental group		P
	Mean	S.D	Mean	S.D	
4 weeks	1.37	0.1358	0.82	0.2496	0.001
8 weeks	1.63	0.1264	0.73	0.4429	<0.001

-Number of animals in each group = 5 animals.
-5 readings were obtained from each group.
- P: student test between E group and C group.
The probability ($P \leq 0.05$ significant)

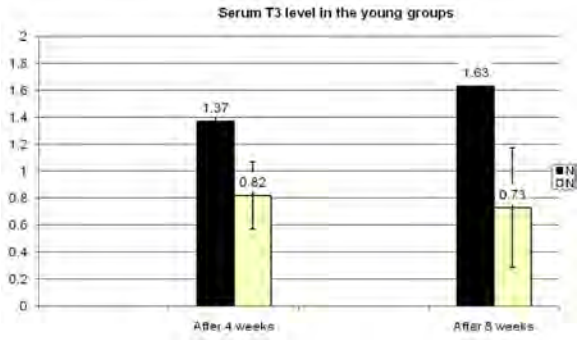


Table (4): Serum T_4 Level

Duration of the experiment	Control group		Experimental group		P
	Mean	S.D	Mean	S.D	
4 weeks	8.62	0.6504	5.90	0.3843	<0.001
8 weeks	9.01	0.3640	4.81	0.3745	<0.001

-Number of animals in each group = 5 animals
-5 readings were obtained from each group
- P: student test between E group and C group.
The probability ($P \leq 0.05$ significant)

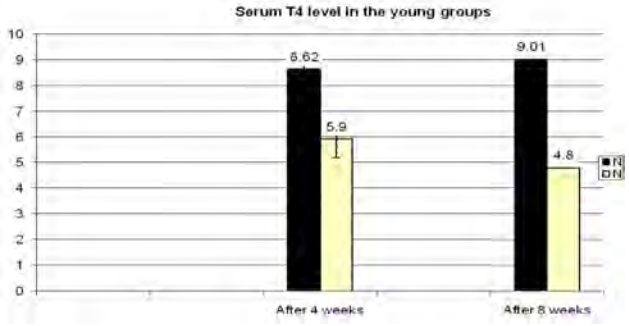


Table (5): The Percentage of C-Cell Surface Area per Field

Duration of the experiment	Control group		Experimental group		P
	Mean	S.D	Mean	S.D	
4 weeks	0.7440	0.3932	1.0430	0.7980	0.003
8 weeks	2.8220	1.5175	4.4700	1.3050	<0.001

-Number of animals in each group = 5 animals
-10 readings were obtained from each group.
- P: student test between E group and C group.
The probability (P ≤ 0.05 significant)

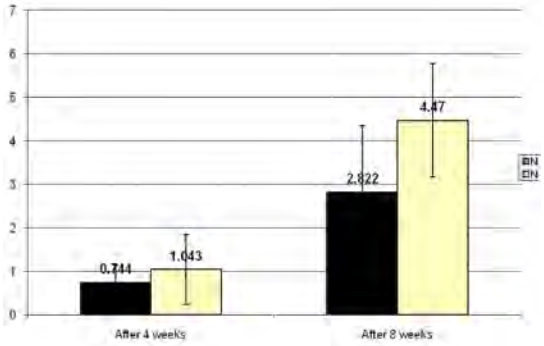
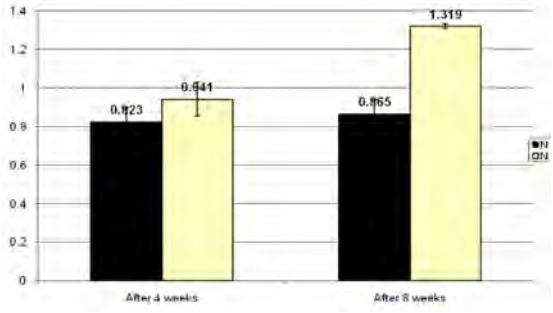


Table (6): The Density of Reaction in C-Cell

Duration of the experiment	Control group		Experimental group		P
	Mean	S.D	Mean	S.D	
4 weeks	0.8230	.03917	0.9410	0.08987	0.03
8 weeks	0.8650	0.06187	1.3190	0.06707	0.005

- Number of animals in each group = 5 animals.
- 10 readings were obtained from each group.
- P: student test between E group and C group.
The probability (P ≤ 0.05 significant)



Discussion

Fluorides are cumulative and build up steadily with ingestion of fluoride from all sources, which include not only water, but also the air we breathe and the food we eat [18].

The thyroid gland has a double endocrine component; follicular cells, responsible for secreting T3 and T4 hormones and C-cells, which produce calcitonin. Thyroid hormones (T3 and T4) are essential for metabolism and growth especially in children [5].

In the present study, radioimmunoassay revealed a significant decrease in blood thyroid hormone levels (T3 and T4) in young rats, four and eight weeks after fluoride ingestion, indicating a hypothyroid condition. Similar results have been reported by many investigators[26,27,28,29,30&31]. It is reported that sodium fluoride depresses the endocytosis of colloid and thyroid secretion by inhibiting aerobic glycolysis in the follicular cells[26]. It was considered that fluoride, by increasing the intracellular cAMP concentration, causes desensitization of the thyroid stimulating hormone receptors[27]. the levels of serum free thyroid hormones were lower than those of the control group at the late stage of their experiment (after 8 months of fluoride treatment); in spite of the early elevation of T3 & T4 at the beginning of their experiment (after 4 months of fluoride treatment). The late decrease was referred to the degenerative changes in the thyroid follicular epithelial cells[18]. Fluoride blocks the uptake and utilization of iodine by target cells is another explanation of such decrease in T3 and T4[28].

Light microscopic examination of thyroid sections of young rats treated with sodium fluoride showed morphologic alterations; increased follicular diameter with accumulation of colloid, especially at the center of the gland, decreased follicular epithelial height and decreased marginal vacuoles in the peripheral follicles. Similar results have been described by[31&32]. However, a decreased amount of luminal colloid with fluoride administration was reported[33 & 34].

The current study demonstrated that sodium fluoride induced hyperplasia of thyroid follicular epithelium. It was seen after four weeks of sodium fluoride administration and was more frequently encountered after eight weeks. A similar finding was reported[30] who even described hyperplastic nodes in the thyroid gland of rats after fluoride intake.

Since hyperplasia is considered as a precancerous change[35], the rate of thyroid cancer may be expected to increase with fluoride administration. This suggestion is confirmed[36] who documented that the rate of thyroid cancer increased with excess fluoride. They explained that fluoride stresses the functional status of the hypothalamo-pituitary thyroid system, thus adversely affecting the synthesis of DNA and RNA in thyroid cells. On the other side, evidence of thyroid cancer in rats with chronic fluoride toxicity has been denied[37]. The possibility of increased risk of thyroid cancer was eliminated, as the level of DNA strand breaks did not increase in blood, liver, kidney, thyroid gland and urinary bladder after acute fluoride exposure of rats and DNA damage is an important step in events leading to carcinogenesis [38].

Follicular deformity in the form of irregularity in the shape of the follicles as well as loss of continuity of their lining epithelium, accompanied with the presence of desquamated cells in the lumen, were observed after four weeks of sodium fluoride treatment. In addition, cells with small darkly stained nuclei and acidophilic cytoplasm were detected. These degenerative changes were more encountered after eight weeks of fluoride administration. Similar degenerative changes in both thyroid follicles and follicular epithelium after fluoride administration was described[18].

Ultrastructural examination of the thyroid follicular cells in fluoride-treated groups revealed loss of apical microvilli, swollen mitochondria with loss of cristae and dilated rER cisternae with loss of attached ribosomes. These ultrastructural changes increased with the duration of fluoride intake and may confirm the hypofunctional

state of the thyroid gland. Similar ultrastructural changes have been described after fluoride intake[30] in the thyroid follicular cells of chicks, in the pancreatic acinar cells of young pigs[39] and in the thyroid follicular cells of piglets[29]. The later also reported that rER dilatation may indicate enhancement of detoxification reactions and its rupture would suppress protein synthesis. The authors also suggested that the destruction of mitochondria and ER could be attributed to oxidative stress induced by fluoride, which can seriously damage the structure of cells and organelles.

Immunohistochemistry revealed a significant and progressive increase in the area percentage of C-cells in sodium fluoride-treated groups. Also, the density of immunohistochemical reaction in C-cells was significantly increased after four weeks of fluoride treatment and a further significant increase was recorded after eight weeks. The significant increase in area percentage and density of reaction of C-cells is similar to that described[40]. Those authors even demonstrated hyperplastic nodules formed of thyroid parafollicular cells (C-cells) in the thyroid gland with fluoride exposure.

The results of the current immunohistochemical study were confirmed by the ultrastructural examination of parafollicular C-cells in young rat groups treated with fluoride which revealed an increase in the number and electron density of C-cell cytoplasmic granules. These results are in accordance with the increased serum calcitonin level after fluoride ingestion[40&41]. Evidence of secondary hyperparathyroidism in rats treated with fluoride was observed[10] that were accompanied with increased calcium level in blood by the parathyroid hormones. Accordingly the increased serum calcium level may be an indirect stimulus to the C-cells for increasing calcitonin secretion in order to counteract the effect of parathyroid hormone. Unfortunately, experimental studies on the effect of fluoride intake on the ultrastructure of C-cells are deficient.

Conclusion

In the light of the present study, it is concluded that fluoride has a deleterious effect on the structure and function of the young-aged thyroid gland.

Recommendation

Since the thyroid of young-aged has been proved to be susceptible to fluoride hazards, therefore, parents must be carefully advised to avoid giving any fluoridated substance to their children.

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EFFECT OF FLUORIDE ON THE THYROID FOLLICLES OF YOUNG MALE ALBINO RATS (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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PSYCHIATRIC SIDE EFFECTS DURING PEGINTERFERON AND RIBAVIRIN THERAPY FOR CHRONIC HEPATITIS C : IS THERE A PREDICTIVE VALUE OF INTERFERON-INDUCED DEPRESSION?

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Abstract

Introduction: *The combination of pegylated interferon (IFN) & ribavirin is the standard treatment of chronic HCV. It is well known that IFN had many side effects. The psychiatric side effects of interferon, often responsible for dose reduction or treatment discontinuation, represent a major limitation in the treatment of chronic hepatitis C (CHC).*

Aims : *To prospectively assess the occurrence, risk factors of psychiatric side effects during peginterferon and ribavirin therapy for CHC and to determine if interferon-induced depression could be a predictive factor of sustained virological response (SVR).*

Materials & Methods: *A prospective follow up study including a convenient sample of 105 patients with CHC attending Mansoura University Hospitals & receiving a standard course of peginterferon alfa 2a plus ribavirin for 48 weeks according to national Egyptian guidelines for treatment of CHC. Neuropsychiatric side effects were monitored prospectively using the Mini-International Neuropsychiatric Interview (MINI), Hamilton Depression Rating Scale (HDRS), Hamilton Anxiety Rating Scale (HARS), Mini Mental state Examination (MMSE).*

Results: *Psychiatric side effects occurred in 28 patients (26.6%), mostly within the first 12 weeks, there was a high incidence of depression occurring in 20 patients (19%), 5 cases developed anxiety (3.8%),*

3 patients (2.8%) developed life-threatening psychiatric symptoms (i.e. psychosis; one of them had suicidal ideation); Twenty three patients did not complete the study as 6 severe cases (3 depression, 3 anxiety) needed psychiatric treatment; they were excluded to avoid its effect on sustained viral response (SVR), 3 cases of psychosis required early discontinuation of antiviral therapy), 3 cases were not available for final evaluation; and 11 cases were non responder ,they stopped treatment after 24 weeks. Only 82 patients completed this study. Psychiatric disorders in the final group were depression (17 cases), anxiety (2 cases). IFN response rates were significantly higher in those patients who developed IFN-induced depression than in those who did not (SVR rates: 70% versus 41.1% respectively; P = 0.04,Odds ratio=3.2,95% CI: 0.9-11.9). No correlation was found between incidence of depression and baseline level of viraemia, ALT level or fibrosis score (P=0.32, 0.41, 0.21 respectively). The only factor that could predict psychiatric disorders at 12 weeks of interferon therapy was the baseline psychiatric scoring HDRS, HARS and MMSE (P< 0.001, P< 0.001, P= 0.04 respectively).

Conclusion: *Interferon-induced psychiatric side effects are important to be monitored and treated if significant symptoms develop for optimizing treatment outcomes in CHC patients. Our findings suggest that IFN-induced depression may be considered as an indicator of optimal dosing rather than a predictor of a positive response to IFN therapy.*

Keywords: *antiviral therapy, depression, HCV, Interferon, Psychiatric disorders, SVR.*

Introduction

The prevalence of hepatitis C virus (HCV) infection varies throughout the world, with the highest number of infections reported in Egypt. The use of parenteral antischistosomal therapy in Egypt is thought to have contributed to a prevalence of antibodies against HCV in various regions ranging from 6% to 28% (mean 22%)[1]. However, transmission continues despite termination of this program and the implementation of measures to reduce infection [2].

An estimated 70% to 85% of infected patients are likely to develop chronic hepatitis, and up to

30% of these cases might progress to cirrhosis[3]. About 1-4% of these cases might develop hepatocellular carcinoma[4,5]. Currently, chronic hepatitis C is the leading indication for liver transplantation [6].

The current standard of care for chronic hepatitis C (CHC) is combination therapy with pegylated interferon-α (IFN-α) and ribavirin, it is well known that IFN have many side effects[7]. Among the numerous side effects reported with IFN-α therapy, psychiatric complications are the most frequent, mainly occurring within the first 12 weeks of therapy [8,9]. Neuropsychiatric side effects include depression, anxiety, insomnia, mood disorders, frank psychosis, suicidal ideation, actual suicide, and homicide.

The most consistent risk factors for developing depression are the presence of mood and anxiety symptoms prior to therapy. A past history of depression and of receiving higher doses of interferon, as well as being female, have been identified as risk factors, but are less reliable ones[10]. Finally, the occurrence of psychiatric side effects during IFN-α therapy is often responsible for dose reduction or treatment discontinuation, thus limiting its therapeutic potential[11,12]. Good treatment adherence is crucial for sustained viral clearance[13], but the impact of IFN-α psychiatric side effects on adherence and virological response has so far received little attention and the results tend to conflict. One study found that the occurrence of psychiatric side effects was associated with a better virological response[14], whereas two other studies[15,16] found that it was associated with less frequent sustained virological response (SVR).

The aim of this study was to prospectively assess the occurrence, risk factors of psychiatric side effects, particularly depression, during peginterferon and ribavirin therapy for CHC and to determine if interferon-induced depression could be a predictive factor of sustained virological response (SVR).

Materials & methods

Patients:

A convenient sample of 105 treatment-nayive Egyptian patients with chronic hepatitis C attending Mansoura University Hospitals were considered for enrolment in this study. Participants were recruited from outpatients in the period between January 2008 and December 2010. Patients received a standard course of peginterferon alfa 2a plus ribavirin for 48 weeks according to the guidelines of the national Committee for Control and Prevention of viral Hepatitis "C" in Egypt[17]. Twenty three patients did not complete the study , the final group included 82 patients. The study protocol was approved by The Ethics Committee for Medical Research at Mansoura University and all participants gave their written informed consent.

Study design:

The study utilized a prospective cohort design for the assessment of depression. The relationship between depression and viral clearance was examined retrospectively. Subjects were psychiatrically evaluated at baseline (prior to initiation of PEG IFN/ribavirin) and at, 12, and 48 weeks of PEG IFN/ ribavirin treatment.

Antiviral therapy:

According to the national Egyptian guidelines for treatment of chronic HCV [17], the participants were treated for 48 weeks with pegylated interferon α-2a (Pegasys, Hoffmann La Roche Ltd., Basel, Switzerland) given at a dose of 180 μg taken once weekly subcutaneously in addition to oral ribavirin taken daily in a dose of 1000-1200 mg (according to bodyweight : 1,000 mg for those who weight ≤75 kg and 1,200 mg for those who weight >75 kg). Chronic viral hepatitis C was serologically and PCR-confirmed (according to reverse transcription-polymerase chain reaction). Exclusion criteria : History of previous treatment with IFN-Ribavirin therapy, patients positive for hepatitis B virus infection (HBsAg) or human immunodeficiency virus infection, autoimmune hepatitis, decompensated cirrhosis, hepatocellular carcinoma, presence of major uncontrolled depressive illness, solid organ transplant (renal, heart, or lung), immunosuppressive drugs,

untreated thyroid disease, sever concurrent medical disease such as severe hypertension, heart failure, significant coronary heart disease, poorly controlled diabetes, chronic obstructive pulmonary disease, CNS trauma or active seizures which requires medication, substance abuse (abstention for the last 12 months), severe pre-existing psychiatric conditions and evidence of cognitive impairment demonstrated by Mini-Mental State Examination (scores of less than 23).

All patients were monitored for at least 6 months after completion of therapy. Sustained virological responders were defined as patients who tested negative for HCV RNA for at least 6 months after completion of IFN therapy. All other patients, who did not show SVR, were considered non-responders.

Liver biopsy:

Transcostal liver biopsy was performed to all patients at baseline with ultrasound guidance by using an 16-gauge needle. The stage of fibrosis was scored according to Ishak scoring system which uses a 6-point scale for staging: stage 0= no fibrosis; stage 1= fibrous expansion of some portal areas; stage 2= fibrous expansion of most portal areas; stage 3= occasional portal-portal bridging fibrosis; stage 4= marked bridging fibrosis; stage 5= incomplete (early) (developing) cirrhosis and stage 6= established cirrhosis [18].

Psychiatric assessment:

Participants were interviewed at baseline, after 12 weeks of therapy and at the end of therapy(at week 48) using the Structured Clinical Interview for DSM-IV Disorders (SCID), Mini International Neuropsychiatric Interview (MINI) and Mini Mental state Examination (MMSE). The severity of depression and anxiety were assessed using Hamilton Depression Rating Scale and Hamilton Anxiety Rating Scale. [In case of occurrence of psychiatric disorders, psychiatric medications were prescribed according to clinical need : for 6 cases (3 depression & 3 anxiety), they were excluded to avoid the effect of psychiatric treatment on SVR, and also for 3 cases who developed psychosis].

MINI: The Mini-International Neuropsychiatric Interview[19] was developed as a short and efficient diagnostic interview to be used in clinical as well as research settings. It follows DSM-IV and the ICD-10 criteria for psychiatric disorders, screening for 17 Axis I disorders, with brief suicidality and antisocial personality modules.

SCID-I-R: The Structured Clinical Interview for DSM–IV Axis I Disorders-Research Vers ion[20] is a well known semistructured interview that provides a framework upon which to make DSM-IV Axis I diagnoses. SCID-I-R was administered in this study in order to determine the validity of MINI diagnoses. The MINI and SCID-I-R were administered in a single session, and each subject was interviewed privately and independently.

Hamilton Depression Rating Scale (HDRS) [21]: Multiple choice questionnaire that used to rate the severity of a patient' depression.

Hamilton Anxiety Rating Scale (HARS)[22]: is a 14 item test measuring the severity of anxiety symptoms.

Mini Mental state Examination (MMSE) [23]: is used to screen for cognitive impairment. It is commonly used in medicine to screen for dementia. It is also used to estimate the severity of cognitive impairment at a given point in time and to follow the course of cognitive changes in an individual over time.

Statistical analysis:

The statistical analysis of data done by using excel program for figures and SPSS (SPSS, Inc, Chicago, IL). program statistical package for social science version 16. To test the normality of data distribution K-S (Kolmogorov-Smirnov) test was done, only significant data revealed to be nonparametric. N.B : all tested data revealed to be parametric. The description of the data was done in form of mean (+/-) SD for quantitative data. And Frequency & 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 compare between two groups. Paired sample t-test to compare one group at different times. Chi square test was used for qualitative data. N.B: P is significant if < or = 0.05 at confidence interval 95%.

Results

This study included a convenient sample of 105 patients with CHC receiving a standard course of peginterferon alfa 2a plus ribavirin for 48 weeks according to national Egyptian guidelines for treatment of CHC. None of the patients was suffered from any psychiatric disorders at the start of treatment of interferon.

Psychiatric side effects occurred in 28 patients (26.6%), mostly within the first 12 weeks, there was a high incidence of depression occurring in 20 patients (19%), 5 cases developed anxiety (3.8%), 3 patients (2.8%) developed life-threatening psychiatric symptoms (i.e. psychosis; one of them had suicidal ideation). Twenty three patients did not complete the study as 6 severe cases (3 depression, 3 anxiety) needed psychiatric treatment; they were excluded to avoid its effect on SVR, 3 cases of psychosis required early discontinuation of antiviral therapy), 3 cases were not available for final evaluation; and 11 cases were non responder so they stopped treatment after 24 weeks. The final group included 82 patients (55 males and 27 females), their age range from (35-52) years. Psychiatric disorders in the final group were depression (17 cases) and anxiety (2 cases).

Table 1 showed baseline characteristics of 82 patients included in this study. Their age ranged from 35-52 years. They were 55 men and 27 women, their mean body mass index (BMI) was 25.2±3.6 and they were scored according to Ishak scoring system into mild, moderate and severe fibrosis stages.

Table 2 showed tested risk factors for depression in the final studied group (82 patients) including age, gender, previous history of depression, ALT level, viraemia level, development of anaemia, fibrosis scoring and baseline psychiatric scoring The only factor that could predict psychiatric dis-

orders at 12 weeks of interferon therapy was the baseline psychiatric scoring HDRS, HARS and MMSE (P< 0.001***, P< 0.001***, P= 0.04* respectively).

Figure1 showed the changes in Hamilton depression rating scale (HDRS), Hamilton anxiety rating scale (HARS) and MMSE scores at baseline, after 12 weeks of treatment with interferon and at the end of treatment .Using paired sample t test., there was significant increase in the depression scores after 12 weeks of IFN treatment compared to baseline scoring (P< 0.001***). Also, significant increase in anxiety scores after 12 weeks was found (P= 0.005**). MMSE scores were decreased significantly after 12 weeks of IFN treatment (p= 0.02*).

Table 3 showed that IFN response rates were significantly higher in those patients who developed IFN-induced depression than in those who did not (sustained viral response (SVR) rates: 70% versus 41.1% respectively).

Table (1) : Base line characteristics of the studied group (82 patients)

Age		44±8.7 (35-52 years)
BMI		25.2±3.6
Gender Male/Female		55/27
Ishak scoring system for liver fibrosis	Mild 1-2	29cases (35.4%)
	Moderate 3-4	49cases (59.8%)
	Severe 5-6	4 cases (4.9%)

Table (2) : Risk factors for depression in the final studied group (82 patients).

Parameter		Depression (17)	No depression (65)	P value	OR 95% CI
Age		43.3±4.1	45.6±4.9	0.81	
Male gender		12/17 (70.6%)	43/65(66.15%)	0.72	1.23[0.34-4.6]
History of depression		4/17(23.5%)	6/65(9.2%)	0.1	3.03[0.6-14.8]
ALT		46.4±9.3	44.03±9.63	0.41	
PCR(IU/ml)		$670\times10^3\pm116\times10^3$	$704\times10^3\pm128\times10^3$	0.32	
Anaemia		9/17(52.9%)	33/65(50.7%)	0.87	1.09[0.33-3.61]
Ishak scoring	Mild (1-2)	9	20	0.21	2.53[0.75-8.61]
	Moderate(3-4)	7	42		0.38[0.11-1.29]
	Severe(5-6)	1	3		1.22[0.21-7.03]
Base line scoring	HDRS	9.2±4.3	6.53±3.4	<0.001***	
	HARS	14.8±2.8	11.62±2.6	<0.001***	
	MMSE	26.5±3.9	28.4±3.2	0.04*	

Figure (1) Follow up of psychiatric scores at base line, week 12 during antiviral therapy and at the end of treatment).

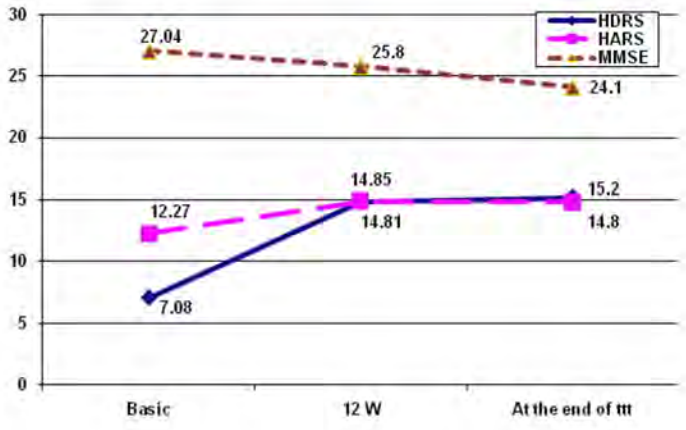

Table (3) : Relation between depression and SVR .

	Depression	No depression	P	OR 95% CI
SVR	12/17 (70%)	27/65 (41.1%)	0.04	3.2[0.9-11.9]

Discussion

Because newer agents for treatment of HCV may require coadministration of interferon, clinical knowledge about the diagnosis and management of psychiatric problems during interferon treatment of chronic hepatitis C will remain important over the next several years[24]. Antiviral treatment with interferon can be associated with several central nervous system changes, notably fatigue, anhedonia, depression, irritability, cognitive, disturbances, psychotic symptoms, delirious syndromes, relapse in alcohol or drug abuse, or suicidal thoughts and attempts[25]. Psychiatric adverse events particularly depression may lead to dose reduction and treatment discontinuation affecting the efficacy of interferon-based therapy. In addition, the patient's quality of life may be markedly reduced during treatment [26].

Previous studies have identified a number of risk factors that may be relevant to the development of depressive symptoms in at-risk populations, including age, gender, and history of depression and/or substance abuse, as well as baseline depression status [27,28, 29,30,31,32-35].

In this study, the only factor that could predict psychiatric disorders at 12 weeks of interferon therapy was the baseline psychiatric scoring HDRS, HARS and MMSE ($P<0.001$, $P<0.001$, $P=0.04$ respectively). These results were in accordance with previous studies,[28,29,36], they reported that depressive symptoms at baseline were associated with higher risk of major depression. A history of depression was not a risk factor in our study, although divergent observations in this regard have previously been reported[28,35,37]. Our study also comprised an analysis of other risk factors associated with the development of depression during HCV therapy including, age which was not a risk factor, in accordance with previous reports[28,37] but in contrast to the observations reported by Horikawa et al.[34]. Previous studies have yielded conflicting data with respect to female sex as a risk factor for development of interferon-induced depression[31,37,38]. In our study, gender was not found to be a predictor of the emergence of depression during HCV therapy.

Depressive ratings were independent of biochemical parameters (AST, ALT), HCV RNA and liver fibrosis stages. A previous study by Malyszczak et al,[39] reported that rise of depressive ratings was independent of any initial biochemical parameters. Also Dan et al,[36] found that Hepatitis C (HCV) infected patients have significant impairment in health-related quality of life (HRQL)scales which worsens during anti-viral therapy, and were not associated with ALT and HCV RNA levels.

Earlier studies show that antidepressive treatment may optimize the outcome of HCV therapy, in particular by reducing the risk of early treatment discontinuation[14,37] In this study we tried to exclude the effect of antidepressants on treatment outcome to detect if interferon-induced depression could be a predictive factor of sustained virological response (SVR).

Current data regarding possible associations between mood changes and response to antiviral treatment of chronic hepatitis C are controversial. In this study, a significant assosiation was found between incidence of depression and sustained virological response (SVR). Loftis and colleagues[14] reported that interferon-induced major depression was a predictor of a positive response to standard interferon alfa-2b plus ribavirin treatment in a prospective study including 39 patients. Overall, 38.5% of patients who developed major depression had an SVR compared with 11.5% of patients who did not develop major depression ($P < 0.05$). In addition, history of depression was not associated with SVR. However, in this trial the high rate of antidepressant treatment (33%) might be responsible for the improved virologic response rate in this group. By contrast, 3 other trials found different results. Leutscher et al.[40] found that approximately 30% of patients receiving IFN-based therapy developed major depressive disorder (MDD). They also reported that IFN-induced depression was associated with a reduced likelihood of achieving SVR. Raison and colleagues[15] followed HCV-infected patients (N = 102) treated with peginterferon alfa-2b and ribavirin and found that patients

with more depressive symptoms during treatment were less likely to have an SVR at the end of treatment. Likewise, Maddock and colleagues[16] prospectively studied 29 patients who received peginterferon alfa-2b and ribavirin for chronic hepatitis C, of whom 18 (64%) had an SVR. Baseline mental state did not predict response; furthermore, nonresponders had higher scores for fatigue and depression during treatment. On the other hand, a prospective study by Evon and colleagues[41] analyzed the relationship between baseline and new-onset depression and treatment outcomes, and the SVR rates did not differ between patients who developed MDD and those who did not. Another retrospective cohort study by Hauser and colleagues[42] supports these findings by comparing the rates of antiviral therapy completion and SVR between patients with hepatitis C virus and MDD and those without MDD. They found that the MDD group had completion and SVR rates similar to those of patients without MDD, in this study, all patients with MDD were on antidepressant medications before and during antiviral therapy.

Given these conflicting results, possible interactions between the development of depressive mood changes during antiviral treatment and SVR remain unclear.

Limitations of our study include the relatively small sample size, thereby limiting power to detect significant number of cases suffering from psychiatric disorders induced by IFN therapy other than depression. The relationship between depression and SVR observed in this study can't be generalized; multi-center study of HCV patients should be conducted to confirm this relationship. In conclusion, management of psychiatric events and cooperation with an experienced psychiatrist are crucial for treatment success. Significant association was found between depression and the efficacy of antiviral treatment in chronic hepatitis C. Interferon-induced depressive symptoms are important to be monitored and treated if necessary; they can be considered as an indicator of optimal dosing. rather than a predictor of SVR

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PSYCHIATRIC SIDE EFFECTS DURING PEGINTERFERON AND RIBAVIRIN THERAPY FOR CHRONIC HEPATITIS C : IS THERE A PREDICTIVE VALUE OF INTERFERON-INDUCED DEPRESSION?

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EFFECT OF PERIPUBERTAL CHRONIC STRESS ON EXPRESSION OF HYPOTHALAMIC KISS-1 mRNA AND HISTOPATHOLOGICAL CHANGES OF THE REPRODUCTIVE TRACT IN FEMALE RAT

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Abstract

Aim of the work: *Kiss-1 gene is involved in the regulation of gonadotropin secretion and in puberty onset. Kiss-1 mRNA expressing neurons are located in the hypothalamus. The main aim of the present study was to investigate whether exposure of female rats to repetitive stressors, while growing from weaning to adolescence, would affect the expression of hypothalamic kiss-1 mRNA and accrodingly alter the histology of the reproductive tract.*

Methods: *2 groups of Sprague-Dawley female pups (at age of 25 days) were assigned as control group (CR; n=10) that housed as usual and stressed group (SR; n=10) that was subjected to daily different stressors for 12 weeks. At the end of stress period, blood samples were taken for plasma cortisol, FSH and LH level determination. The brains, ovaries and uterine horns were dissected out for Kiss-1 mRNA determination and histopathological examination.*

Results: *Quantitative RT-PCR of hypothalamic kiss-1 mRNA revealed a significantly lower level in SR than in CR ($p<0.0001$). Serum cortisol was significantly higher in SR than in CR ($p<0.001$), while FSH and LH levels were significantly less in SR than in CR ($p<0.01$, 0.0001 respectively). The ovarian sections of SR showed significantly increased number of preantral and atretic follicles. The uterine sections of SR showed*

hyperplesia of the endometrium with polyps formation while some sections showed thinning and atrophy of the uterine wall.

Conclusion: *the present results suggest that peripubertal exposure to repetitive stressors, seems to markedly reduce the hypothalamic Kiss-1 mRNA expression that was associated with reduced gonadotropins secretion, polycystic ovarian histological changes and dysorganised uterine proliferative changes.*

Introduction

With the demanding life nowadays, exposure to either acute or chronic stressors may have its impact on health and well-being. Evidence from previous studies has documented the effect of stress on the reproductive function. Thus, an early study by Gonzalez et al.[1] has reported that exposing female rats to stressors such as tail pinch or cold stress led to persistent diestrus. On the other hand, others[2] found that chronic stress by foot shock initially affected body weights and elevated ACTH level but did not affect significantly estradiol, progesterone or the length of estrus cycle in Sprague Dawley female rats. By contrast, Baker and co-workers[3] reported a disruption in the cyclicity of estrus cycle with reduction in number of regular cycles in Long Evans and Sprague Dawley female rats following a three weeks of repetitive mild stressors. Despite the fact that the time of puberty is one of the most challenging periods of life, studies about how the pubertal development of hypopthalamo - pituitary gonadal axis is affected by exposure to stress are few and conducted mainly on male adult animals.

The primary driving force for the reproductive axis is known to be provided by Gonadotropin-releasing hormone (GnRH) cells of the hypothalamus as it regulates the biosynthesis and secretion of anterior pituitary gonadotropins; LH and FSH that by their turn stimulate ovarian function[4]. Recently, kisspeptins, the peptide products of the Kiss-1 gene that act via G protein-coupled receptor 54 (GPR-54), have emerged as an essential hypothalamic conduit for the generation of the preovulatory GnRH-LH surge, an event that occurs only in females[5]. Kiss-1 mRNA is expressed in different

brain areas; of them are the anteroventral periventricular nucleus (AVPN), the periventricular nucleus (PVN), and the arcuate nucleus of hypothalamus[6], in the pituitary gland and the placenta[7]. Many studies have shown that Kiss-1 mRNA expression increases at puberty in rodents and primates[8,9] indicating that the hypothalamic Kiss1-GPR54 system is a major player in the activation of the gonadotropic axis at puberty. In support of this, Navarro et al.[10] have shown that central administration of Kiss-1 peptide, induced precocious activation of gonadotropic axis in immature female rats. Also, kisspeptin administration has been shown to stimulate LH secretion in all phases of the estrous cycle in rats[11,12] .

Thus, the main aim of the present study is to investigate how prolonged exposure to a variety of stressors, before and after the onset of puberty, would affect the hypothalamic Kiss-1 mRNA expression in female rat. Further, the stress-related change in the histological structure of ovarian and uterine tissues was studied.

Materials & Methods

Animals & Experimental Groups :

Sprague Dawely Females' pups were obtained from breeding at the animal house, Medical Experimental Research Centre, Mansoura Faculty of Medicine, Mansoura University. During breeding, adult rats were fed normal rat chow ad libitum and were maintained on 12:12 h light-dark cycle and temperature was kept at 22^{o}C. After weaning (21 post natal day), female pups (n=20) were identified, weighted and isolated in four cages (5 per each) under the normal housing conditions. Then, at post natal day (PND) 25, pups were randomly divided into 2 groups; control non stressed rats (CR; n= 10), and stressed rats (SR; n= 10). Both groups were fed normal rat chow ad libitum. CR group was housed and manipulated as usual while SR group was housed in a separate room where they were subjected to chronic stress protocol for 12 weeks.

Starting from the post natal day 30, the animals were examined daily for vaginal opening as the external indicative of onset pu-

berty in female rat[13] The date of vaginal opening was recorded for each animal and then daily vaginal smears were performed and examined under light microscope for follow up of the cyclicity of 4-5 days estrous cycle {proestrus (nucleated epithelial cells), estrus (cornified cells), diestrus (leukocytes)}[13]. At the end of experimental protocol, vaginal smear was performed on the morning and animals were sacrified at estrus or ealry diestrus as the level of kiss-1 mRNA expression was found to be not significantly different during these two stages of estrus cycle in rat[12]. All procedures were approved by and conducted according to the ethical laws of Research Committee of Mansoura Faculty of Medicine.

Chronic Stress Model

The stressed female pups were subjected to to the following stressors in random order: continuous overnight illumination, 40^{o} cage tilt for 2 hours daily, damp bedding for 2 hours (300 ml water spilled into bedding), exposure to an empty water bottle immediately following a period of acute water deprivation[14]. The stressors were applied in a cyclic way and repeated each of the following weeks for a total of 12 weeks. Control animals are left undisturbed in the home cages with the exception of regular handling (i.e., regular cage cleaning and measuring body weight).

Specimen Collection

Rats were killed by overdose of sodium thiopental and trunk blood was collected on dry vials and left to clot. Then, serum was centrifuged, divided into aliquots and frozen at -20^{o}C until subsequent hormonal assay. Brains were immediately dissected out and placed in iced saline and then the hypothalamus and forebrain areas were snapped in liquid nitrogen for immediate RNA extraction. The hypothalamus is dissected out to a depth of approximately 3 mm with the following borders: the anterior edge of the optic chiasma, the anterior edge of the mammillary bodies, and the two hypothalamic sulci on either lateral side. Then, the ovaries and uterus were carefully dissected out and placed in 10% buffered neutral formalin for subsequent histological assessment .

*** Hormonal Assay**

Plasma cortisol was measured using ELISA kit[15]. Plasma FSH and LH were measured by immulite chemilluminescent immunometric assay[16] .

*** Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for brain areas**

Expression of kiss-1 mRNA by quantitative RT-PCR was evaluated in 2 brain areas; the hypothalamus; the target area for comparison between the two experimental gropus and the forebrain as reference control samples.

- Isolation of total RNA

DNA-free total RNA was purified from the brain tissues using RNA extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, Total cellular RNA was initially isolated from the tissue blocks by homogenizing in QIAGEN buffer . Each homogenate was further extracted for RNA using the QIAGEN RNeasy kit and treated with ribonuclease-free deoxyribonuclease I. The integrity of the RNA was checked by the visualization of the ethidium bromide-stained bands. The concentration of RNA was measured spectrophotometrically by absorbance at 260 nm in a Model Smartspec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

- Reverse transcription and real-time quantitative PCR

Total RNA (1 µg) from each preparation was transcripted and amplified in one step using 96-well plates on an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA) according to method described by[17]. Real-time PCR was performed in 25-µl reactions containing 5 µl total, 500 nm primer pairs, and 1 x SYBR green PCR master mix The primers for the PCR are rat $KiSS^{-1}$ (GenBank accession no.{"type":"entrez nucleotide","attrs":{"text":"AY196983","term_id":"31744922"}}AY196983; forward 5_-GCTGCTGCTTCTCCTCTGTGT-3_ and reverse 5_-CTGTTGGCCTGTGGGTTCA-3_; product size 88 bp) the probe 5_-(FAM,6-carboxy-fluorescein) TGCTTCTCCTCTGTGTGGCCTCTTTTGG- (TAMRA, 6-carboxytetramethyl-rhodamine)-3, respectively. The PCR cycling conditions were 95 C for 10

min, followed by 40 cycles at 95 C for 15 sec and 60 C for 1 min. Dissociation curve analysis was also done for each gene at the end of the PCR. In this regard, each amplicon generated a single peak and did not show any peak when the template was not included in the PCR. Additionally, each PCR product was electrophoresed onto a 2% agarose gel containing 0.5 µg/ml ethidium bromide, which showed a single band of the expected size. Expression levels were analyzed and normalized to the weight of the dissected tissue[18] .

- Data analysis

Data analysis of relative gene expression was performed with the Taq-Man SDS analysis software on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems), and the results were exported to Excel sheets for further processing. Fluorescence emission data were determined as CT values for each reaction and, for each sample, duplicate CT values were averaged. Gene expression was calculated as described[18] .

***Histopathological Evaluation**

Samples from the ovaries and uteruses were fixed in 10% buffered neutral formalin for at least 5 days. Then, the tissue was briefly washed with saline, passed through a series of ascending concentrations of ethanol and cleared with xylene. The samples were embedded in paraffin and sectioned at 5-µm thick. The sections from each paraffin block were stained with Hematoxylin and Eosin (H&E) for histopathological assessment and estrogen receptor-α (ERα) immunoperoxidase stain[19]. The sections then examined and photographed with Olympus light microscopy. Upon microscopic examination of the whole ovary, the presence and number of abnormal corpora lutea cysts, preantral, antral and atretic follicles were determined in order to assess reproductive function.

Statistical Analysis

All values were expressed as mean ± SEM. Real-time RT-PCR analyses were conducted in duplicate. Results were analyzed using unpaired student t-test (GraphPad Prism 5; Graphpad Software, Inc.,

USA). P< 0.05 was considered significant.

Results

Effect of chronic stress on body weights & Vaginal opening

At the end of stress protocol, body weights of SR (210 ±1.34 gm) was significantly less than that of CR (238.8 ± 2. 63 gm; p<0.0001, unpaired t-test; Table 1). Regarding the date of vaginal opening, it occurred at 37.1 ± 0.31 PND in the stressed group and at 39.3 ± 0.26 PND in the control group (p<0.001).

Plasma cortisol, FSH & LH Levels

Serum levels of cortisol, FSH & LH from control and stressed rats are shown in Table (1). Cortisol level was significantly higher in samples from stressed female rats when compared to that of CR (2.79 ± 0.31 vs. 1.43 ± 0.08 ng mL^{-1} respectively; p<0.001; unpaired t test). On the other hand, plasma level of FSH was significantly reduced in the SR (7.87 ± 0.33) than in CR (9.27 ± 0.22 ng mL^{-1}; p<0.01; unpaired t-test). Also, plasma LH level was significantly reduced in SR (0.85 ± 0.04 ng mL^{-1}) when compared with CR (1.731 ± 0.06 ng mL^{-1} ; p<0.0001; unpaired t-test).

Kiss-1 mRNA expression in the hypothalmus

Expression of $kiss^{-1}$ mRNA was detected in the hypothalamic areas from female rats in both groups, while it was not detected in the forebrain areas that were taken as a reference. The hypothalamic expression was confirmed by real time RT-PCR. The quantitative RT-PCR revealed a significantly lower level of kiss-1 mRNA in hypothalamic areas isolated from the stressed female rats (147.2 ± 32.24) when compared to that of control ones (2552 ± 202.3; p<0.0001; unpaired t-test; Fig. 1).

Histological assessment

ovarian histology

The ovaries of control rats showed healthy follicles with intact oocytes and normal granulosa, theca cells and corpus luteum (Figs. 2a, 2b & 2c). By contrast, the ovaries of the stressed rats showed a thick tunica albuginea (Fig. 2f & 2h), numerous atretic follicles and disorganized granulo-

sa cell layers. The atretic follicles were characterized by degenerating oocytes, partially or completely separated from the granulosa cells (Figs. 2d, 2e & 2f). The secondary follicles were numerous (Fig. 2g). The tertiary follicles were considerably distended and cystic (Fig. 2d). As shown in Table 2, the ovaries of the stressed group contained significantly more preantral ($p = 0.006$) and atretic ($p = 0.001$) follicles than did those of the control group. In addition, the stressed group showed a significantly increased number of abnoraml corpora lutea cysts ($p = 0.002$). No significant difference was observed in the number of antral follicles of the two groups ($p > 0.05$).

Uterine histology

Microscopic examination of uteri from the control group revealed normal endometrium, with normal glands lined with simple cuboidal epithelium. The glands appeared in the sections as round, oval or elongated with a narrow lumen, and had no branches or daughter glands (Figs. 3a, b). on the other hand, sections from stressed rats revealed cystic endometrial glands with more than average or large size (Fig. 3f); glands with daughter glands and glands forming conglomerates with a very complex architecture in which individual glands are closely disposed to each other almost without intervening stroma and have multiple interconnecting lumens (Fig. 3c). Some sections showed disordered proliferation of the endometrium in the form of hyperplesia of the endometrium with polyps formation (Figs.3, d & e), while some sections showed thinning and atrophy of the uterine wall (Fig. 3e).

ER immunostaining

In the ovary of both groups, some scattered positive cells were observed among granulosa and thecal cells in all the stages of the follicle. Positive immunostaining for ER was also found in granulosa-lutein cells of corpora lutea. No positive ER immunostaining was found in the stromal cells, or the theca lutein cells or in the cells in the center of the newly formed corpus luteum (Figs. 4a, b). The staining was more intense in the stressed group in comparison to the control.

ER immunostaining was observed in the stroma, epithelium and glands of the uterus of the control group Fig. 4c), while it was less immunoreactive in tissue from the stressed group (Fig. 4d).

Table (1) : Body weight (gm) and plasma concentrations of cortisol, FSH & LH (ng /mL) in control (CR) and stressed female rats (SR) (*n* =10 each).

	Body weight	Cortisol	FSH	LH
CR	238.8 ± 2.63	1.43 ± 0.08	9.27 ± 0.22	1.731 ± 0.06
SR	210 ± 1.34***	2.79 ± 0.31**	7.87 ± 0.33**	0.85 ± 0.04***

Values are mean ± SEM. *, **, ***; p<0.05,0.001, 0.0001 respectively; stressed rats vs. control rats (unpaired t-test).

Table (2): Comparison of number of various follicles and corpora lutea in ovaries from control rats (CR) and stressed female rats (SR) (n =10 each).

	Preantral follicles	Antral follicles	Atretic follicles	Corpus luteum cysts
CR	1 ± 0.26	1.6 ± 0.2	0.2 ± 0.16	0.2 ± 0.16
SR	6.6 ± 1.73 ***	2.1 ± 0.52	2 ± 0.42 **	3.1 ± 0.75 **

Values are mean mean ± SEM. **, ***: p<0.01, 0.001 respectively; stressed rats vs. control rats (unpaired t-test).

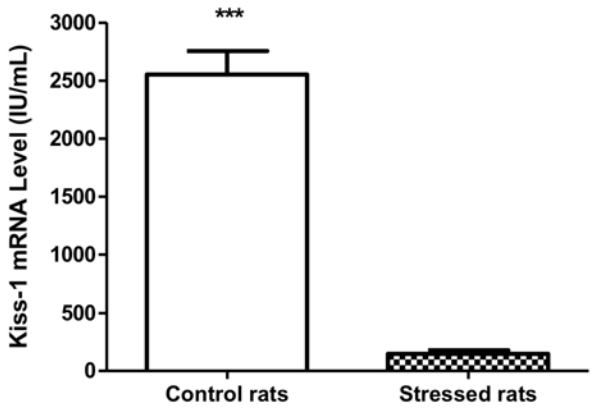

Fig. 1: Quantitative value of hypothalamic *kiss-1* mRNA level in control and stressed female rat (n=10 each). Values are mean ± SEM. *;p<0.0001 vs. stressed rats (unpaired t-test).**

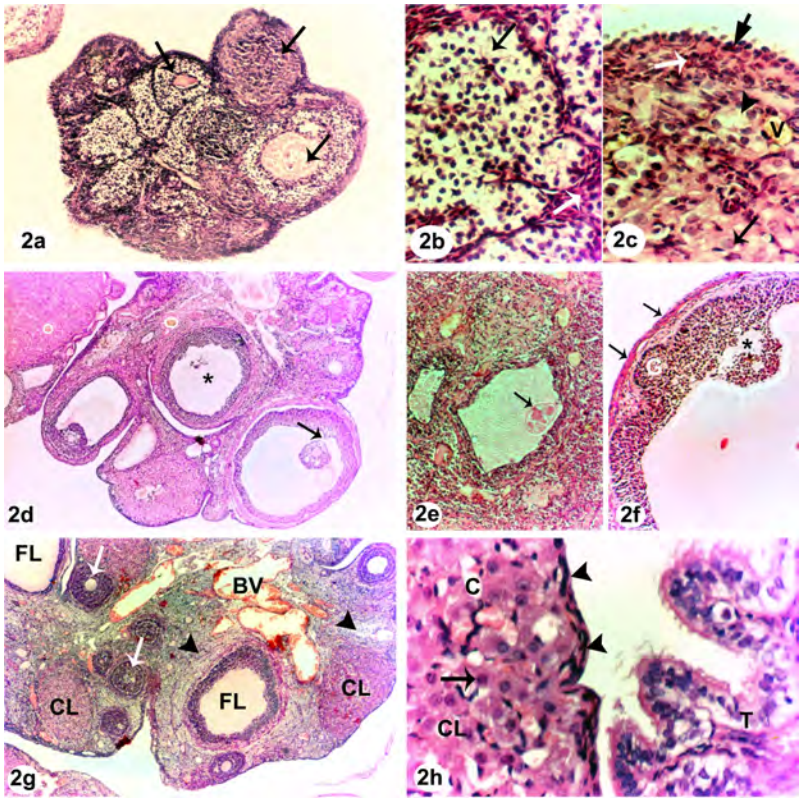

Fig. (2): Photomicrographs of the rat ovary, control (a-c), stressed (d-h)
a- Section showing many corpora lutea at different stages of development (arrows). **b-** Sections showing the degenerating corpus luteum. The granulosa-lutein cells have pyknotic nuclei and abundant cytoplasmic lipid (arrow). The theca-lutein cells (white arrow) form the outer covering and ensheath the septa that extend into the center of the corpus luteum. **c-** Sections showing the germinal layer of surface epithelium (short arrow), large polygonal granulosa-lutein cells (arrow) with abundant, pale, eosinophilic cytoplasm, some cells contain lipid droplets (arrowhead). The theca-lutein cells (white arrows) appear small; contain a round to oval nucleus with a less abundant, more darkly staining cytoplasm and form the irregular outer covering of the corpus luteum. They ensheath the septa which contain the blood vessels (V). **d-** Section showing many tertiary atretric follicles, with degenerated ovum (*). Note its detachment from the granulose cell layer (arrow). **e-** Section showing irregular atretric follicle with degenerated ovum (arrow). **f-** Section showing atretric tertiary follicle, note the thick tunica albuginea (arrows), irregular granulose cells layer (G) and degenerated ova (*). **g-** Section showing many secondary follicles (arrows), early formed corpora lutea (CL), corpora albicans (arrowheads) and folliculo-luteal cyst (FL). Note the dilated blood vessels (BV). **h-** Section showing early formed corpus luteum (CL) with large polygonal granulosa-lutein cells (arrow) with abundant, pale, eosinophilic cytoplasm, and a large rounded nucleus with prominent nuclei. Note the thick tunica albuginea (arrowheads) and rich capillaries (C) in between the granulosa lutein cells. Note the vacuolization of the tubal epithelium and the thick irregular cilia (T). (Stain: Hx & E; Magnification: (d, g) X40 (a, e, f) X100 (b, c, h) X400).

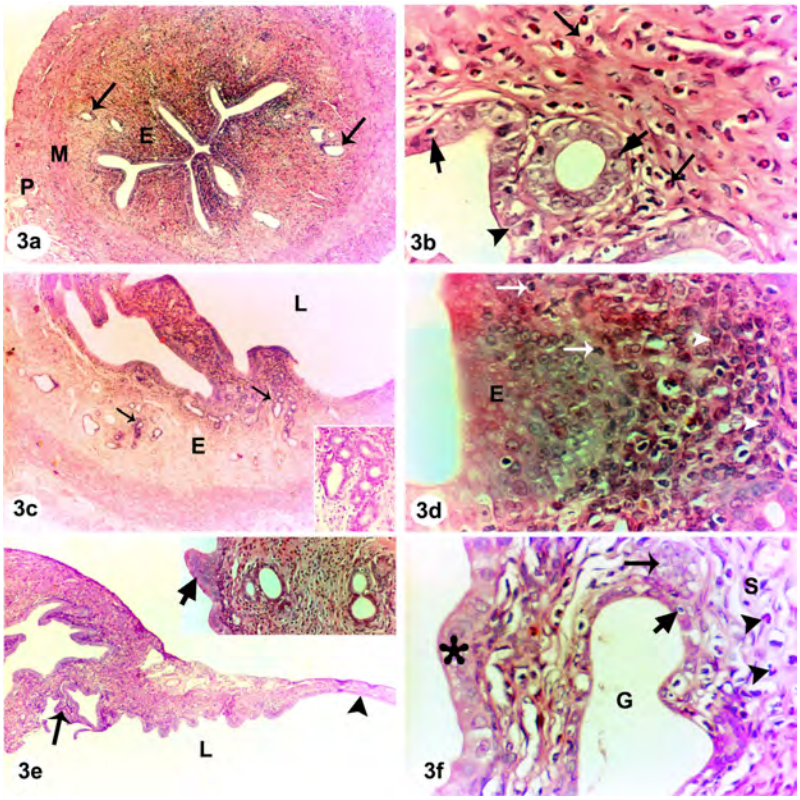

Fig. (3): Photomicrographs of the uterus, control (a, b), stressed (c-f)
a- Section showing its layers, endometrium (E), myometrium (M) and the loose connective tissue layer the parametrium (P). Note the endometrial glands are simple with narrow lumen (arrows). **b-** High magnification of the endometrium of the photograph above showing the surface epithelium formed of columnar cells with vacuolar degeneration (arrowhead) and apoptosis (short arrows) are frequently observed within the luminal and glandular epithelium, but mitotic figures are rare. Large numbers of polymorphonuclear cells (arrows) infiltrate the lamina propria and endometrial glands. **c-** Sections showing disordered proliferation of the endometrium which is a sign of anovulatory cycles. There is nonsynchronous growth of the glands (arrows) in relation to the stoma and papillary metaplesia of the endometrium (E). Note the dilated lumen (L). Inset; showing the proliferative glands. **d-** Higher magnification showing some degree of pseudostratification of the surface epithelium (E) with increase in mitotic activity (arrows). Note the plump, round to oval stromal fibroblasts (arrowheads). **e-** Section showing widening of the lumen of the uterus (L), atrophic endometrium and thinning of the wall of the uterus on the right side (arrowhead) and disordered proliferation of the endometrium with nonsynchronous growth of the endometrium with papillary projections within the lumen on the left side (arrow). Inset; showing cystic dilatation of the glands. The uterine polyp is formed of pseudostratified epithelium (short arrow). **f-** Higher magnification of the atrophic endometrium composed of a single layer of cuboidal cells (*). Mitotic figures are absent and the nucleus-to-cytoplasm ratio is high. There is cystic dilatation of the gland (G) with abnormal budding (arrow). The gland is lined by low cubical epithelium with increased apoptosis (short arrow). The stroma cells are spindled shape with pyknotic nuclei (S). Note the leukocyte infiltration (arrowheads). (Stain: Hx & E; Magnification: (a, c, e) X40 (b, d, f) X400 (Insets) X100).

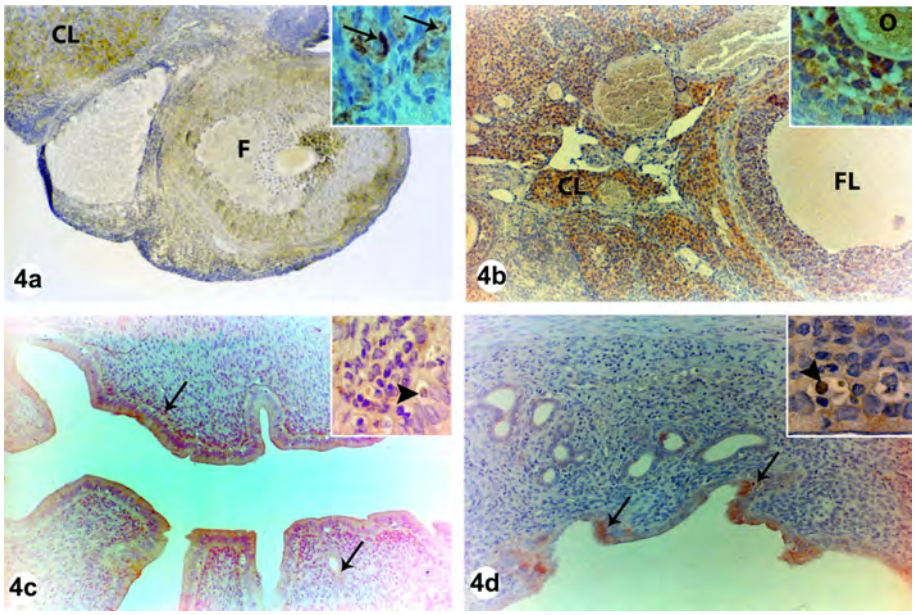

Fig. (4): Expression of estrogen receptors in rat ovary and uterus
a- Section of the control ovary showing some immunoreactive granulosa and theca cells of the tertiary follicle (F), immunoreactivity is also observed in the granulosa-leutin cells of the corpus luteum (CL). Inset; showing the immunoreactivity is observed both in the nucleus and in the cytoplasm of the granulose lutein cells (arrows) while the theca lutein cells were negative. **b-** Section of the stressed ovary showing immunoreactive cells of the atrophic corpus luteum (CL), in few granulosa-lutein and theca-lutein cells of the folliculo-luteal cyst (FL). Inset; showing the immunoreactivity is observed in some granulosa cells surruounding the ovum (O) of the secondary follicle, note that the theca cells are negatively stained. **c-** Section of the control uterus showing some immunoreactive cells in the stroma, endometrial epithelium and the glands (arrows). Inset; showing positive cells in the stroma and in the apoptotic endometrial epithelium (arrowhead). **d-** Section of the stressed uterus showing few immunoreactive cells in the endometrial epithelial cells (arrows). Inset; showing positive cells in the apoptotic cells of the endometrial epithelium (arrowhead). (Stain: Anti ER immunoperoxidase; Magnification: X100, insets; X400)

Discussion

The present study, to our knowledge, represents the first attempt to investigate the effect of peripubertal repeated exposure to different stressors on the expression of hypothalmic kiss-1 mRNA and correlating this with the histological changes in the reproductive system in female rat. Thus, the current results provides evidence that exposure of female rat to repetitive stressors, while growing from weaning to adolescence, lead to significant reduction in expression of hypothalamic kiss-1 mRNA and evident histopathological changes in the reproductive tract. The repetitive exposure to stressors resulted in a significant reduction in body weights of SR, when compared to that of CR. On the other hand, the date of vaginal opening, the external index of puberty onset, in the stressed rats (37.1 ± 0.31 PND) was not delayed and even occurred earlier when compared with that of CR (39.3 ± 0.26 PND; $p<0.001$). However, it was in the normal range recorded for vaginal opening in rats[13], and therefore it is difficult to speculate whether it is stress-related actual acceleration of puberty onset or not. Also, the present study showed that the SR had regular cycles after the onset of pubery (as followed by vaginal smears) suggesting no effect of stress, from post weaning (25 PND) to date of vaginal opening (37 PND), on the neuronal mechanisms contributing to the onset of puberty and the attainment of cyclicity. However, the significant molecular and histological findings observed at the end of experimental protocol suggests that these changes are related to the longer postpubertal exposure to stress. On the other hand, the higher serum level of cortisol in SR correlates with previous findings that chronic stress is associated with a significant activation of hypothalamo-adrenal axis . Recently, Nemeto et al.[20] reported that the activation of hypothalamo-adrenal axis in response to stress might contribute to gonadal dysfunction through the pituitary corticotrophs secreting urocortin 2 that may supress gonadotropin secretion. Such possibility was not tested in the present study but it seems interesting to investigate such link in future experiments.

In line with the significant reduction of kiss-1 mRNA expression, the hormonal assay results showed a marked reduction in mean plasma levels of FSH and LH in stressed female rats indicating the disruption in hypothalamo-pituitary axis (HPA) that regulates their secretion. Furthermore, the ovarian histology of stressed rats showed increased number of ovarian cysts of many types within the ovarian cortex with many atretic follicles which is a sign of anovulation as reported by Willoughby et al [21]. In correlation with this disrupted ovarian histology, there was disordered proliferation of the endometrium with non synchronous growth of the endometrium with papillary projections confirming further the disruption in phasic release of gonadotropins that resulted in such adverse effects on the morphology of the reproductive tract in the stressed rats. The role of kisspeptins, peptides ligands coded by kiss-1. in mediating LH surge in female rat was reported by Kinoshita et al.[22] who showed that injection of anti-rat kisspeptin antibody blocked spontaneous preovulatory LH surge. Also, Smith et al.[23] proposed that hypothalamic kisspeptin neurons are involved in regulating tonic LH release. Thus, it seems that repeated exposure to different stressors led to a reduction in kiss-1 mRNA with subsequent reduction in LH secretion and such ovarian morphology indicative of anovulation. However, the presence of signs of folliculogenesis in the stressed rats, though disturbed, represents a marker of of partially patent FSH derive for follicular phase and hence estrogen secretion. Indeed, FSH level was not reduced markedly as was the case with LH. In fact, previous studies have indicated that the gonadotropin secretion is regulated by a complex neuronal mechanisms that might differently affect LH & FSH responsivness to GnRH input[24, 25]. In line with this, Navarro et al.[10] suggested a dissociated role of Kiss-1 system in regulating gonadotropin secretion such that Kiss-1 peptide preferentially stimulate LH release.

Expression of hypothalamic Kiss-1 mRNA has been found to be regulated by estrogen as ER alpha colocalise in Kiss-1 mRNA

positive neurons[12]. Also, Navarro et al.[26] have demonstrated that hypothalamic kiss-1 expression increased significantly after gonadectomy and that increase was prevented by sex steroid replacement in male and female rats. Thus, this negative correlation between estrogen and hypothalamic kiss-1 expression might be a possible mechanism for the current stress-related reduction in kiss-1 mRNA. In the present experiments, the plasma estrogen level was not measured as the focus was on FSH and LH as an index for HPA. But in view of the evidence that polycystic ovary syndrome is characterised by a high estrogen level and low progestrone level[27], one might expect a high estrogen level that might negatively regulated kiss-1 mRNA expression. In support of this, the higher ER immunoreactivity observed in ovarian sections from SR as estrogen has been reported to upregulate ER mRNA[28]. Indeed, Navarro et al.[26] has shown that neonatal exposure to high dose of estrogen led to a persistent reduction in expression of hypothalamic Kiss1 mRNA and LH level in young adult rat. Furthermore, Tsai et al.[29] have indicated that disruption in the neural mechanisms controlling GnRH could lead to abnormal gonadotropic stimulation of the ovary resulting in follicular disruption suggesting that it is an estrogen specific effect.

The present experiments focused on the effect of stress on HPA in female animals by adoption of different stressors for a relatively long period to simulate what is happening in real life when girls around age of puberty can be exposed to different daily stressors. Thus, the stressors applied varied from severe psychological one like water deprivation followed by exposure to an empty bottle, to a milder physical cage tilt. Earlier studies about the effect of stress on HPA mainly adopted restraint stress as psychological and physical stressor and found either no change in mean value of LH or an increase or initial increase followed by reduction[30,31,32]. on the other hand, a more recent study has shown that LH pusle reduction in response to repeated restraint stress (60 min for 4 times each, at 6 day intervals) varied according

to rat species in that Fisher and Lewis rats showed complete habituation by the 4th session while wistar rats showed no habituation[33]. No studies, to our knowledge, have investigated the effect of peripubertal repetitive stressors on kiss-1 mRNA expression.

In conclusion, the present study provides an evidence that peripubertal exposure of female rat to repetitive stressors, as they grow from weaning to adolescence, seems to markedly reduce the hypothalamic Kiss-1 mRNA expression that was associated with polycystic ovarian histological changes, and dysorganised uterine proliferative changes. It seems interesting to study, in the future, the link between kiss-1 expression and fertility problems in women, especially those described as being primary infertile with no obvious reason.

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EFFECT OF PERIPUBERTAL CHRONIC STRESS ON EXPRESSION OF HYPOTHALAMIC KISS-1 mRNA AND HISTOPATHOLOGICAL CHANGES OF THE REPRODUCTIVE TRACT IN FEMALE RAT

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INFLUENCE OF CELL-CYCLE REGULATORY GENES IN HCV POSITIVE BLADDER CANCER PATIENTS

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Abstract

Objective: *This work was carried out to investigate the possible role of HCV infection in pathogenesis of bladder cancer and its impact on expression of cell cycle regulators P53 and P21 genes as well as to correlate the findings with clinicopathological parameters (histopathogical grade and staging).* ***Materials and Methods:*** *Participants were 80 patients (10 females and 70 males). Group I: 10 (age range 17-80), with normal bladder urothelium with HCV -ve served as control group. Group II: 10 patients (age range 46-65) with normal bladder urothelium with HCV positive: Group III: 30 malignant patients (age range 44-75) without HCV. Group IV: 30 malignant patients (age range 45-73) (with HCV).* ***Results:*** *Tumors associated with HCV infection were TCC rather than SCC, high grade rather than low grade, invasive tumors rather than non-invasive tumors. Altered immunoreactivity for p53 protein accounted for 43.33 % in bladder tumors not associated with HCV, and 80 % in bladder tumors associated with HCV. p21 immunostaining positivity was 30% in normal urothelium associated with HCV, 60% in bladder tumors not associated with HCV, and 23.33% in bladder tumors associated with HCV. p53 expression by real time PCR was significantly increased in bladder tissues associated with HCV when compared to normal urothelium in non-HCV infected subjects. There was a significant positive correlation between HCV infection and p53 expression in contrast to p21 which showed significant negative correlation with HCV.* ***Conclusion:*** *HCV infection was associated with TCC of high grade. HCV may play a significant role in the development of bladder*

cancer through increasing the expression of p53 and decreasing the expression of p21 genes.

Introduction

Elucidation of molecular pathways involved in urothelial carcinoma of the bladder is essential for understanding of carcinogenesis and disease progression. Mutations of cell cycle regulatory genes are the genetic alterations most commonly found in human neoplasia, including bladder cancer[1-4]. Alterations in the p53 and retinoblastoma (RB) tumor suppressor genes have been shown to play an important role in the development of urothelial carcinoma. However, downstream pathways that contribute to urothelial transformation are not completely defined and are key factors too [5-6]. Members of the kinase inhibitor protein (KIP) family, p21WAF1/CIP1 and p27Kip1 are both p53-inducible and p53-independent cyclin-dependent kinase inhibitors that can arrest the cell by inhibiting DNA replication. Alteration of p53, pRB, p21 and p27 expression has prognostic significance in patients treated with radical cystectomy for bladder cancer [7-15].

Pagano et al.[16] came to the finding that association between a given virus and a specific malignancy is ranged between 15% and 100%. This association range varies according to the virus, the cancer and the geographic location. The author pointed out that viruses are one of the potential causes of various types of malignancies.

As for hepatitis C virus (HCV), many investigators[17-18] considered this virus as an etiologic agent of hepatocellular carcinoma (HCC). The lifetime risk of HCC in patients chronically infected with HCV is estimated to be between 5% to 20%. Hepetitis C virus infects not only in hepatocytes, but also infects and proliferates in various other cells[19-24]. In this concern, Nagao et al.[25] proved the presence of a relationship between HCV infection and oral squamous cell carcinoma. In a recent study, Gordon et al.[26] found high risk (nearly double) for renal cell carcinoma in patient with chronic hepatitis C infection.

So, the present study was planned to find if there is a possi-

ble role for HCV infection in the pathogenesis of bladder cancer. Such correlation was tested by investigating the impact of HCV on the expression of p53 and p21 regulatory genes that are known by their role in the development of bladder cancer. Findings were correlated with clinicopathological parameters including histopathogical grade and staging of the tumor.

Subjects and Methods

Subjects:

This study involved 80 subjects (10 females and 70 males) admitted to the urology and nephrology center during the period from Jan 2009 to Jan 2010. Subjects were divided into four main groups: Group I: 10 HCV negative subjects with normal bladder urothelium served as control group. Group II: 10 HCV positive subjects with normal bladder urothelium. Group III: 30 HCV negative patients with bladder cancer. Group IV: 30 HCV positive patients with bladder cancer. Patients with history of smoking, and those with chronic cystitis and +ve Bilhariaziasis were excluded in this study.

Prior to the procedure all patients were subjected to full clinical examination, routine laboratory investigations (liver function test and serum creatinine), complete urine analysis, abdominal and pelvic ultrasonography, general and abdominal examination, digital rectal examination (DRE), bimanual examination under anesthesia, plain X-ray of the urinary tract, intravenous urography (IVU), cystoscopy and transurethral resection (TUR) biopsies were taken from apparent growths. HCV infection was assessed by detection of HCV antibodies by ELISA assay and HCV RNA by PCR. Also serum creatinine was measured.

Tumor specimens were taken by cystoscopy (transurethral resection biopsies, TUR) and cystectomy. The control normal urothelium was collected by transurethral resection biopsy(TURB). The study protocol was approved by the Ethical Committee of TBRI according to the Institutional Committee for the Protection of Human Subjects and adopted by the 18th World Medical Assembly, Helsinki, Finland. An informed consent from

all patients who underwent cystoscopy and biopsy from apparent growth and lesions was taken.

Methods

Routine histopathologic examination:

Each bladder biopsy specimens from malignant tissues and normal urothelium were divided into two parts: a small fresh part was frozen for PCR and the large portion was fixed in 10% formalin. The paraffin blocks were retrieved and 3 μm thickness sections were prepared for routine H&E. Other sections were prepared on coated slides for immunohistochemistry. In all cases a histopathological diagnosis was made according to the World Health Organization (WHO) histological classification of urothelial tumors [27].

Immunohistochemistry :

Deparaffinized sections of all cases were incubated for 30min with 0.3% hydrogen peroxide in methanol and microwave heated in citrate buffer (pH 6.0) for 20 minutes. Subsequently, an indirect immunoperoxidase technique was applied, using monoclonal antibodies for p53 (monoclonal mouse anti-human antibody DO-7; Dako, Carpinteria, CA; dilution 1:4000), p21 (monoclonal mouse anti-human, SX118, Dako; dilution 1:2000. Immunostaining was performed using ImmunoPure Ultra-Sensitive ABC Peroxidase (Thermo Scientific Cat. # 32052), with (DAB) as chromogen. Positive control is breast carcinoma for p53 and colorectal carcinoma for p21.

Interpretation of immunohistochemical staining :

Markers were placed in one of two categories, altered or normal. Nuclear p53 immunoreactivity was considered altered when samples demonstrated at least 10% nuclear reactivity. p21 immunoreactivity was considered altered when samples demonstrated no detectable or only very low levels of p21 nuclear staining[15]. Quantitative assessment was done in five different fields at a magnification of 400X. Labeled cells were expressed as a percentage of tumor cells with positively stained nuclei divided by the total number of tumor cell nuclei counted [28].

Quantitative real time polyme-

rase chain reaction (q PCR) for p53 and p21 genes

RNA extraction and cDNA synthesis :

The total RNA from frozen tumor and corresponding non-cancerous tissue specimens was isolated by disruption of 50-100 mg tissues in 1 ml of Trizol (Invitrogen Corporation, Grand Island, NY, USA). RNA was quantified spectrophotometrically, and its quality was determined by agarose gel electrophoresis and ethidium bromide staining. Only samples that were not degraded and showed clear 18 S and 28 S bands under ultraviolet light were used for real-time RT-PCR. Reverse transcription was done using 1 µg total RNA and a cDNA kit (high-capacity cDNA archive kit.

Primers and probes :

The primers and probe were purched from Applied Biosystems. All of primers and probes are depicted in table 1.

The reaction was performed for in a total volume of 50µl containing 25 µl From 1x TaqMan® Universal PCR with 25 µl from 20X TaqMan® Gene Expression Assay Mix and 22.5 µl of cDNA diluted in RNase-free water.The cycling parameters were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation 95°C for 15 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute. Data analysis was carried out using ABI prism 7000 by equation 2-$\Delta\Delta$ ct [29].

Statistical analysis :

All statistical analyses were done using Statistical Program for Social Sciences (SPSS 16.0). Descriptive statistics were done.

Table (1): Sequences of primers and probes used in this study.

Genes	Forward primer	Reverse primer	Probe
TP53	CGTCTGGGCTTCTTGCATTC	AAGACCTGCCCTGTGCAGC	5′ FAM- CTGTGACTTGCACGTACTCCCCTGCC-(TAMRA) - 3′
P21	CTGGAGACTCTCAGGGTCGAAA	GAGGAAGCCTAATCCGCC	5′ FAM-CGGCAGACCAGCATGACAGATTTCTACCA-TAMRA - 3′
GAPDH	GAAGGTGAAGGTCGTAGTC	GAAGATGGTGATGGGATTTC	5′ (FAM) CAAGCTTCCCGTTCTCAGCC-TAMRA - 3′

TP53, **P21**, **GAPDH** (Glyceraldehyde-3- phosphate dehydrogenase).

Quantitative data were tested for normality and found to be of nonparametric distribution. Qualitative data was presented as number and percent. Analytic statistics were done to test statistical significance in relation between different variables. Chi-square test of independence was used for evaluating the significant association of histopathology type, tumor grade, tumor invasiveness, staging, immunohistochemical staining of tumor for p53 protein and p21 with HCV infected and non-HCV patients. Pearson´s correlation was used to measure the relation between 2 variables. A significant correlation between two variables was taken at the 95% confidence interval. Comparison between means of different groups was done using one way ANOVA.

Results

The histopathological features of HCV-associated and non-HCV-associated bladder cancers:

Chi square test for comparing the pathological criteria between HCV associated bladder cancer vs non-HCV bladder cancer revealed that bladder tumors with HCV infection were significantly associated with TCC rather than SCC, high grade rather than low grade ($p< 0.05$). Bladder tumors with HCV infection were associated with invasive tumors rather than non-invasive tumors, late stage than early stage but unfortunately, not reaching a statistically significant value ($p> 0.05$). At the same time, there is no association between HCV-associated tumors and non-HCV-associated tumors and lymophvascular invasion and the presence of positive LN ($p> 0.05$) (Table 2).

Effect of HCV infection on p53 protein in normal and malignant bladder tissues :

Table 3 demonstrated p53 protein immunoreactivity in all studied groups. The normal urothelium from subject without HCV infection showed no immunoreactivity for p53 protein, while altered p53 protein immunoreactivity is 30% in normal urothelium associated with HCV, 43.33 % in bladder tumors not associated with HCV, and 80 % in bladder tumors associated with HCV ($p< 0.001$) (Figure 1). Quantitative real time

PCR showed minimal expression of mRNA of p53 gene in normal urothelium from subjects have no HCV infection, HCV infection significantly increased p53 gene expression in normal endothelium ($p< 0.001$). Also, p53 mRNA expression was significantly increased in tumors tissues from patients having HCV infection when compared to tumors from patients have no HCV infection (Table 3 and Figure 2).

Effect of HCV infection on p21 in normal and malignant bladder tissues :

Table 4 shows p21 immunostaining and the relative expression of p21 gene in normal and malignant bladder urothelium. Immunohistochemistry of p21 in bladder tissue sections of the 80 cases showed maximal p21 positivity in normal urothelium from subjects without HCV, p21 positivity was decreased (30%) in normal urothelium associated with HCV, and it was 60% in bladder tumors not associated with HCV with, and 23.33% in bladder tumors associated with HCV ($p< 0.001$). p21 gene expression by real time PCR was significantly decreased in bladder tissues associated with HCV when compared to normal urothelium in non-HCV infected subjects ($p< 0.001$). Also, this expression became significantly decreased in bladder tumors associated with HCV infection when compared to that from non-HCV infected patients ($p< 0.001$) (Table 4 and Figure 4).

Correlations between p53 and p21 expression and HCV infections, histopathogical type, grade and invasiveness

Results of the statistical analysis showed that there is a positive correlation between HCV infection and p53 expression in contrast to p21 which showed negative correlation with HCV. Also, p53 expression showed positive correlation with TCC, while it had a negative correlation with grading and invasiveness of the tumour. On the other hand, p21 displayed significant negative correlation with HCV infection, and TCC, while, it showed positive correlation with the grade and invasiveness of bladder cancer (Table 5).

Table (2): Histopathological features of bladder cancer in HCV-infected patient vs Non-HCV patients

Criteria	HCV infected patients	Non-HCV patients	P value
Histopathological type • **TCC** (44) • **SCC** (16)	 26 (59.09%) 4 (25%)	 18 (40.90%) 12 (75%)	0.020
Tumour grade • **GI** (16) • **GII** (26) • **GIII** (18)	 4 (25%) 14(53.85%) 12 (66.67%)	 12 (75%) 12 (46.15) 6 (33.33%)	0.046
Tumour invasiveness • **Non-invasive** (23) • **Invasive** (37)	 10 (43.48%) 20 (54.05%)	 13 (56.52%) 17 (45.95%)	0.426
Lymphovascular invasion 7- ***Absent*** (37) 8- ***Present*** (23)	 17 (45.95%) 13 (56.52%)	 20 (54.05%) 10 (43.48%)	0.426
Lymph Node (LN) • ***Absent or negative LNs*** (46) • ***Positive LNs*** (14)	 21 (46.67%) 9 (60 %)	 25 (54.33%) 5 (40 %)	0.222
Tumour staging • **Stage (I)** (23) • **Stage (II)** (14) • **Stage (III)** (9) • **Stage (IV)** (14)	 10 (43.48%) 6 (42.86%) 5 (55.56%) 9 (64.29%)	 13 (56.52%) 8 (57.14%) 4(44.44%) 5 (36.71%)	0.587

Table (3) : Effect of HCV infection on P_{53} expression in normal and malignant tissues.

	Immunohistochemistry of P53 protein		$2^{-\Delta\Delta}$ ct of P53 by RT-PCR
	No. of +ve cases		Mean ± SD
	No.	%	
Group I (Normal urothelium without HCV) (n=10)	0	0 %	0.04 ± 0.004
Group II (Normal urothelium with HCV) (n=10)	3	30 % [a]	1.75 ± 0.25[*]
Group III (Malignant patients without HCV) (n=30)	13	43.33 % [b]	3.35 ± 0.35[#]
Group IV(Malignant patients with HCV) (n=30)	24	80 % [a, b]	13.49 ± 0.58[*#]

[*]$p < 0.001$ compared to non-HCV infected patients, [#]$p < 0.001$ compared to normal urothelium (One-way anova test). [a]$p < 0.001$ compared to non-HCV infected patients. [b]$p < 0.001$ compared to normal urothelium (Chi square test).

Table (4) : Effect of HCV infection on p21 in normal and malignant tissues

	Immunohistochemistry of p21 protein		2-$^{\Delta\Delta}$ ct of p21 by RT-PCR
	No. of +ve cases	%	Mean ± SD
Group I (Normal urothelium without HCV) (n=10)	10	100%	9.76 ± 0.48
Group II (Normal urothelium with HCV) (n=10)	3	30% [a]	4.95 ± 0.69 [*]
Group III (Malignant patients without HCV) (n=30)	18	60% [b]	3.53 ± 0.37 [#]
Group IV(Malignant patients with HCV) (n=30)	7	23.33 [a,b]	0.89 ± 0.064 [*#]

*$p < 0.001$ compared to non-HCV infected patients, #$p < 0.001$ compared to normal urothelium (One-way Anova test). [a]$p < 0.001$ compared to non-HCV infected patients. [b]$p < 0.001$ compared to normal urothelium (Chi square test).

Table (5) : Correlations of p53 and p21 protein to HCV infection, urothelial carcinomas, grade, and invasiveness

	r = correlation coefficient	p value
• p53 expression vs HCV infection	0.730	0.000
• p21 expression vs HCV infection	-0.567	0.000
• p53 expression vs TCC	0.283	0.029
• p21 expression vs TCC	-0.328	0.010
• p53 expression vs grade	-0.545	0.000
• p21 expression vs grade	0.597	0.000
• p53 expression vs invasiveness	- 0.040	0.764
• p21 expression vs invasiveness	0.048	0.716

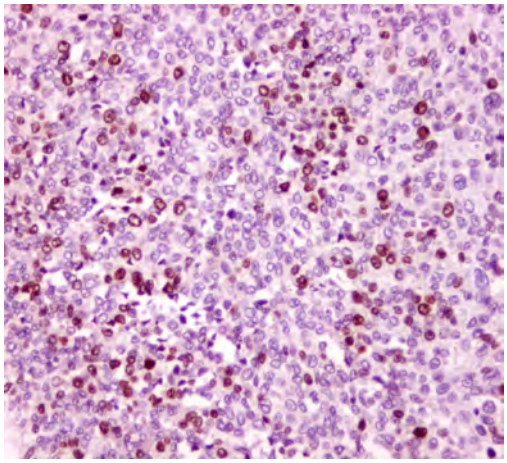

Fig. (1): Transitional cell carcinoma with nuclear immunoreactivity for p53 in more than 10% of tumor cells (Immunoperoxidase DAP X 200).

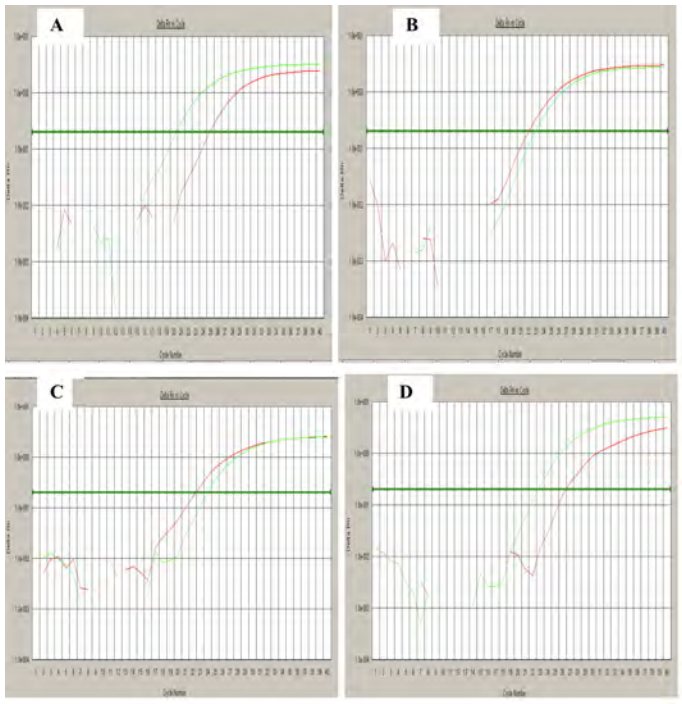

Fig. (2): Curves of real time PCR for p53 gene and control gene (GAPDH) in different groups ; healthy control group without HCV(A), HCV patients without bladder cancer (B), patients with bladder cancer without HCV(C) , and bladder cancer with HCV(D).

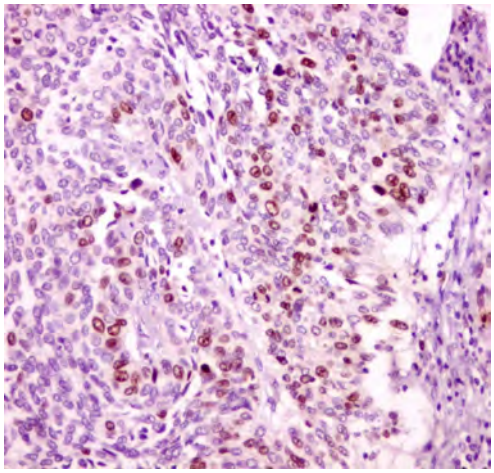

Fig. (3): Transitional cell carcinoma with nuclear immunoreactivity for p21 in more than 10% of tumor cells (Immunoperoxidase DAP X 200).

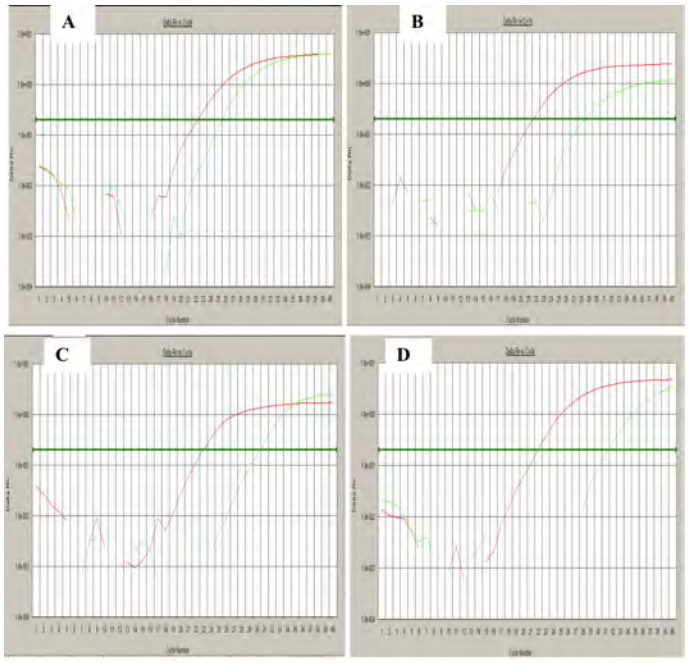

Fig. (4): Curves of real time PCR for p21 and control gene (GAPDH) in different groups ; healthy control group without HCV(A), HCV patients without bladder cancer (B), patients with bladder cancer without HCV(C) , and bladder cancer with HCV(D).

Discussion

It has been established that HCV infection plays a major pathogenetic role in the induction of hepatocellular carcinoma[17-18]. Earlier, Huang et al. [30] confirmed that some HCV-associated hepatocellular carcinomas (HCCs) have mutations in the tumor suppressor p53, the proto-oncogene β-catenin and several other genes. The biological behavior of bladder cancer is determined by many key events including alterations in cell cycle, oncogenic, tumour suppressor genes, apoptotic proteins, telomerase [31-33]. In the context of the many associations between a virus and a given malignancy, the distinction between associated versus causative agent frequently arises and may be difficult to make [34]. To our knowledge, no previous studies have explored the impact of HCV infection on the expression of cell cycle regulators p53 and p21. In the present study the impact of HCV infection on 2 cell cycle regulator genes p53 and p21 were tested.

The present study demonstrated that the bladder cancers associated with HCV infection were predominantly of transitional cell carcinoma (TCC) type (26 cases out of 30), high grade, and more invasive while, non-HCV-associated cancers were associated with more squamous cell carcinoma (SCC) type (12 cases out of 30), low grade, and non-invasive tumors. Ghoneim et al. (35) reported that squamous cell carcinoma (SCC) including the schistosoma-induced bladder cancer predominated (59.3%) over transitional cell carcinoma [TCC] (22.2%), adenocarcinoma (11.4 %). Results of Mokhtar et al. (36) confirmed the occurrence of a significant change in the histopathological profile of bladder carcinoma. Thus, the relative frequency of bladder carcinomas was reversed where TCC represented (64%), SCC represented (29%), adenocarcinoma represented (5%) and the undifferentiated carcinoma represented (2%). However, the present study showed that HCV infection was significantly associated with more TCC and less SCC types, suggesting that this reversal in frequency of histopathological type in bladder cancer might point to change in the risk factor claiming a role for HCV.

p21 is a cyclin kinase inhibitor

that induces growth arrest by preventing phosphorylation of pRB in the G1/S phase transition. Initially, p21 was identified as an effector of p53 response that provoked cell cycle arrest. It has become evident that control of p21 expression is more complex and can be mediated by p53-independent pathways [37]. A previous study of patients with advanced bladder carcinoma undergoing radical surgery showed that patients with tumors that maintained p21 expression had increased survival relative to patients with loss of p21 expression[10]. In the present study, immunohistochemical detection of p21 protein in tissue samples showed significant p21 altered negative expression in bladder tumors associated with HCV infection when compared with those not associated with HCV infection. In addition, it was found that p21 expression by RT-PCR was diminished in bladder tumors associated with HCV infection and that not associated with HCV infection when compared to control healthy groups and was significantly lower in bladder tumors associated with HCV infection than that not associated with HCV infection. Moreover, p21 had a significant negative correlation with HCV infection, and TCC, while, it has positive correlation with the grade and invasiveness of bladder cancer. The immunohistochemical methods were used to evaluate p53 and p21 status because of its affordability and feasibility in the routine clinical setting. However, due to limitations that are associated with this technique related to tissue handling and processing which may affect immunostaining, we used real time PCR to confirm the relative expression of the p21 gene.

Functional inactivation of p53 is the most common event in human malignancies, occurring in at least half of all tumors [38]. In the present study, immunohistochemical detection of p53 in tissue samples from normal healthy urothelium revealed negative staining. In normal urothelium with HCV-infection, the positivity was 30 % of samples, while in malignant tissues the positivity was 43.33 % in non-HCV associated bladder tumors, and 80% in HCV-associated bladder tumors. In consistence with immunohisto-

chemical findings, assay of p53 by RT-PCR demonstrated increase in p53 expression in normal urothelium with HCV-infection when compared to normal healthy urothelium, and significant increase in p53 expression in malignant tissues with HCV infection when compared with that without HCV infection. Moreover, p53 expression showed positive correlation with HCV infection and TCC, while it had a negative correlation with grading and invasiveness of the tumour.

One of the primary functions of the p53 protein as a cell cycle regulatory protein, is to upregulate the expression of p21, a universal cyclin/ cyclin dependent kinase inhibitor with an important role in G1 arrest [39]. In response to DNA damage, p53 induces the expression of p21, which inhibits cyclin/ CDK complexes, and thereby prevents phosphorylation of pRb proteins[7]. In its unphosphorylated form, pRb can bind to and sequester the transcription factor E2F/ DP[40], which is known to activate the expression of genes required for transit from G1 to S phase. Thus p53, p21, and pRb are critical components in a series of highly interrelated pathways.

Conclusion

Transitional cell carcinoma (TCC) of high grade and invasive was associated with HCV infection. p21 and p53 proteins may be of significance in the development of bladder cancer. HCV infection increased the expression of p53 gene and decreased the expression of p21 in bladder cancers. Moreover, p21 expression showed negative correlation with TCC and HCV infection and positive correlation with tumour grading and invasiveness. On the other hand, p53 showed significant positive correlation with TCC and HCV infection and negative correlation with tumour grading and invasiveness. To establish the possible role of HCV infection in pathogenesis of bladder cancer additional work is to be achieved.

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INFLUENCE OF CELL-CYCLE REGULATORY GENES IN HCV POSITIVE BLADDER CANCER PATIENTS

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HYPOMAGNESEMIA IN CRITICALLY ILL CHILDREN

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Abstract

Magnesium (Mg) plays an essential role in a wide range of fundamental cellular reactions. It has been reported that in rodents Mg-deficient diet-induced hypomagnesemia results in an early inflammation.

This is a prospective study had included all patients with sepsis and Multiple Organ Dysfunction Syndrome (MODS) admitted to the (Pediatric Intensive Care Unit) PICU from February 2008 to October 2010. Age, sex, number of organ failure, PRISM Score, probability of death and admission serum magnesium were recorded.

Our results showed 66.9% of patients having hypomagnesaemia at the time of admission 1.7±0.12), with significant difference (p<.001) compared to the normal magnesium level patients (2.09±0.4). The serum level of magnesium was negatively correlated with each of the number of failing organs (r= -0.541, p<0.001), inotrope requirement (r=0.428, p<0.001) and the probability of death calculated from PRISM-II score (r=-0.273, p=0.002).

The group of patients who died had a significantly lower mean serum level of Mg of 1.72 mg/dL (SD 0.24) compared to the group of patients who survived to PICU discharge who had a mean serum level of Mg of 1.87 mg/dL (SD 0.32) with a p value of 0.04

We had concluded that hypomagnesemia is common in critically ill children. The association of hypomagnesemia with higher mortality and morbidity warrants routine screening of critically ill children for hypomagnesemia especially those with high inotrope requirement or progressive organ failure.

Introduction

Disturbances in fluid and electrolytes are among the most common clinical problems encountered in the intensive care unit (ICU). Recent studies have reported that fluid and electrolyte imbalances are associated with increased morbidity and mortality among critically ill patients. To provide optimal care, health care providers should be familiar with the principles and practice of fluid and electrolyte physiology and pathophysiology.[1]

Critically ill patients in the intensive care unit have a high mortality. Modern critical care provides comprehensive life support for patients with multi-organ failure. [2].

Magnesium (Mg) is the second most abundant intracellular cation and the fourth most common cation in the body [3]. Mg was considered the "forgotten cation" in clinical practice. Estimates of Mg deficiency range from 20% to 61%[4], and another study found that reductions in total serum Mg on admission are associated with increased mortality [5].

Magnesium-deficient animals exhibit circulating cytokine levels which are indicative of a generalized inflammatory state. Dramatic elevations of the macrophage-derived cytokines, IL-1, IL-6, and TNF-alpha together with significantly elevated levels of the endothelial cell-derived cytokine, endothelin, were detected in the plasma of these animals, the pathophysiological effects caused by the action of these cytokines may play a role in the promotion of cardiovascular pathology associated with magnesium deficiency [6].

Patients and Methods

This prospective study and data collection were approved by the local institutional ethics committee. Excluding patients with chronic renal impairment, severe malnutrition and those who received aminoglycosides or diuretics, all patients with sepsis and multiple organ dysfunction syndrome (MODS) admitted to the pediatric intensive care unit (PICU) were included. The study was conducted at the PICU at Mansoura University children hospital, data collection extended from February 2008 to October 2010.

Age and sex of patients were recorded. Severity of illness was assessed using PRISM II Score and probability of death[7] and number of failing organs were recorded according to the criteria defined by the international consensus in 2005[8]. All patients had an admission measurement of serum Mg level. Serum magnesium level was estimated by titan yellow method. Serum magnesium levels were expressed as milligram per deciliter[9].

Inotrope infusion requirement was recorded and patients were classified according to inotrope need into 4 grades: grade 0: no inotropes required, grade I: Dopamine < 10 mcg/kg/min +/- dobutamine, grade II: Dopamine >10 mcg/kg/min and/or adrenaline <0.5, grade III adrenaline >0.5 mcg/kg/min or noradrenaline at any dose (10). Primary outcome measure was survival to PICU discharge.

Analysis of data was conducted using the Statistical Package of Social Science (SPSS) version 17 (SPSS incorporation, Illinois, Chicago, USA). In all analyses, p-value of <0.05 was considered statistically significant.

Results

During the study period, 121 patients (70 males, 57.9% and 51 female, 42.1%) fulfilled inclusion criteria. The mean age was 9.1±4.2 months.

Normal serum magnesium was considered 1.8-3 mg/dl [11]. From the studied 121 patients, 81 patients (66.9%) showed hypomagnesaemia with a mean serum Mg level of 1.7±0.12 mg/dL while 40 patients (33.1%) showed normal Mg level with a mean value of 2.09±0.4 mg/dL.

There was no statistically difference between the two groups as regard age (p=0.102) or sex differentiation (p=0.263) (Table 1).

The serum level of magnesium was negatively correlated with each of the number of failing organs (r= -0.541, p<0.001), inotrope requirement (r=0.428, p<0.001) and the probability of death calculated from PRISM-II score (r=-0.273, p=0.002). Figure 1 (A, B & C).

ROC Curve analysis of serum magnesium and need for inotrope, it was noticed that patients with Mg level below 1.77 mg/dL were more likely to require inotrope infusion with sensitivity 52.1% and specificity 74.1%. Area under the curve was 0.637 (Figure 2).

The group of patients who died (25/121, 20.7%) had a significantly lower mean serum level of Mg of 1.72 mg/dL (SD 0.24) compared to the group of patients who survived to PICU discharge who had a mean serum level of Mg of 1.87 mg/dL (SD 0.32) with a p value of 0.04 (Figure 3).

Table (1) Comparison between hypo-magnesemic group and normo-magnesemic group as regard age and sex.

	Hypo-magnesemic Group	**Normo-magnesemic Group**	**p value**
Age	8.64±4.61	9.97±3.04	0.102(1)
Sex			
Males	44 (54.3%)	26 (65%)	0.263(2)
Females	37 (45.7%)	14 (35%)	
Data expressed as mean±SD and frequency (%) **1) Student's t test** **2) Chi square test**			

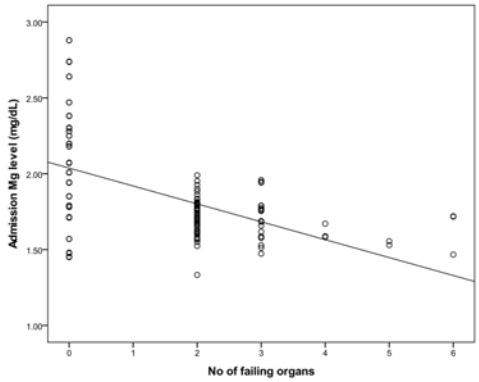

Figure (1A) scatter plot for the correlation between serum Mg level and the number of failing organs.

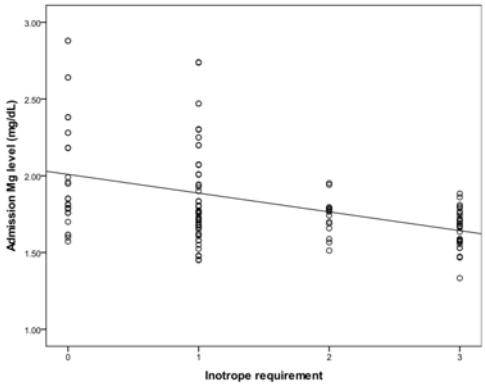

Figure (1B) scatter plot for the correlation between serum Mg level and the inotrope requirement.

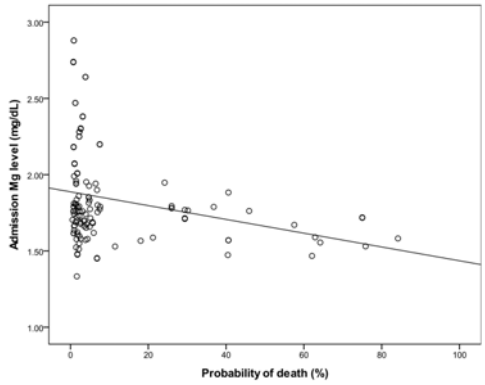

Figure (1C) scatter plot for the correlation between serum Mg level and the probability of death.

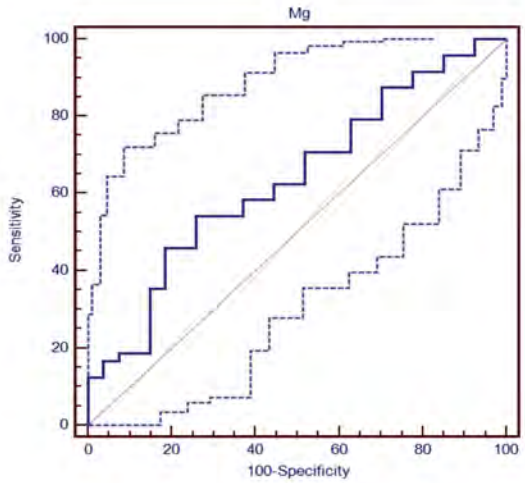

Figure (2) ROC curve analysis for Magnesium level and use of inotrope.

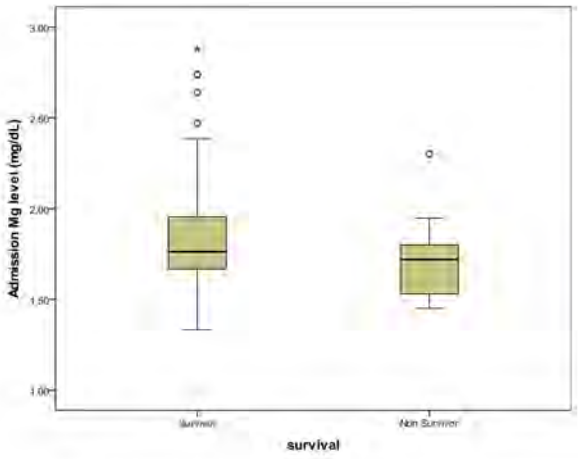

Figure (3). Serum Mg levels in survivors and non survivors

Discussion

Magnesium is the fourth most abundant cation in the body and plays an important physiological role in many of its functions. Magnesium balance is maintained by a complex mechanism including renal and hormonal. Magnesium deficiency is a common problem in hospital patients, with a prevalence of about 10% [12].

Magnesium is essential for bone stability, neuronal excitability, muscular relaxation and many other metabolic functions. Despite its fundamental biological importance, mechanisms controlling systemic magnesium homeostasis are only partially understood [13].

One of the major findings of this study are the lower serum Mg levels in the group of patients who did not survive to PICU (P = .04). This finding is in agreement with Tong and Rude, 2005 who found that Mg deficiency correlated with a higher mortality and worse clinical outcome in ICU patients[14]. Rubiz et al., 1993 also found that hypomagnesemia detected at the time of admission of acutely ill medical patients was associated with an increased mortality rate for both ward and medical ICU patients[5]. Explanation of this association is possible in the light of the findings of Weglicki, and Phillips 1992, who reported that Mg deficiency in a rodent model, had a dramatic increases in serum levels of inflammatory cytokines as IL-1, IL-6, and TNF-alpha[6]. A state of impaired balance in the inflammatory response favoring the proinflammatory limb is the possible explanation of the association between higher mortality and lower admission Mg level reported in this study and others [6&14].

The negative correlation between the magnesium level on admission and number of failing organs during PICU stay (Figure 1A) is in similarity to what have been noticed in adult ICU patients. Safavi and Honarmand (2007) reported that patients with low magnesium at admission had higher Acute Physiology And Chronic Health Evaluation II (APACHE II) and Sequential Organ Failure Assessment (SOFA) scores at admission and a higher maximum SOFA score during their ICU stay[15].

Endothelium injury, exaggerated in presence of low serum Mg, might be responsible for the higher number of failing organs in those with low serum Mg on admission. In a large animal experiment Salem et al., 1995 found that progressive magnesium deficiency was strongly associated with increased mortality from endotoxin challenge. They also found that magnesium replacement therapy provided significant protection from endotoxin challenge [16].

In this study, it was found that the lower the magnesium level the more we need for inotrope use and higher doses of inotropes ($r=-0.428$, $P<.001$). The explanation comes from the findings of Weglicki et al., 2010, who found that in cases of hypomagnesemia there is neurogenic inflammation leading to release of neuronal sources of the neuropeptide, substance P (SP). This neurogenic inflammation is systemic in nature, affecting blood cells, cardiovascular, intestinal, and other tissues, leading to impaired cardiac contractility[17]. Another author Kurabayashi 2005 documented that magnesium deficiency induces an increase in intracellular Ca^{++} concentration in cardiac myocytes, formation of reactive oxygen species, production of inflammatory cytokines, leading to the development of ischemic heart disease, congestive heart disease, and sudden cardiac death [18].

Conclusion

Hypomagnesemia is common in critically ill children. The association of hypomagnesemia with higher mortality and morbidity warrants routine screening of critically ill children for hypomagnesemia especially those with high inotrope requirement or progressive organ failure. Prompt correction of hypomagnesemia might contribute to reduction in those morbidities.

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HYPOMAGNESEMIA IN CRITICALLY ILL CHILDREN

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EFFECTS OF NEONATAL ESTROGEN EXPOSURE ON TESTES OF MALE ALBINO RAT HISTOLOGICAL AND ULTRASTRUCTURAL STUDIES

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Abstract

Objective: *to study the histological and the ultrasturctural changes in the reproductive organs of the adult male albino rats after neonatal estrogen exposure.*

Materials and methods: *the male pups of forty pregnant female albino rats were divided into three equal groups: Group I: control group, Group II: male pups that were injected by low dose of estrogen, Group III: male pups that were injected by high dose of estrogen.*

One testis, from each animal, was fixed in Bouin's fixative and processed to obtain 5 µm thick paraffin sections for histological study. For electron microscopic study, perfusion was done, then the testes were trimmed to obtain very small fragments to be processed for examination by transmission electron microscope.

Results: *On histological examination in low dose group (II): The majority of the tubules exhibited a normal histological appearance, yet some of them, showed structural variations. Some tubules revealed absence of spermatogenic series on some areas and preserved series at others. Some tubules showed spaces in between the spermatogenic cells. In high dose group (III): Examination of the testes revealed that several tubules showed decrease in the thickness of the seminiferous epithelium. In some tubules, there was complete arrest of spermatogenesis and, only spermatogonia were lying on the basement membrane.*

The degenerated tubule showed few sperms attached to the Sertoli cells and in the lumen.

Conclusion: *From the present study we concluded that neonatal estrogen exposure of male albino rat leads to major histological abnormalities in their testes which will persist until adulthood interfering with the normal functions of the testis and thereafter sperm production.*

Introduction

The most dramatic male fertility change that appeared to have occurred over the past fifty years or so is a marked decline in sperm count and semen quality which is one of the causes of male infertility[1]. Abnormalities in male reproductive health are also becoming more frequent. Comparable effects are also occurring in a range of wild life and aquatic animals[2]. It is suggested that there is generally increased human exposure to chemicals that mimic hormones. The chemicals that mimic estrogen are called xenoestrogens or endocrine disrupters which include polychlorinated biphenyls a byproduct in the production of plastics (PCBs), organochlorine pesticides, alkylphenoles, phthalates, dioxin are estrogen like and anti androgenic chemicals in the environment[3]. These compounds of anthropogenic or natural origin, inhibit the action of hormones or alter the normal regulatory function of endocrine system and have potential hazardous effect on male reproductive axis causing infertility[4]. The endocrine and reproductive effects of these chemicals are believed to be due to their ability to: (1) mimic the effect of endogenous hormones, (2) antagonize the effect of endogenous hormones, (3) disrupt the synthesis and metabolism of endogenous hormones, and (4) disrupt the synthesis and metabolism of hormone receptors[5].

Epidemiological, clinical, and experimental studies have suggested that excessive exposure to estrogens and xenoestrogens during fetal and neonatal development may induce testicular developmental disorders, leading to alterations in the adult male fertility[6]. Testicular cancer, which is the most prevalent cancer in young men, has steadily increased in many studies allover the world, rising from 3.4% in 1973 to 5.5%

in 1997 in North America. Hypospadias and cryptorchidism also increased from 0.2 and 2% respectively in 1970 to 0.38 and 3.5% respectively in 1991[7].

Aim of The Work

The present study was designed to determine the histological effects of neonatal estrogen exposure on the testes in male albino rats at adulthood .

Materials and Methods

Animals used : 40 female albino rats and 20 male albino rats were brought from Mansoura Nephrology and Urology center. Every 2 females were kept in a separate cage with one male. Every morning, vaginal smears were taken from the female rats to be sure that the sperms are in their vaginae. Then the female rats were monitored until the occurrence of pregnancy. We examined females daily and caged them separately when a vaginal sperm plug was observed [defined as gestational day (GD) 1]. All the pregnant rats was monitored daily for delivery of pups. The day of birth was considered day 0.

The male pups of pregnant rats was divided into the following groups:

Group I: Control group.

Group II: were injected subcutaneously with 10 µg folone on days 10,15 postnatally.

Group III: were injected subcutaneously with 25 µg folone on days 10,15 postnatally.

Specimen Collection :

When the male pups were 3 months old. 2 male rats from each group were anaesthetized, and then the testes were dissected out and isolated. One testis, from each animal, was fixed in Bouin's fixative and processed to obtain 5 µm thick paraffin sections for histological study and staining with Hematoxylin and Eosin stain (Hx, E. stain)[8] .

Another 2 male rats from each group were anaesthetized and processed for preparation of ultrathin sections for transmission electron microscopic examination 8. The animals were perfused through the heart apex with 200 ml saline followed by 300 ml 2.5% gluteraldhyde in 0.1 mol cacodylate buffer (ph 7.3).

* The testes were fixed in the same fixative for 4 hours.
* Rinse the fixative with cacodylate buffer for 20 minutes with 2 changes.
* Post fix in a fume cupboard with osmium tetroxide for 2h at 4°C.
* Change cacodylate buffer, two times, and each one 20 minutes.
* The specimens were dehydrated in 5 ascending grades of ethyl alcohol.
* The specimens then passed into two changes of propylene oxide to be lastly embedded in Epon.
* The capsules were trimmed, sectioned at 1 μm (semi-thin sections) and stained with toludine blue to evaluate fixation quality and select sites for ultrathin sections.
* Ultra-thin sections of 60-70 nm thickness each, were cut with glass knife and stained with 2% uranyl acetate and lead citrate.
* Stained sections were examined in a Jeol 100 S E/M, in Electron Microscope Unit, Tanta University.

Results

Hematoxylin and Eosin stain (Hx, E. stain)

Group I: Control group, the wall of the seminiferous tubule is lined by stratified spermatogenic cells and Sertoli cells. Some mature sperms are attached to the apex of Sertoli cell. The tunica albuginea is composed of interlacing collagen fibers enclosing fibroblasts (Fig.1).

Group II: Testes of rats exposed to low dose of estrogen showed minimal changes. The majority of the tubules exhibited a normal histological appearance, yet some of them, showed structural variations. Some tubules revealed absence of spermatogenic series on one side and preserved series at the other side (Fig.2).

Group III: In testes from rats exposed to high dose of estrogen, the number of damaged tubules increased as compared to the previous group. Several tubules showed decrease in the thickness of the seminiferous epithelium, there was spaces in between the cells replacing the lost ones. The

degenerated tubule showed few sperms attached to the Sertoli cells and in the lumen (Fig.3).

Transmission electron microscopic examination

Group I: Control group, examination of the head of the mature sperm revealed that it had a pyramidal dark nucleus covered by acrosomal cap. Transverse section in the middle piece showed centrally located microtubules which form the axoneme of the flagellum. The axoneme was surrounded by the nine coarse longitudinal fibers. Mitochondria aggregated and surrounded the nine coarse fibers to form the mitochondrial sheath. The cytoplasm became reduced in amount and pale with no obvious organelles (Fig.4).

Group II: Low dose exposure, the sperm are more or less normal in shape but present in abnormal position it was present in between the spermatogonia (Fig.5).

Group III: High dose exposure, The head of mature sperm showed abnormally elongated nucleus and acrosomal cap. Vacuoles appeared in the cytoplasm. Other sperms showed degenerated heads with loss of the nucleus and vacuolated cytoplasm (Fig.6).

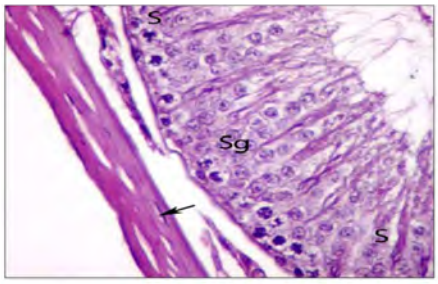

Fig 1: A Photomicrograph of a section of control rat testis. It demonstrates the thick connective tissue capsule of collagen fibers and fibroblasts " tunica albuginea" (arrow). Segment of a seminiferous tubule is seen lined by stratified spermatogenic cells (Sg) and Sertoli cell (S). (H. & E. stain, X400)

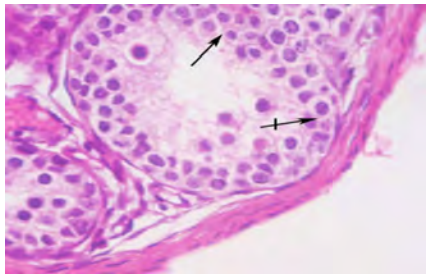

Fig 2 : A photomicrograph of neonatally estrogenized rat with low dose of estrogen showing preserved cells on one side (arrow) and partial loss of spermatogenic cells on the other side (crossed arrow). Notice thickened tunica albugenia. (H.&E. stain X400)

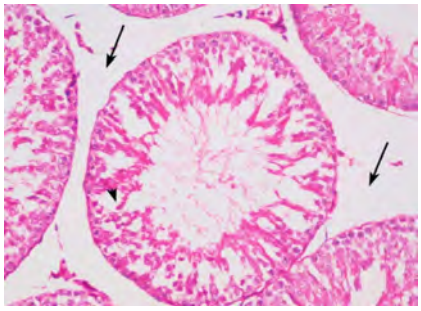

Fig 3: A photomicrograph of rat testis exposed neonatally to high dose of estrogen showing the seminiferous tubules with wide inerterstitial spaces (arrows) and many spaces in between the seminiferous epithelium (arrow heads).
(H. & E. stain x 250)

Fig 5 : An electron micrograph of rat testis exposed neonatally to low dose of estrogen showing parts of spermatogonia with prominent nucleolus (Nu) and part of sperms (S) , found abnormally near the basal compartment.
(TEM x4000)

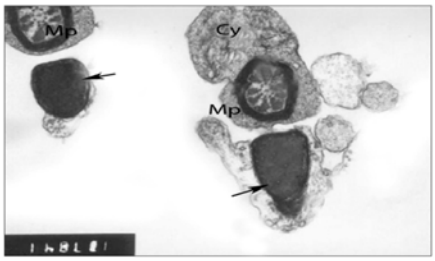

Fig 4 : An electron micrograph demonstrating parts of normal spermatozoa. Sperm heads (arrows), middle piece (Mp), parts of cytoplasm that will be phagacytosed (cy).
(TEM. X 13 000)

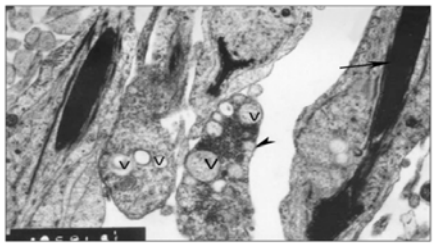

Fig 6 : An electron micrograph of rat testis exposed neonatally to high dose of estrogen showing a head of mature sperm with elongated nucleus (arrow) and the cytoplasm contains vacuoles (V). The other sperm shows degenerated head (arrow head) and vacuolated cytopplsm (V). (TEM x 13000)

Discussion

In recent years, evidences have accumulated that exposure to environmental components with estrogenic activity causes reproductive disorders in human populations. Studies conducted over the past 50 years have clearly shown a continual decline in semen quality accompanied by an increase in male reproductive disorders during this period in industrial countries. As healthy gametes are a prerequisite for healthy children, such disorders are a significant problem not only for the current society, but also for future generations[9].

In the present study, adult male rats which were neonatally estrogenized with low dose of estrogen (stilboestrol dipropionate, folone,), and examination of their testes showed that the seminiferous tubules were either rounded or oval in shape. The majority of the tubules exhibited a normal histological appearance, yet some of them, showed structural variations. Some tubules revealed absence of spermatogenic series on some areas and preserved series at others. Some tubules revealed complete loss of all series apart from the spermatogonia. The tunica albuginea was thick and formed of a dense fibrous membrane. there were spermatogonia and some primary spermatocytes as well as some sperms were attached to Sertoli cells. Some tubules showed spaces in between the spermatogenic cells by transmissiom electron microscope, sperms were found abnormally near the spermatogonia. These sperms seemed to be normal in structure. These results are consistent with the finding of Atanassova et al.[10] and Cook JC et al.[11], who noticed that the basement membrane of the seminiferous tubules remain intact, however disruption of spermatogenesisis was observed at different stages. In these animals, the spermatogenesis was seen up to spermatids while no mature spermatozoa were seen in any of seminiferous tubules of these animals. The Sertoli cells were intact in the seminiferous tubules of all animals. Harmful effects of ethinylestradiol (EE) exposure on spermatogenesis depend on the amount of given dose. This finding coincides with Atanassova et al.[10], who observed

depression of spermatogenesis in the experimental albino rats with the dose of 10 µg of ethinylestradiol but they also found that the lower does in the range of 1 µg do not have significant effects on the spermatogenesis. While in other study dose effect was observed and significant effects were seen with variable doses of EE.

In the present study, the second experimental group included the adult male rats which were neonatally estrogenized with high dose of estrogen (folone). Examination of the testes revealed the number of damaged tubules increased as compared to the previous group. Such damaged tubules were randomly distributed and adjoined the normal ones. Several tubules showed decrease in the thickness of the seminiferous epithelium. There was spaces in between the cells replacing the lost ones. The degenerated tubules showed few sperms attached to the Sertoli cells and in the lumen. Transmission electron micrograph revealed sperms of treated animals to abnormally elongated nucleus and acrosomal cap. Vacuoles appeared in the cytoplasm. Other sperms showed degenerated heads with loss of the nucleus and vacuolated cytoplasm. Transverse section in the middle piece showed multiple vacuoles These results are consistent with the finding of Atanassova et al.[12], Goyal et al.[13] and Dalia m et al.[14] (2007), they noticed disruption of basement membrane of seminiferous tubules and complete arrest of spermatogenesis with loss of Sertoli cells. The spermatogenesis was seen at the level of Spermatogonia but there was marked degenerative changes. Similar picture was observed by Aceitero et al. in his experiment on rats who gave estradiol benzoate 0.5mg/5gm body weight. The later author observed a significant dose dependent rise in the tubule percentage lined by sertoli cells only and presence of multinucleate germ cells in a thin epithelium and sloughed into widened tubular lumen reflecting spermatogenesis impairment.[15]

Summary and Conclusion

This work was undertaken to study the histological and the ultrasturctural changes in the testes of adult male albino rats

after neonatal estrogen exposure.

The male pups of forty pregnant female albino rats were divided into three equal groups: Group I: control group, Group II: male pups that were injected by low dose of estrogen, Group III: male pups that were injected by high dose of estrogen.

Specimens from the tsetes were taken and fixed in Bouin's fixative; paraffin sections (5 μm thick) prepared and stained with Hematoxylin and Eosin stain.

Small fragments from the testes were processed for examination with transmission electron microscope.

After neonatal exposure of the male albino rats to estrogen, it was noticed that testes showedlong term abnormalities that persist till sexual maturation. There were marked histological abnormalities in the seminiferous tubules depending on the dose of the estrogen used. The number of the affected tubules increased with the increase in the dose. The spermatogenic lining was significantly decreased. In the degenerating tubules, some spaces were found in between the cells replacing the lost ones. There were arrest of spermatogenesis at early stages apart from few malformed sperms.

From the present study we concluded that neonatal estrogen exposure of the male albino rat leads to major histological abnormalities in their testes which will persist until adulthood interfering with the normal functions of the testis and thereafter sperm production.

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EFFECTS OF NEONATAL ESTROGEN EXPOSURE ON TESTES OF MALE ALBINO RAT HISTOLOGICAL AND ULTRASTRUCTURAL STUDIES

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HAEMODYNAMIC DISTRUBANCES DURING HEMODIALYSIS

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Abstract

The aim of this study was to study the factors which predict the occurance of itradialytic hypotension either cardiac predictors "Electrocardiographic ECG", Echocardiographic" or non cardiac predictors. Methods: the study included 100 patients on regular hemodialysis who were classified into 2 groups, group 1 included 53 patients, who were liable to intradialytic hypotension, group 2 included 47 patients who were not liable to intradialytic hypotension. All patients were subjected to full history and clinical examination, laboratory investigation, resting ECG and echo Doppler study with stress on assssemnt of E/A ratio of the mitral flow and deceleration time "DT" of the E wave.

Results: *the incidence of intradialytic hypotension was significantly higher in females "p = 0.01", with aging " p< 0.001", with diabetes "P= 0.007, higher predialytic systolic blood pressure "BP" "P< 0.001", lower predialytic diastolic BP "P< 0.001", hypoalbuminemia "P = 0.04", Anemia "P = 0.002". QTc intervals were prolonged in both groups with no predictive value. ECG ischemic changes were significantly higher in group 1 "P = 0.005".*

Inverted E/A ratio diminished its value and prolongation of the DT of E wave were an important predictor of introdialytic hypotension.

Conclusion: *we concluded that the predictors of intradialytic hypotension "IDH" are female sex, aging, diabetes mellitus, higher predialytic systolic BP, lower predialytic diastolic BP, hypoalbuminamia, Anemia, ECG changes of ischemia, inverted E/A ratio with lowering its value and prolongation of DT of E wave.*

Introduction

Hypotension during dialysis is one of the most common complications encountered during hemodialysis.

Although, there is big progress in the process of hemodialysis including the machines, filters and dialysate, the incidence of hypotension during dialysis is still high. About 20-30% of hemodialysis sessions are complicated by hypotension. Healthy individuals can tolerate a decrease in circulating blood volume up to 20% before hypotension occurs [1,2]. However, in a dialysis patient, hypotension may occure with a much smaller decrease in blood volume [3]. Hypotension during dialysis is an important clinical challenge because associated symptoms such as nausea and cramps have a negative impact on quality of life [4]. Also, it results in early termination of hemodialysis session and administration of fluids and hence prevents fluid removal. So, patient may ultimately may experience the effects of volume overload and chronic underdisalysis [5]. Hypotension during dialysis is a multifactorial complication included factors related to the patient and others to the technique of hemodialysis.

Cardiac abnormalities may increase the risk for dialysis hypotension. The decrease in B.P was larger in patients with systolic dysfunction compared with patients with normal systolic function [6]. Also, diastolic dysfunction may increase the risk of hypotension during dialysis.

Echocardiography is a well established non invasive technique to assess cardiac dimensions, volumes and function in an accurate fashion. The mitral inflow Doppler spectrum effects left ventricular filling and it is a very load dependent in patients with various cardiac diseases [7]. So, it has the potential to complement the present methods of fluid status assessment.

Prediction of intradialytic hypotension is an important issue as it results in better control, and outcome of hemodialysis.

The aim of this study was to predict the occurance of intradial-

ysis hypotension either by cardiac predictors "electrocradiography or echocardiography" or non cardiac predictors.

Subjects & Methods

This study was carried out on a total of 100 patients on regular hemoidalysis from multicenters. A hypotensive episode was defined as a symptomatic reduction of mean blood pressure > 20% of the basal predialytic value and requiring intravenous saline administration.

Cardiovascular collapse was defined as a fall in the systolic BP below 85 mmhg with symptoms related to hypotension including profuse sweating, loss of consciousness necessitating sessation of the dialysis session.

Patients were classified into 2 groups:

Group I, included 53 patients on regular hemodialysis and liable to intradialysis hypotension. In these patients at least 3 monthly episodes of hypotension and at least one episode of cardiovascular collapse were recorded in 3 months.

Group II, included 47 patients on regular hemodialysis not liable to intradialytic (hypotension) "as a control group". In these patients no more than one hypotensive episode during the same period without signs of cardiovascular collapse was noted.

Inclusion criteria: patients on hemodialysis for more than 6 months.

Exclusion criteria: patients with hepatic diseases, cancer, valvular heart diseases, congenital heart diseases, atrial fibrillation and patients with left ventricular ejection fraction <50% were excluded from the study.

Methods:

All patients of the two groups were subjected to:

1- Full history and complete clinical examination with stress on cardiac examination and blood pressure assessment.

All patients were not allowed to take any medication up to 6 hours before hemodialysis session.

2- Laboratory investigation

with stress on blood urea, serum creatinin, serum albumin and hemoglobin.

3- Resting electrocardiogram: was recorded just before hemodialysis session and analysed with stress on:
   a. St segment deviation.
   b. Corrected QT interval "GTc".

The QT was measured from the onset of the QRS complex to the end of the T wave. When the T wave was inverted, the end was taken at the point of the return to the baseline. When the U wave was present, the end of the measurement at the nadir between the T and U waves. Three successive QT intervals were measured and the mean was used.

The QTc was measured for heart rate using Bazzett's formula which is as follows:

$$QTc = \frac{QT}{\sqrt{RR}}$$

Where RR is the interval between two consecutive R waves.

4- Echocardiography was performed with Hp "1500" machine with 2-2.5 MHz phased array transducer. Recordings were made with the patient in left lateral position with the transducer at the cardiac apex. The Doppler spectrum of the mitral inflow was recorded between the tips of the mitral leaflets in the 4 chamber view which include E waves of early filling in ms, A wave of the atrial contraction in ms, the E/A ratio, deceleration time of the E wave in ms "DT".

During hemodialysis, the following were measured:

- Duration of the session.
- BP to detect hypotesion.

During Dialysis BP was measured automatically every 15 min. The net volume loss of ultrafiltrate in ml was measured by the machine.

Patients underwent dialysis 3 times weekly, 3.5-5 hour each time.

Statistics :

Statistical analysis was conducted using the mean, standard

error, student T test "unpaired, paired T test", analysis of variance, "ANOVA", test Chi square by SPSS v. 13.

Results

This study was carried out on 100 patients who were classified into 2 groups, group 1 included 53 patients (11 males and 42 females) and group 2, included 47 patients (23 males, and 24 females).

There was significant difference in group 1 and 2 as regard sex "p = 0.01" (more females in group 1).

As regard age, in group 1, the range was "39-69 years" and "Mean ± SD" "58±11", in group 2, the range was "20-55 years" and "Mean ± SD" "35±8". There was significant difference between the two groups "P<0.001".

35 patients in group 1 "66%" were diabetic, whereas 14 patients "29%" in group 2 had diabetes. So, there was significant difference in group 1 and 2 as regard diabetes "P = 0.007". The incidence is higher in group I.

The range of predialytic systolic BP in group 2 was 105-140 mmhg and "Mean ± SD" "115±10".

In group 1, the range was 110-160 mmhg and "Mean ± SD" "137±11". There was significant difference between the two groups where P value <0.001.

Regarding predialytic diastolic BP in group 2, the range was 65-90 and "Mean + SD" "70±5", in group 1, the range was 80-115 and "Mean ± SD" "97±11". There was significant difference between the two groups p<0.001.

In group 1, the range of duration of hemodialysis session was 2.5-4 hours and "Mean ± SD" "3.5±5", in group 2, the range was 3.5-4 and "Mean ± SD" "3.8±0.15". There was significant differences between the two group where p<0.001.

Comparison between group 1 and 2 as regard number of hypotension episodes per month.

There was significant difference between the two groups as regard number of hypotension episodes

per month which found higher in group 1 than group 2.

Predialytic urea "Mean ± SD" in group 1 was "150±35", and in group 2 was "135±35", where there was no significant difference between the two groups P=0.6.

In post dialytic urea mean+SD in group 1 was 75±20 and in group 2 "60±20", where there was significant difference between the two groups. P = 0.004.

Predialytic creatinin Mean+SD in group 1 was "9.1±2.6" and in group 2 was "9.2±2.5".

There was no significant difference between the two groups p=0.66. In postdialysis mean ± SD in group 1 was 7.4±1.6 and in group 2 was 5.5±2. There was significant difference between the two groups p=0.002.

The level of predialysis serum albumin in group 1 "Mean±SD" "3.2±0.7" in group 2 "3.6±0.5". There was significant difference between the two groups p = 0.04.

There was significant difference between the 2 groups as regard bevel of hemoglobin, in group 1, the range was 5.5 - 12 and "mean+SD" "8.9±1.6", in group 2 the range was 9.1-12.5 and mean ± SD "10.5 ± 0.9", p = 0.002.

Comparison between predialytic length of QTc in both groups.

Although QTc in both groups is prolonged than normal, there was no significant difference between the two groups.

Regarding ECG, in group 1, 26 patients "50%" showed st depression more than 1 mm, whereas in group 2 only 14 patients "30%" showed st depression p = 0.005, in group 1, 30 patients "56%" showed t wave inversion while this occurred in 14 patient "30%" in group 2 p = 0.008. This means that patients in group 1 had ischaemic ECG signs significantly more than patients in group 2.

There was significant difference between both groups as regard predialytic level of E/A ratio which was lower in group 1 than in group 2.

There was significant difference between both groups as regard predialysis value of DT of E waves which was larger in group 1 than in group 2.

Statistical analysis showed significant correlation between DT of E wave and number of hypotension attacks in group 1, r=0.75, p<0.002.

Table (1): Comparison between group 1 and 2 as regard number of hypotension episodes per month.

	No. of episodes		P value
	Range	Mean±SD	
Group 1	4-6	3.9±1.1	0.001
Group 2	0-1	0.3±0.4	

Table (2): Comparison between predialytic length of QTc in both groups.

Group	No. of episodes		
	Range	Mean±SD	P value
Group 1	440 – 505	480±20	0.32
Group 2	445 – 500	475±15	

Table (3):

ECG abnormality	Group 1		Group 2		Total 100		Chisquare p value
	No.	%	No.	%	No.	%	
St dep > 1 mm	26	50	14	30	40	40	0.005
T wave inversion	30	56	14	30	44	44	0.008
PVC	16	30	4	8	20	20	0.007
PAC	15	28	13	27	28	28	0.44

Table 4: Shows comparison between predialytic E/A ratio in both groups.

Group	E/A ratio		P value
	Range	Mean±SD	
Group 1	0.4 – 1.0	0.6 ± 0.12	< 0.001
Group 2	0.7 – 1.4	1 ± 0.3	

Table 5: Shows comparison between predialysis deceleration time "DT" of E wave in both groups:

Group	Deceleration time		P value
	Range	Mean±SD	
Group 1	210 – 260	235 ± 15	< 0.002
Group 2	190 – 220	200 ± 20	

Discussion

Hemodynamic disturbance either hypotension or cardiovascular collapse is the most common complication of hemodialysis occurring in 20-30% of patients [8]. Several mechanisms may be responsible for this phenomenon. First, the normal cardiac response to hypovolemia with increased myocardial contractility and heart rate may be impaired. It has been shown that the presence of cardiac disease leading to systolic or diastolic dysfunction, increase the risk for this phenomenon. At comparable ultrafiltration rate, decline in Bp was larger in patients with systolic dysfunction compared with patients with normal systolic function [9]. Also, diastolic dysfunction may increase the risk for this phenomenon. Those with frequent hypotensive episodes had more severe left ventricular hypertrophy and impaired diastolic left ventricular filling [9].

Factors associated with hemodynamic disturbance during dialysis in this study were female sex, old age, the presence of diabetes, higher

predialytic systolic Bp, lower predialytic diastolic Bp, hypoalbaminemia, Anemia, inverted E/A ratio with lowering of its value, prolonged DT of E waves and ECG changes indicative of ischemia.

In this study, we found that patients in group 1 had higher post dialytic urea and creatinine, short hemodalysis session, all of this were due to interruption of hemodialysis session and its early termiration. Female patients were found to have more incidence of hypotension during dialysis than males and this agree with Tisler A. et al. (2002) [10], Claudia Barth et al. (2003) [11]. This may be due to that female patient are mostly diabetic, and autonomic nervous system and Bp regulation are impaired in diabetic patients.

Aging was important risk factor for hypotension during dialysis in this study and we can refer this to vascular, cardiac factors and decreased physiologic reserve of the body. Vascular factors are attributed to low compliance of the vascular system due to atherosclerosis of the walls.

Predialytic systolic Bp was higher in those who are liable to hypotension. This was found in this study and agrees with Claudia Barth et al. (2003) [11].

Low predialytic diastolic Bp was also an indicator for liability of hypotension during dialysis in this study and this agree with Blocher et al (2001)[12]. This may be due to the low compliance of the vascular system because of calcification and stiffness of the vessels. Hypoalbuminemia was associated with higher incidence of hypotension and this agrees with Tisler et al. (2002) [10] as albumin is an important factor for preservation of oncotic pressure of blood.

Anemia is another risk factor for hypotension during dialysis in this study. Hemoglobin is an important factor for preservation of oncotic pressure of the plasma and its lowering in ane-

mia is associated with higher incidence of hypotension during dialysis. Also, anemia is associated with decreased cardiac performance and hence insufficient response to hypotension.

Myocardial ischemia is a important risk factor of intradialytic hypotension. ECG changes indicative of ischemia were significantly prevalent in group 1 than 2. myocardial ischemia definitely depress cardiac performance and response to hypovolemia.

Predialytic values of QTc were prolonged in all patients on hemodialysis with no predictive value of intradialytic hypotension in this study. This agree with Morris Stw et al. (1999) [13] predialysis electrolyte disturbance such as hypocalcemia and hypomagnesemia can lead to prolongation of QT intervals.

Echocardiographic predictors in this study were invereted E/A ratio and prolonged DT of E wave. They were directly proportioned to the incidence of intradialytic hypotension. This also agrees with Gagliordi et al. (2006) [14].

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HAEMODYNAMIC DISTRUBANCES DURING HEMODIALYSIS

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THORACIC TRAUMA : INCIDENCE AND MANAGEMENT

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Abstract

From January 2006 to July 2010, 350 patients with chest injuries were assessed for the incidence, presentation, and outcome of thoracic trauma. The majority (82%) were less than 45 years of age and 300 (85.7%) were male. The mode and extent of injury, specific intrathoracic organ injuries, associated injuries, flail chest, ventilatory requirements, management, and mortality were analyzed Blunt injuries were seen in 298 (89.3%) and penetrating injuries in 52 (10.7%). Multiple rib fractures with haemopeumothorax was the most frequent presentation with head, orthopedic, and abdominal injuries being most commonly associated Patients with partial pressure of arterial oxygen less than 60 mm Hg, lung contusion, flail chest and those with more than 6 rib fractures most often required ventilation but the majority of chest injuries (55.7%) were treated with a chest drain alone. Emergency or delayed thoracotomy was required in 47(13.4%) patients. The morality rate was 7.7% (27 patients) mainly due to respiratory insufficiency and head trauma. Chest injuries were of major concern in multisystem trauma patients and early planned management is recommended in a mostly vulnerable section of our population in an age of violence and vehicular accidents.

Introduction

Trauma is one of the most sudden, dramatic, and often irreversible medical conditions, and it is associated with significant mortality.1 Chest injury alone is responsible for 25% of trauma-related deaths and is a contributing factor in another 25%. [1]. Twenty–five of every 100.000 trauma victims die

following the trauma[2]. Associated organ injuries raise the mortality. Heamodynamically unstable "patients with cardiac and great vessel injuries, tracheobronchial or diaphragmatic rupture may need urgent surgical intervention[2,3].

The most serious pathologies that affect mortality in isolated thoracic traumas are pulmonary contusion and flail chest[4,5].

Nearly 20% of fatal-trauma victims die at the scene of the accident. Therefore, early effective intervention at the accident site by qualified personnel with the necessary equipment as well as early transportation of victims can obviously reduce morbidity and mortality to a considerable degree. In addition, public education and provision of appropriate road conditions are of paramount importance in preventing accidents[3].

This study was undertaken to assess the patteren and the incidence of trauma, nature of chest injuries, management and outcome.

Patients and Methods

All patients with chest trauma with or without associated injuries, admitted to our emergency department between January 1998 and July 2002, were included in this study. The injuries were classified as blunt (inclusive of injuries without loss of skin continuity) or penetrating.

Diagnostic methods employed during the initial assessment and treatment in the emergency room included physical examination, chest radiography, arterial blood gas analysis, electrocardiography, paracentesis, tube thoracostomy, and intravenous fluids. Associated injuries, specific intrathoracic organ injuries, and pathology were noted. Patients requiring ventilation were studied with respect to flail chest, tachypnea, cyanosis, blood gas analysis, rib fractures, surgical procedure, and intrapleural pathology. Patients with rib fractures were anesthetized epidurally or intercostals nerve blockade was performed. In patients with abdominal injuries, analgesics were avoided until a conclusive diagnosis was made. Massive intrathorcic hemorrhage or air

leakage, and vascular injuries were indications for emergency thoracotomy. In the presence of pulmonary contusion, prophylactic treatment was provided to avoid adult respiratory distress syndrome (ARDS).

Results

Three hundred and fifty patients with thoracic trauma managed through a period of 4 years. Of the patients, 300(85.7%) were males and 50(14.3%) were females. The majority (82%) were less than 45 years of age with 9 (2.6%) less than 15 years old, 278 (79.4%) in the age range 16 to 45 years, 41(11.7%) between 46 and 60 years and 13(3.7%) over 60 years old.

Blunt injuries, mostly resulting from vehicular accidents, were seen in 268 (89.3%) patients. Penetrating chest trauma occurred in 32 (10.7%) patients, with stab injuries being the most common (Table).

aType of thoracic injuries which occurred are shown in table (2). Associated extrathoracic injuries occurred in 161(53.7%) patients (table 3). Forty-four (14.7%) patients required ventilation, of whom 13 had flail chest. Fourteen patients with fail chest did not require ventilation. The other 31 patients needed ventilatory support because of respiratory insufficiency with abnormal blood gases, lung contusion, shock or after surgical procedures (table 4). One hundred ninty (55.7%) patients were managed by chest drain alone (as regards chest injuries only) and 61 (17.4%) patients did not require any invasive procedure.

Forty - seven (13.4%) patients underwent thoracic surgical intervention that was performed on an emergency or urgency basis in 14 (37.8%) (Table 5).

Nine patients explored due to large volume of blood drained through chest tube in the first 2 hours (rang from 700-1200, mean 843 ml), two of them were in haemorrhagic shock, three of them had severe haemopysis and one patient had hypoxaemia with PaO2 of 60 mm Hg or less despite inhaling oxygen on arrival.

Double - lumen endotracheal tube was used in patients with heamoptysis to prevent the danger of aspiration into the undamaged lung. During exploration 8 patients had deep pulmonary laceration that required suture repairs in 5 patients and lobotomies in other 3 patients, of them two patients died due to uncontrollable haemorrhage from associated liver rupture. In another 4 patients haemothorax was due to cardiac injury caused by stap, two tears were in left ventricle and the other two were in right atrium. Both tears were repaired with large, tephlon-reinforced mattress sutures. In another 2 patients haemothorax was due to splenic rupture associated with diaphragmatic tear, splenectormy was performed and tear was sutured.

Three patients explored due to massive air leak and failure of lung expansion, bronchoscope confirmed the presence of partial tear in left main bronchus in 2 patients and right main bronchus in the third patients. Tears were sutured and full - lung expansion was achieved. Two patients had sternal fractures with severe dislocatios that required open reduction and fixation with wire sutures. Delayed thoracotomy was performed in 16 patients to evacuate clotted haemothorax, and in 10 patients with empyema for decortications, of them one patient died due to post operative septicemia. In one patients a diagnosis of traumatic diaphragmatic hernia was made on suspicion because of an unusual appearance in chest x- ray and was confirmed by barium - enema (Fig.1). the defect was repaired by nonabsorbable interrupted sutrures after reduction of herniated viscera into the abdomen.

The last patient who required delayed thoracotomy was a 12-years old female who presented by total right lung atelectasis (Fig. 2) with history of blunt trauma 2 months before presentation.

Diagnosis of complete right main bronchus transaction was confirmed by bronchoscope. During thoracotomy the right main bronchus was completely disrupted from lung hilum, leaving

only 0.5 cm of distal stump and a collapsed lung. Both disrupted ends were lying approximately 4 cm apart, completely surrounded by fibrous tissue they were mobilized after dividing the azygos vien. Secretions from the collapsed lung were sterile on culture. Inflation of right lung was carried out by a separate endotracheal tube inserted into the distal disrupted end. Repeated suctioning. Normal saline irrigation, and inflation of the right lung were performed. Bronchoplasty was carried out by performing an end - to - end anastomosis with interrupted 4-0 vicryl sutures, and the site was covered with mobilized surrounding parietal pleura. A postoperative chest radiograph showed a completely inflated lung. Repeat chest radiograph at 6 weeks and bronchoscope at 3 months postoperatively showed no abnormality.

Twenty-seven (7.7%) patients died from different causes (table 6) of which respiratory insufficiency resulting from adult respiratory distress syndrome and lung contusion accounted for one third of the mortality.

Table (1): Causes if thoracic trauma.

Cause	No of patients	%
Blunt trauma		
Vehicle accident	260	81%
Being beaten	20	4.3%
Compression	10	1.7%
Animal kick	5	1.3%
Fall	3	1%
Total	298	89.3%
Penetrating trauma		
Stab injury	36	8.7%
Gun – shot	16	2%
Total	52	10.7%

Table (2): types of chest injuries in 350 patients.

Type of injury	No of patients	%
Single rib fracture	38	9.3%
Multiple rib fracture	238	72.6%
1st and 2nd rib fracture	4	1.3%
Sternal facture	2	0.6%
Flail chest	37	9%
Haemopneumothorax	152	50.6%
Pneumothorax	90	30%
Haemothorax	63	14.3%
Lung contusion	42	14%
Deep lung laceration	9	1.7%
Cardiac contusion	11	3.7%
Cardiac tear	5	0.6%
Pneumomediastinum	6	2%
Bronchial injury	4	1.3%
Diaphragmatic injury	3	1%

Table (3): Other organ trauma accompanying thoracic trauma.

Extrathoracic trauma	No of patients	%
Head trauma	57	16.3%
Abdominal trauma	43	12.3%
Pelvic fracture	7	2%
Fracture of extremity	52	14.9%
Spinal fracture	2	0.6%
Total	161	46%

Table (4): Criteria for ventilatory supporting 44(12.6%) patients.

Criteria for ventilation	No of patients	%
Pa O2 < 60 mm Hg	25	56.8%
PCO2 > 50 mm Hg	25	56.8%
Flail chest	13	29.5%
Lung contusion	22	50%
Rib fractures > 6	20	45.4%
Shock	10	22.7%
After surgical procedure	19	44%

Pa O_2 = partial pressure of arterial oxygen. PCO_2 = pressure of carbon dioxide.

Table (5): Surgical management in 47 (13.4%) patients.

Surgical Management	No of patients	%
Emergent or urgent		
Lung laceration	8	17%
Cardiac injury	4	8.5%
Rupture spleen & diaphragm	2	4.3%
Bronchial rupture	3	6.4%
Sternal fracture	2	4.3%
Delayed		
Clotted haemothorax	16	34%
Decortication for empyema	10	21.3%
Diaphragmatic injury	2	4.3%
Bronchial rupture	1	2.1%

Table (6): Causes of mortality in patients with trauma.

Cause	No of patients	%
Respiratory insufficiency	9	33.4%
Head trauma	7	28.9%
Abdominal trauma	5	18.5%
Multi-system organ failure	5	18.5%
Septicemia	1	3.7%
Total	27	7.7%

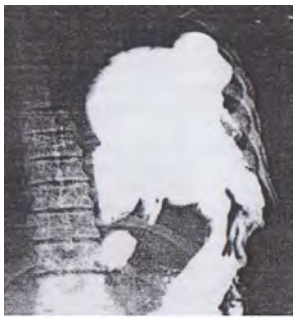

Fig. (1): Barium enema showing traumatic diaphragmatic hernia.

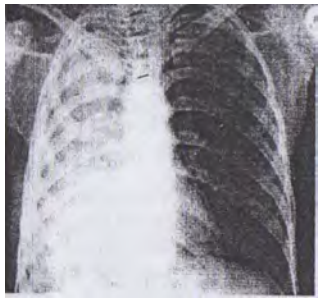

Fig. (2): Chest X-ray showing total right lung atelectasis.

Discussion

Chest trauma is an important cause of morbidity and mortality throughout the world. Although the overall survival rate has improved, deaths are often due to airway obstruction, hemorrhage, flail chest, tension pneumothorax, cardiac tampondade, and associated intra-abdominal and skeletal injuries[6].

Males predominated in our study probably because of their more mobile lifestyle and use of high - speed vehicles. The most vulnerable age group was 16 to 45 years, in which there was a higher incidence of trauma due to active lifestyle.

Intra-abdominal pathologies may be observed in the lower thorax or upper abdomen as a result of blunt thoracic traumas or from a high place[7,8]. Intra-abdominal injury accompanied thoracic traumas in 14.3% of patients in our series. Such injuries can be missed because of severe chest pain. Therefore, the patients should be repeatedly assessed for possible pathologies. Two cases of hemothorax were operated on and nothing abnormal was detected in spite of intra-abdominal bleeding that passed into the thorax through the ruptured diaphragm.

Of the specific thoracic injuries, multiple rib fractures were most

common with over 90% resulting in haemothorax, similar to the findings of locicero and mattox(9). The diagnosis was clinical rather than radiographic, with pain on respiration and tenderness on palpation. A very low threshold should be adopted for chest tube placement in cases of haemothorax because blood in the chest increases the risk of empyema with loss of lung function. Delay in placement of a chest drain allows the blood to clot, making later attempts at drainage more difficult[10].

We performed decortications in 21 patients with clotted haemothorax or pleural thickening due to inadequate drainage. The timing of decortication was consistent with the criteria adopted by the authors for early decortication[11,12].

Of 37 patients with fail chest only 13 required mechanical ventilation indicating that lung contusion rather than altered mechanics was the major factor causing a need for ventilation. However, fail chest can serve as a marker of significant intrathoracic injury associated with pulmonary contusion. Without marked derangement of the basic pulmonary architecture, the contused lung is more amenable to the sequestration of fluid that leads to adult respiratory distress syndrome[13,14,15].

Other authors showed open fixation of flail segment is necessary in cases such as impairment of stability in a large segment of the thoracic wall and loss of pulmonary function in spite of ventilation. They concluded that the procedure not only corrects the deformity and restores pulmonary function but also shortens ventilation time and relieves pain[1,4].

The fact that 55.7% of our patients were managed by chest drain alone emphasizes, like others, the usefulness of tube thoracostomy that can be a lifesaving and versatile technique, affording a margin of safety for patients in the emergency room[16,17].

Exploratory thoracotomy was performed due to deep pulmonary laceration (DPL) in 8 patients, of them 2 patients presented with shock. These patients died in early

postoperative. It has been shown that poor prognostic factors of DPL were (1) a stage of shock with systolic blood pressure less than 80 mmHg on arrival and (2) the volume of blood loss through the chest tube of more than 1000 ml within 2 hours after arrival[18].

Cardiac injuries rank highest for mortality among thoracic traumas. Since most of such victims die at the place of the accident, only a small proportion reach hospital[19]. In penetrating trauma the proximity of the wound from heat should raise the possibility of cardiac involvements. Diagnosis can be difficult in some patients with multiple traumas and the possibility must be kept in mind. Urgent thoracotomy is indicated if cardiac arrest has occurred on arrival and more than half of the patients in this condition can be saved[19].

Tracheobronchial rupture is multifactorial. Rapid deceleration or compression may give rise to ruptures in fixed regions such as the carina and cricoid cartilage, and a sharp increase in the intrabrochial pressure may lead to bronchial rupture. The location of the injuries has been found to be relatively consistent: 80% occur within 2.5 cm of the carina, 80% involve the main stem of the bronchi, 15% involve the trachea, and 5% involve the distal bronchi, and serious associated injuries occur in 50% of the cases[20].

Massive continuous air-leak with failure of lung expansion should rise the presence of bronchial rupture and bronchoscope is mandatory[21]. In case of partial bronchial tear early sutures result in full-lung expansion, but in case of complete distruption, prognosis depends on three factors: the time intervals between injury, diagnosis, and treatment; associated vessel injury; and the condition of the distal transected lung[22-25]. Although successful bronchoplasty has been done as long as 8 to 15 years after injury[26], the time interval between injury and repair will effect the postoperative outcome. A longer duration will delay the post expansion and clearance of haziness on chest radiograph; additionally, lung maturation is delayed in children[23-24]. The optimal surgical procedure is debrid-

ment of the injured tissue with end-to-end anastomosis; results are satisfactory in 90% of the cases[27].

Diaphragmatic ruptures can occur as a result of blunt or penetrating injuries to the lower chest and upper abdomen[28]. They are usually missed in the early period after blunt trauma. Diagnosis can be made with further investigation upon suspicion[8].

The mortality rate in our series (7.7%) lies between reported rates of 7.7% to 14%. The principal factors affecting mortality were respiratory failure, head trauma, multisystem organ failure and abdominal trauma[8,12].

Conclusions

Trauma, principally from traffic accidents, is responsible for thousands of deaths and handicaps every year. The number can be minimized only through better driver training and awareness of the risks of speeding as well as by providing motorways of a high standard. A great responsibility falls on mass-media organizations in this respect. Success in treatment, however, can be increased with initial effective intervention at the site of the accident. Outcomes can be improved if appropriately trained hospital staff are available and priority given to chest trauma in a case of polytrauma, in addition to minimization of pre-hospital delays. Acute stabilization in the emergency room of a non-trauma-specialist hospital should be carried out before transportation to specialist centers, according to the Advanced Trauma Life Support protocols of the American College of Surgeons, as prompt institution of shock resuscitation can reduce many unnecessary deaths[29].

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CHANGES IN PERIPAPILLARY NERVE FIBER LAYER THICKNESS AND GLOBAL VISUAL FIELD INDICES AFTER INTRAOCULAR PRESSURE REDUCTION IN HIGH TENSION GLAUCOMA

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Abstract

Purpose: *To assess any functional changes in the peripapillary retinal nerve fiber layer (RNFL) thickness and global visual field (VF) indices in patients with high tension glaucoma after reduction of intraocular pressure (TOP).*

Design: *Prospective observational case series.*

Patients & Methods: *Thirty-five consecutive patients who underwent monocular deep sclerectomy (surgery group) and medically treated fellow eyes (control group). Quantitative -analysis of the peripapillary RNFL by OCT and global VF indices by program 24-2 automated perimetry were performed before surgery and 6 months after surgery in both eyes. The changes in RNFL thickness overall and by quadrant were evaluated and studied with respect to age, best-corrected visual acuity (BCVA), preoperative global FV indices, postoperative changes in TOP, and postoperative changes in global VF indices. Changes observed in RNFL thickness and VF indices were compared between eyes after surgery and in fellow eyes.*

Results: *The IOP decreased from a baseline mean of 24.5+3.2 mmHg to 11.5±2.7 mmHg (P<0.001) 6 months after surgery at the time of OCT testing. A significant increase in the overall mean RNFL thickness was observed after surgery (P<0.001).The mean preoperative visual field mean deviation (M.D) was significantly correlated with a postoperative*

increase in the RNFL thickness (P<0.075). No correlation was found between RNFL thickness changes and age, BCVA, or changes in the global VF indices. There was no significant difference between eyes with an IOP reduction of more than 50% and those with a reduction in IOP less than 30% (P = 0.312).

Conclusions: *A significant increase of the peripapillary RNFL thickness, was associated with IOP reduction by glaucoma filtration surgery as measured by OCT. Post the glaucoma surgery, a new baseline needs to be established for assessing the longitudinal follow-up of a glaucoma patient. Key Words: Peripapillary RNFL, OCT, VF indices, Glaucoma medication, Glaucoma surgery.*

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Introduction

Glaucoma goes along with the clinically detectable tissue loss in the optic nerve head and the RNF layer. Defects in the peripapillary RNFL may even precede changes in optic nerve head appearance and visual field loss. The optic disc sometimes is seen to be less excavated when the intraocular pressure (IOP) falls. This anatomic change has been documented previously after trabeculectomy by stereoscopic disc photographs, computer-assisted planimetry, optic nerve head analysis, and confocal scanning laser ophthalmoscopy (CSLO) [1-4].

There is controversy about the effect of IOP reduction on the peripapillary retinal nerve fiber layer (RNFL), which also is considered a marker of structural optic nerve damage[5]. However, the effect of IOP reduction on. peripapillary NFL is still unclear[6]. Some studies have found no significant changes in the retinal cross-sectional area using CSLO and an optic nerve analyzer [4-7], whereas others have shown a significant increase of mean retinal height at the optic disc margin with CSLO [8-10].

Optical coherence tomography

(OCT) evaluates and quantifies the peripapillary RNFL thickness in vivo[5]. Aydin et al[11] reported a significant increase in the mean peripapillary RNFL thickness assessed by OCT scans performed with a noncommercial, prototype - device in eyes undergoing filtering surgery. While, 'Leung et al [12] reported structural and functional recovery in a patient with juvenile open-angle glaucoma, which was documented quantitatively by OCT after trabeculectomy.

Deep sclerectomy is a non-penetrating filtering procedure that facilitates IOP control with fewer complications than trabeculectomy[13-14]. The purpose of the present study was to assess changes in the peripapillary RNFL thickness and global VF indices in a prospective manner using a third generation OCT device after TOP lowering by surgical or medical treatment in high tension glaucoma (HTG).

Patients and Methods

Consecutive patients with bilateral high tension glaucoma scheduled for unilateral deep sclerectomy were enrolled. Informed consent was obtained from each patient, and the study protocol was approved in accordance to the Declaration of Helsinki. Eyes were scheduled for deep sclerectomy when the IOP exceeded the target pressure and/or visual field defect and glaucomatous optic nerve damage showed progression despite maximum tolerated medical therapy.

Full ophthalmic examination was done including: assessing visual acuity, slit-lamp anterior and posterior segment biomicroscopy, lop measurement by Goldmann applanation tonometry, gonioscopy using Goldmann three. mirror contact lens, optical coherence tomography, 24-2 program Humphrey visual field analyzer, and cup/disc ratio estimation. A detailed medical history included age, gender, glaucoma medications, systemic hypertension, systemic medications, and previous ocular surgery were recorded.

The lop measurements were measured at least five times on different times of the day from 8 AM to 5 PM. Highest and lowest measured lop values were used to

determine TOP diurnal range. The visual field categories were: (I) normal; [11], mild, an arcuate defect; (III) moderate, abnormal in one hemifield and not within 5 degrees of fixation; and (IV) severe, abnormal in both hemifields or within 5 degrees of -fixation. Assessment of visual field loss was done the basis of the last reliable Humphrey visual field tests before elective ocular surgery.,

Changes in the RNFL thickness 'and visual field indices between eyes undergoing deep sclerectomy (surgery group) were .compared with those of the contralateral eyes in which the IOP was controlled medically (control group). The RNFL thickness postoperative change was analyzed for several potential related factors (age, preoperative overall RNFL thickness, postoperative IOP change, and visual field global indices).

Patients were selected based on their ability to perform perimetry reliability and on the clarity of the ocular media. Only patients with high tension glaucoma were included in this study. excluion criteria included; (1) patients had ocular pathologic features other than glaucoma such as diabetic retinopathy, and age-related macular degeneration, (2) zeripapillary atrophy extending beyond L7 mm from the disc center, (3) inability to obtain adequate OCT images, and (4) patients unwilling to participate.

Visual Field Testing :

All patients underwent Humphrey visual field testing using standard Humphrey 24-2 full threshold perimetry (Humphrey Instruments, Carl Zeiss Meditec, San leandro, Dublin). A reliable visual field test was defined as one with less than 30% fixation loss and false-positive or false-negative responses. The preoperative and postoperative mean deviation (MD) and pattern standard deviation (PSD) were used for the analysis.

Optical Coherence Tomography Scanning :

Cross-sectional image of peripapillary RNL was performed with the Stratus OCT (model 3000, software version 4.4; Carl Zeiss Meditec, Dublin, CA) after pupillary dilation with 1 tropicamide to

a minimum diameter of 5 mm. Circular 360° OCT scans were obtained using the fast RNFL thickness scan, with a diameter of 3.46 mm on the peripapillary RNFL. The scans include the single mean RNFL thickness, the average thickness within each of four quadrants (temporal, superior, nasal, and inferior), and average thickness within each of 12 sectors corresponding to clock hours.

Good scans were defined as focused images from the ocular fundus, with an adequate signal-to-noise ratio and a centered, circular ring around the optic disc. Images with less than 90% satisfactory A scan or a signal-to-noise ratio of less than 25 dB were excluded (1.5). If the amount of pripapillary atrophy exceeded the scan circle, which was visible and controlled by the operator, the patient was excluded. The average of. the three qualified circular scans was used to calculate the mean and quadrantic RNFL thickness.

Surgical Technique :

Local anesthesia was achieved using a peribulbar injection of 4 cc of a mixture of 4% xylocaine, and 0.75% marcaine. A fornix based conjunctival flap was made, the sclera was exposed, and hemostasis by wet-field cautery was performed. A one-third scleral thickness superficial flap (5.0X5.0 mm) was dissected at the 12-o'clock position at least 1.0 mm into the clear cornea. A second flap of deep sclera was dissected, Schlemm's canal was deroofed, and a trabeculo-Descemet membrane window was created. The deep scleral flap was excised, the juxta-canalicular trabeculum and Schlemm's endothelium were removed using small blunt forceps. The superficial scleral flap was sutured with 2 to 4 interrupted nylon 10-0 buried sutures.

Postoperative treatment included a combination of dexamethasone and tobramycin 4 times daily for 2 weeks. The dosage was tapered by 1 drop weekly until discontinuation after 8 weeks. When the vessel density increased or flattening occurred, we intensified the postoperative anti-inflammatory treatment (prednisolone acetate every 1 to 2 hours during waking hours). When filtra-

tion through the trabeculo - Descemet membrane was insufficient because of an elevated IOP, a goniopuncture was performed with the neodymium : yttrium-aluminum-garnet (ND : YAG) laser in the thin driest anterior portion of the trabeculo-Descemet membrane.

Statistical Analysis

A statistics program (SPSS version 15.0 for Windows; SPSS Inc., Chicago, IL) was used for all analyses. A paired t test was used to analyze RNFL thickness differences in individual eyes basis and to compare parameters before and after surgery. A comparison between the 2 study groups was carried out, and Pearson's correlation was used to analyze the association between parameters. The number of glaucoma medications and the visual acuity were compared using the Wilcoxon signed rank test. A P value of 0.05 or less was considered to be significant.

Results

Thirty-five eyes of 35 patients were qualified for this study; 35 eyes underwent deep sclerectomy and the fellow eyes served as the control eyes. The patient demographics and baseline characteristics for both groups are presented in Table 1.

The mean IOP, mean number of medications used, VF indices before surgery were significantly lower in the control group (P=0.001). Six months after surgery, the mean preoperative IOP in the surgery group was decreased from 23.4+6.1 mmHg to 10.6+25 mmHg (P<0.001). The mean percent lOP change was 45.4+16.5% (range, 16% to 65%). In 24 eyes (68.6%), the IOP reduction exceeded 30%.

At six months, the complete success defined as IOP of 21 mmHg or less and IOP reduction of greater than or equal to 20% without anti-glaucoma medication was 91.4% (32/35), qualified success, defined as having an IOP of 21 mm Hg or less and an IOP reduction of greater than or equal to 20% with anti-glaucoma medication was 5.7% (2/35), and failed defined as having an additional surgery was 2.8% (1/35). ND : YAG laser goni-

opuncture was performed in 6 eyes (17.1%).

The mean number of medications used decreased significantly after deep sclerectomy from 3.1+0.5 to 0.9+0.2 after surgery (P<0.001). No significant change was found between the visual acuity before and 6 months after surgery (P= 0.365).

The mean pre and postoperative MD was -9.2+ 5.3 and -8.6+4.6, respectively (P=0.361). The mean preoperative and postoperative PSDs were 5.7+3.1 and 5.3±3.1, respectively (P = 0.325).These results revealed no difference between preoperative and postoperative MD and PSD of the visual field results in the study group.

Table 2 summarizes the mean peripapillary RNFL thickness measured by OCT for the entire study. A significant increase was found in the mean overall and all quadrants of RNFL thickness in the surgery group. The mean overall RNFL thickness change in the surgery group was 7.5±9.6 urn (range, -28.3 to 15.2 urn; median, -1.35 um; P= 0.001). In the surgery group, the RNFL thickness increased in 32 eyes (91.4%) after surgery. In the control group, the mean overall RNFL thickness change was -4.3±5.4 um (range, -17.8 to 10.5 um; P = 0.145).

The correlations between the RNFL changes after surgery and age, IOP change (mmHg), percent IOP change, preoperative MD, PSD, BCVA, and the change in the visual field MD and PSD are shown in Table 3. A significant correlation was found between the RNFL thickness changes after surgery and the preoperative MD (P= 0.374; P= 0.075). There was no significant change in the control group in the MD (P=0.132, PSD (P= 0.145), or IOP (P = 0.127) six months follow up.

Table (1): Demographic and clinical features of the studied patients.

Parameters	Surgery group	Control group	*P. value
No. of patients	35		
Age (year)	62.2 ± 2.5		
Gender **Male** **Female**	18 (51%) 17 (49%)		
Diabetes mellitus	**8**		
Hypertension	**25**		
Cup/Disc ratio	**0.76±2.4**	**0.57**±2.1	**F= 3.52 P= 0.012***
IOP diurnal range**	12.17±2.21	8.26±3.31	F= 4.64 P= 0.015*
Preoperative IOP (mmhg)	23.4±6.1	18.5±2.3	F= 3.54 P= 0.015
Preoperative medication	**3.1** ± 0.5	**1.7** ± 0.4	**F= 2.56** **P= 0.021**
Preoperative VF MD(dB)	-9.2±5.3	-4.5±4.1	F=4.21 P=0.017
Preoperative VF PSD (dB)	5.7±3.1	4.8±2.9	F=4.56 P=0.25
Preoperative VA (logMAR)	0.73±0.4	0.86±0.2	F=3.61 P=0.36

No. = Number, F= One Way Annova test, X_2= Chi-square test

* Significant at P < 0.05

* * IOP diurnal range is the difference between the lowest and highest recorded IOP

VF = visual field, MD= mean deviation, PSD= pattern standard deviation. VA = Visual acuity.

logMAR = logarithm of minimal angle of resolution.

Table 2. Mean peripapillary RNFL thickness measurements in eyes that underwent deep sclerectomy compared with the contralateral eye of the same patient as measured by OCT.

Parameters	Preoperative (µm)	Postoperative (µm)	P*
Overall			
Surgery group	71.6±18.6	89.5±21.2	0.015*
Control group	72.5±17.8	71.7±20.3	0:165
Superior quadrant			
Surgery group	85.8±25.1	96.7±23.2	0.021
Control group	85.7±24.1	86.5±21.5	0.114
Nasal quadrant			
Surgery group	54.6±21.5	72.0±25.1	0.014*
Control group	56.5±18.9	58.2±22.1	0.356
Inferior quadrant			
Surgery group	81.3±25.1	69.2±22.5	0.023
Control group	83.1±18.2	83.3±20.1	0.453
Temporal			
quadrant	56.2±19.2	75.2±20.1	0.016
Surgery group	57.0±18.5	58.1±21.5	0.451
Control group			

RNFL= retinal nerve fiber layer, OCT= optical coherence tomography.

* significant at P< 0.05

Table 3. Pearson's correlation between change in mean RNFL thickness and age, intraocular pressure, and global visual field indices parameters in the surgery group.

	r	P*
Age	-0.164	0.276
IOP change (mmhg)	-0.235	0.023
IOP change (%)	-0.453	0.036
BCVA	*-0.153*	0.216
Preoperative VF MD **(dB)**	0.374	0.075
Preoperative VF PSD (dB)	-0.182	0.383
Change in VF MD	0.395	0.064
Change in VF PSD	0.186	0.625

RNFL = retinal nerve fiber layer.

VF= visual field, MD= mean deviation, PSD= pattern standard deviation. BCVA = best corrected visual acuity.

* significant at P< 0.05

Discussion

Several studies reported less cupping of the optic disc after IOP reduction in some patients after glaucoma surgery[1-4]. This observation is more likely to be due to a simple shift in anatomic structures rather than recovery or reversal of damage. When IOP is lowered, there is less stretch on the lamina cribrosa, and the disc is able to return to its normal position. However, there is no consensus regarding whether the changes associated with IOP reduction occur only in the optic nerve head or also in the peripapillary RNFL[4].

Until recently, the assessment of the RNFL has been argely subjective. Optical coherence tomography, developed to assess tissue thickness in vivo, is a noninvasive imaging technique, allows high-resolution cross-sectional ocular imaging, and evaluates and quantifies the peripapillary RNFL thickness. Optical coherence tomography provides real-time, immediate, objective, and reproducible quantitative measurements of the RN FL within a short time during first visit and offers a reproducible technique with a standard deviation of measurements of 10 to 20 um for the mean overall RNFL thickness [16-17].

In the current study, we prospectively assessed the functional changes in peripapillary RNFL thickness by OCT and global VF indices by automated perimetry in 3 5 patients who underwent monocular deep sclerectomy. In addition, our purporse was to evaluate the correlation of global indices with the structural glaucomatous damage. We used OCT with fast RNFL thickness scan, which reduces the examination time and improves the accuracy and centration of the scans [16].

We found a significant changes in the peripapillary RNFL thickness in the surgery group. The RNFL thickening was significant for the overall measurement and in all quadrants. These results consistent with Aydin et al[11] who reported a significant increase in the overall peripapillary RNFL from 72.8 to 81.7 um after filtration surgery measured by OCT in 18 eyes that underwent trabeculectomy and in 20 eyes that un-

derwent combined trabeculectomy and cataract extraction.

Several studies reported no significant changes in the pripapillaey RNFL thickness such as Sogano et al [7] who used a Rodenstock optic nerve head analyzer and reported that although the cup volume decreased and the rim area increased significantly after trabeculectomy, the RNFL height did not change 2 to 6 months after surgery. Moreover, Irak et al [4] used confocal scanning laser ophthalmoscopy to evaluate 49 eyes 3 months after filtration surgery and did not find a significant change in the RNFL cross-sectional area.

The differences between our findings and those of Aydin et al [11] could be attributed to several factors. First, the absence of a control group in the study which precludes achieving definite conclusions. Moreover, this study was retrospective and the results obtained should be interpreted cautiously. In addition, those authors assumed that the RNFL thickness does not change after cataract surgery, and they combined the data obtained from trabeculectomy or combined cataract extraction and trabeculectomy. In addition, changes in the ocular media, such as posterior subcapsular and cortical cataracts, could impair the ability to perform OCT. To avoid these artifacts, we included only eyes with clear ocular media.

The mean preoperative RNFL thickness was 6.2 um lower in our study than in the study of Aydin et al [11]. Curiously, despite a higher mean thickness before surgery than in our patients, the mean preoperative MD was worse, a finding that can be explained partially by an increase in the diffuse visual field defects as the rvMli, of cataract artifacts. In fact, to avoid the effect of cataract removal on the visual field test, they analyzed the data in only 35 eyes that had undergone only deep sclerectomy. A significant correlation between the RNFL and preoperative visual field MD global indices.

It has been reported that there is a greater likelihood of glaucomatous progression by OCT compared with automated perimetry. This may reflect OCT hypersensi-

tivity or true damage identified by OCT before detection by conventional methods [18]. In addition, the differences may be the result of different degrees of preexisting glaucomatous damage. Some experimental and clinical studies have shown that restoration of anatomic position is more likely to occur in the early stages of glaucoma [19-20].

Our results revealed that overall and segmental RNFL thickness seems to be more reliable index. Deep structural alterations with OCT examination constitute an important indication of early functional changes. The visual field MD seems to be more sensitive for the patients with HTG.

Several studies have shown a high correlation between the degree of improvement in the optic nerve head morphologic features and the percent of IOP reduction [2-4]. Aydin et al [11] reported that after filtration surgery, a 0.5-um increase in the mean RNFL could be expected for each 1-mmHg decrease in IOP. Although a difference in IOP reduction could explain different results, the mean IOP change after surgery obtained in our study was 1.5 mmHg more than that obtained by Aydin et al (I1) Moreover, in the present study, the mean percent of IOP change was 45.3±16.4%. Twenty-seven eyes (77.1%) had an IOP reduction of more than 30%, similar to data (73.7%) reported by Aydin et al [11].

In the present study we did not found a significant, correlation between BCVA and overall or segmental RNFL thickness in the study group. There was significant correlation between BCVA and overall RNFL thickness, and also between the BCVA and the RNFL thickness quadrants (temporal, inferior, superior) in the control group. The highest correlation between BCVA and the RNFL thickness in the temporal sector ($r=0.432$, $P<0.001$) was most likely to be the location of the maculopapillary bundle in this region of the optic disc.

Mechanisms that explain an improvement in RNFL thickness with IOP reduction are unclear. After the retinal nerve fiber is

damaged, it cannot regenerate. One possible explanation is recovery of compressed RNFL. Moreover, it is possible that some axons are able to function marginally while the IOP is high and can recover some physiologic functions when the IOP is lowered, but this is a biomechanical or physiologic restoration, not an anatomic one.

In the present study, we found a significant increase in peripapillary RNFL thickness after successful deep sclerectomy. The only factor significantly correlated with changes in the RNFL thickness was the preoperative visual field MD.

In conclusion, the present study showed an increase in RNFL thickness after deep sclerectomy that correlated with IOP reduction. OCT measurements are affected by IOP reduction after deep sclerectomy as these changes may have any clinical significance in long¬term follow up. Thus, we recommend obtaining OCT images after deep sclerectomy procedure as a base-line for follow-up of HTG patients.

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CHANGES IN PERIPAPILLARY NERVE FIBER LAYER THICKNESS AND GLOBAL VISUAL FIELD INDICES AFTER INTRAOCULAR PRESSURE REDUCTION IN HIGH TENSION GLAUCOMA

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STUDY OF SOME PULMONARY ALTERATIONS IN PATIENTS WITH CHRONIC HEPATITIS C VIRUS INFECTION

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Abstract

Background/aim: *Chronic hepatitis C virus infection (CHC) has been reported in association with several extrahepatic manifestations, including pulmonary abnormalities. The aim of this study was to elucidate the association of CHC with interstitial pulmonary involvement and its impact on pulmonary functional and radiological changes. Also to evaluate the CHC effect on pulmonary function tests (PFTs) of patients with chronic obstructive pulmonary disease (COPD) or bronchial asthma.* ***Patients/Methods:*** *Hundred patients were divided into 3 groups,* ***(Gr I):*** *Thirty patients proved to be CHC, for each patient PFTs, liver biopsy & high resolution CT (HRCT) of chest were done, and its scores were calculated. (****Gr 2):*** *Forty patients with COPD were subdivided into 4 subgroups (10 for each), (A & B): HCV-ve non- and current-smokers, (C & D): HCV+ve non- and current-smokers, respectively. For each patient Forced expiratory volume in the first second (FEV1) and Diffusion capacity of the lung for carbon monoxide (DLco) were followed up for 3 years.* ***(Gr 3):*** *Thirty patients with bronchial asthma (17 HCV-ve and 13 HCV+ve), FEV1 was measured before and after bronchodilator (salbutamol) and followed up for 3 years.* ***Results: Gr 1:*** *Abnormal ventilatory functions and HRCT positive finding were found in 10(33.3%) and 17(56.7%) of cases respectively. There was significant correlation for Metavir stage (F) with FEV1/FVC (Forced vital capacity) & HRCT score (p value <0.05). While neither correlation was found for Metavir activity grade (A), viremia level or HRCT score with PFTs nor in*

HRCT score with Metavir grade (A) and viremia level. ***Gr 2:*** *Baseline spirometry revealed obstructive changes in all patients. Baseline FEV1 & DLco were significantly lower in subgroups B and D (smokers) as compared to A and C (nonsmokers), with p value <0.05. Annual rate of decline (Δ) of FEV1 & DLco were significantly higher in subgroup D than in B and C which in turn were higher than A. There was significant negative & positive correlations between smoking index with baseline (FEV1 & DLco) and (Δ of FEV1 & DLco) in subgroups B and D respectively. No significant correlation between viremia level or liver profile tests with baseline FEV1 & DLco was found in subgroups C & D.* ***Gr 3:*** *There was significant difference for postbronchodilator FEV1 & reversibility between HCV+ve and HCV-ve groups at the third year of the study, also between third year and baseline measures in HCV+ve group.* C***onclusion & Recommendations:*** *Hepatitis C virus can cause interstitial pulmonary abnormalities which are correlated with liver fibrosis stage. So HRCT is recommended in follow up patients with CHC for early detection of interstitial pulmonary fibrosis. HCV infection may accelerate decline of lung functions in patients with COPD and bronchial asthma, and can impair the response to salbutamol. Evaluation of the relation between liver fibrosis with annual decline of lung function, and documentation of interstitial abnormalities in CHC patients by transbronchial lung biopsy is warranted.*

Introduction

Over the last decade, an increasing number of reports have suggested that chronic HCV infection is associated with both direct and indirect effects on pulmonary tissue. The direct effects of HCV on the lung may present as worsening of lung function in some patients with preexisting asthma and/or COPD. In other patients, HCV may present with an interstitial pneumonitis and/or pulmonary fibrosis. Since HCV is well known to induce chronic inflammation and fibrosis in the liver, it was thought that HCV may play a similar role in the lung and be involved in the pathogenesis of pulmonary fibrosis [1].

In across-sectional study the

prevalence of HCV infection in COPD patients was 7.5% and in blood donors was 0.41% & patients with HCV had a lower FEV1 than without HCV[2]. A patient with bronchial asthma and CHC, was improved after IFN therapy and the authors suggested that CHC could be the cause, especially in patients with late appearance of asthma[3]. So the aim of this work was to elucidate the association of HCV infection with interstitial pulmonary involvement and to investigate the relationship of severity of hepatic affection with respiratory functional and radiological changes. Also to test if CHC infection is associated with accelerated decline of lung function in patients with COPD and finally, to determine whether there is a decline in lung function and airway response to salbutamol in asthmatic patients with CHC.

Patients and Methods

This study was carried out on 100 patients chosen from Chest and Hepatology, Gastroenterology & Infectious diseases departments, Benha university hospital in the period from September 2007 to September 2010. Patients were classified into 3 groups: Gr. I included 30 HCV +ve patients (diagnosed on the basis of positive HCV antibodies with ELISA and/or HCV RNA by PCR). Gr. II included 40 patients satisfying the American thoracic society criteria for COPD. They were either non or current smokers, on regular medication. They were classified into 4 subgroups, 10 for each. (A) non smoker HCV -ve, (B) current smoker HCV -ve, (C) non smoker HCV +ve, (D) current smoker HCV +ve. Gr. III included 30 patients, (13) HCV +ve, and (17) HCV -ve with controlled bronchial asthma under treatment, they were non smokers. No treatment 12 hours prior to the spirometric study was given for both Gr II & Gr III.

Exclusion criteria: Patients with decompensated liver cirrhosis or previously treated with interferon, congestive heart failure, chronic pulmonary infections or renal disorders. Patients receiving medications with potential to cause pulmonary alterations e.g amiodarone, cytotoxic drugs and patients with occupational risk factors for lung disease.

For all the patients: Full history, physical examination, CBC, ESR, CXR, Liver profile tests and abdominal ultrasound were done.

HRCT chest was done for Gr I by Toshiba activion 16, 120KV-200MA, slice thickness 2mm, scan spacing 10 mm, in supine position during full inspiration, no IV contrast material was used [4]. HRCT scores were determined by visual estimation of the extent of changes. In each zone if ≤ 25% of pulmonary parenchyma was involved this was given point (1), 26-50% (2), 51-75% (3), 76% and above (4). Global extent score of each abnormality ranges from 0-12. The overall HRCT score of disease severity of the lung is ranging from 0-84.

Liver biopsy for Gr I and interpretation using METAVIR scoring system[5] was done, where A= histological activity (A0- A3) and F= fibrosis (F0- F4).

Spirometry and (DLco) [6] were done once for Gr I. For Gr II, FEV1 & DLco were followed up every 6 months for 3 successive years when the patients were stable and controlled. Average and annual rate of decline for FEV1 and DLco were calculated. FEV1 was chosen, because it is the most reproducible lung function test and excessive decline of FEV1 is the hallmark of COPD [7]. For Gr III bronchodilator reversibility test with salbutamol using 2.5mg in 1cm saline by ultrasound nebulizer was done. FEV1 was recorded thereafter at 10-minutes intervals for 30 minutes. Post-bronchodilator FEV1 was evaluated as the maximal increase in FEV1 after salbutamol administration [8]. Follow up every 6 months for three successive years was done.

Statistical analysis: Statistical package (SPSS, version 10.0) was used for data management. Descriptive statistics were presented as mean ± standard deviations (SD) for continuous variables & number and percentage for categorical variables (frequency distribution). Unpaired Student t-test (two sided) was used to test the significance of difference between the mean value of studied groups and chi-square test was used for comparison of categorical vari-

ables. Pearson correlation test was used to identify the correlation between studied variables. The significance level was set at $p < 0.05$.

Results

Group I : The demographic data, laboratory, histological, HRCT and PFTs criteria for Gr I patients are shown in table 1.

The results of ventilatory functions obtained from flow volume loop revealed obstructive pattern in 1 (3.33%), restrictive pattern in 6 (20%), small airway disease in 4 (13.3%) and normal ventilatory functions in 20 (66.7%) of all cases. The abnormal finding of PFTs were, FEV1% in 4/30, FVC % in 5/30, FEV1/ FVC in 1/30, FEF 25-75 (<60%) in 5/30 and DLco in 2/22 of the patients. DLco was performed in 22 cases due to technical problems, percentage of predicted DLco was (100.9±15.8) %. DLco was decreased (<80%) in 2 (6.7%).

In HRCT, interstitial patterns were detected in 9 (30%), changes other than interstitial pattern in 8 (26.7%) of cases and normal HRCT in 13 (43.3%) of cases. The maximum HRCT score was 68, the commonest abnormalities were ground glass attenuation in 8 (26.7) cases (Fig 1 & 2).

There was significant correlation for metavir fibrosis stage with FEV1/FVC & HRCT score (p value <0.05). While neither significant correlation of Metavir activety grade (A), HRCT score or Viremia level with PFTs nor in HRCT score with Metavir grade (A) or Viremia level (p value >0.05). Moreover there was no significant correlation between viremia level and liver fibrosis stage.

Group II : Four sub-groups of COPD were matched as regard age and duration of COPD. AST& ALT were significantly higher in group C and D than groups A and B.

Spirometry revealed obstructive changes in all patients. Baseline FEV & DLco were significantly lower in group B and D as compared to group A and C, with p value <0.05. Annual rate of decline of FEV1 & DLco were significantly higher in group D than in group B and C which in turn were higher than group A, table (4).

There was significant negative & positive correlations between smoking index with baseline (FEV1 & DLco) and (Δ FEV1 & DLco) in subgroups B and D respectively. While there was no significant correlation between platelets, liver profile tests or viraemia levels with baseline FEV1 & DLco in group C or D.

Group III : Demographic and laboratory data of Gr 3 were shown in table 5. Baseline ALT & AST were significantly higher in HCV +ve than HCV-ve group with p value <0.05 & <0.001.

Comparing each of pre- and post-salbutamol FEV1 at baseline and for 3 years in both groups, There was significant difference for postbronchodilator FEV1 between HCV+ve and HCV-ve groups at the third year of the study (p8), also between 3rd yr and baseline measures in HCV+ve group (p12) (table 6). Comparing levels of increased FEV1(ml) & reversibility % after salbutamol in both groups, there was statistically significant decline at the 3rd yr for HCV+ve group (p4 & p8) and between baseline and 3rd yr measures for the same group (p10 & p12). (table 7).

DATA		Mean±SD/ No (%)	DATA	Mean±SD/ No (%)
Age: (mean±SD)		45.8±7.2		
Sex:Male: No (%)		16 (53.3%)		
Female: No (%)		14 (46.7%)		
Laboratory tests(mean±SD):				
platelet count (cells/mm3)		213 ±63 x 10^6	PFT (mean±SD) :	
AST (IU/L)		58.8 ± 11.725	FVC% prediction.	88.17±9.3995
ALT(IU/L)		80.9 ±82.9	FEV1 pred.	93.13±13.3771
albumin, g/dL;		4.1 ± 0.50	FEV1/FVC pred.	81.83±7.29
total bilirubin, mg/dL		0.70 ± 0.35	FEF25-75% pred.	81.10±17.285
Viremia (viral copies/ml)		1,641,196.7± 3,032,228.5	DLCO pred.	100.91±15.796
Liver biopsy				
Activity grading (A)	A0-1	14 (46.7%)	HRCT pattern No (%) :	
	A2	13 (43.4%)	Ground glass attenuation	8(26.7)
	A3	3 (10%) .	Septal lines	2(6.7)
Fibrosis staging (F)	F0-1	12 (40%)	Non septal lines	2(6.7)
	F2	11 (36.7%)	Nodular areas of high attenuation	1(3.3)
	F3	5 (16.7%)	Consolidation	1(3.3)
	F4	2 (6.7%)	honey combing	1(3.3)

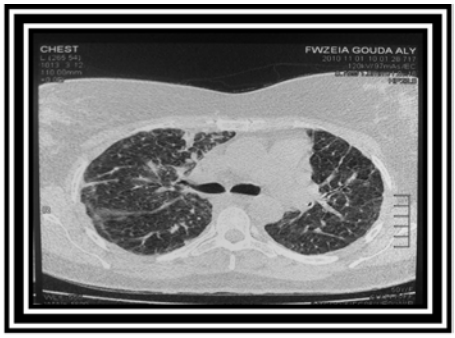

Fig (1) : HRCT score (13) of 40 years old female patient with CHC infection, and no chest complaint, HRCT showed bilateral septal and non septal linear thickening in upper, middle & lower lung zones and anterior lower zonal consolidation.

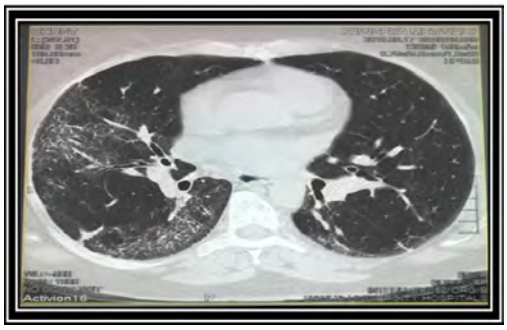

Fig (2) : HRCT score of 9 , it showed bilatera septal and non septal lines with, ground glass attenuation.

Table (2): Correlation of Metavir activity grade A & Stage F , viremia level and HRCT score with PFTs in G I.

Variant	Metavir activity grade(A)		Metavir fibrosis stage (F)		HRCT global extent score		Viremia level	
	r	P	r	p	r	p	r	p
FEV1	0.1	>0.05	0.3	>0.05	-0.1	>0.05	0.01	>0.05
FVC	0.1	>0.05	0.3	>0.05	-0.3	>0.05	-0.1	>0.05
FEV1/FVC	0.1	>0.05	0.4	<0.05*	-0.1	>0.05	0.1	>0.05
FEF25-75%	0.1	>0.05	-0.1	>0.05	-0.02	>0.05	0.2	>0.05
DLCO	-0.1	>0.05	0.2	>0.05	-0.3	>0.05	0.3	>0.05
HRCT score	0.1	>0.05	0.7	<0.05*	----------		0.2	>0.05

Table (3): Overview of group II critera.

	Group A	Group B	Group C	Group D	P value
Age (yrs)	56.9±9.4	53.8±8.9	53±5	51.8±7.9	>0.05
Smoking index	----	505±186.3	-----	497±198.2	>0.05
Duration of COPD (yrs)	12.8 ± 4.3	11.8 ± 5.1	19.1 ± 5.8	17.1 ± 9.9	>0.05
platelet (cells/mm3)	209 ±53 x 10^6	198 ±43 x 10^6	189 ±33 x 10^6	201 ±89 x 10^6	>0.05
ALT(IU/L)	22.5±12.02	23.7±8.6	43.8±11.2	55.5±11.4	<0.05*
AST(IU/L)	24.5±12.02	29.7±8.8	63.8±13.2	75.5±21.4	<0.001*
bilirubin, mg/dL	0.90 ± 0.55	0.80 ± 0.45	0.70 ± 0.15	0.80 ± 0.25	>0.05
Viraemia level Copies/ml	----------	----------	613201.54± 565750.31	68041.34± 594780.95	>0.05

Table (4) : Comparisons between subgroups as regard baseline, follow up and Δ (annual decline) of FEV1 (L) and DLco (ml/mm/mmHg) for three years.

	Base line	1st year	2nd year	3rd year	Δ (annual decline)
Group A					
FEV1	1.833±.590	1.800±.590	1.765±.590	1.738±.593	31.5±2.54
DLco	13.9±3.3	13.3±3.2	12.8±3.1	12.56±3.27	2.6±0.64
Group B					
FEV1	1.693±.431*	1.631.9±.430*	1.573±.429	1.517.5±.428	58.50±6.9 #
DLCO	11.8±1.8 *	11.1±1.7*	10.4±1.68	9.73±1.7	3.4±0.4 #
Group C					
FEV1	1.873±.324	1.822±.329	1.772.9±.333	1.725.8±.335	49.03±4.2 #
DLco	14.3±1.6	12.2±4.3	12.5±1.3	11.94±1.05	3.2±0.4 #
Group D					
FEV1	1.620±.231*	1.537±.233*	1.459±.233	1.394.8±.216	75.2±13.8 #
DLco	12.1±1.9 *	10.7±1.5*	9.5±1.5	8.63±1.45	4.9±1.7 #
P value	B & D compared				# compared to A
FEV1	to gr A&C	--------	----------	----------	B,C vs A<0.05
DLco	*P<0.05				D vs A<0.001

Table (5): Overview criteria of Gr III patients.

	HCV –ve(a) (n=17)	HCV +ve(b) (n=13)	P value
Age (yrs)	44.7±9.3	47.4±9.8	>0.05
Sex (M:F%)	41.2:58.8%	46.2:53.8%	>0.05
Duration of BA (yrs)	12.9±4.9	13.4±4.3	>0.05
platelet (cells/mm3)	223 ±83 x 10^6	201 ±43 x 10^6	>0.05
ALT(IU/L)	21.7±7.9	43.8±11.2	<0.05
AST(IU/L)	22.7±6.9	90±15.7	<0.001
bilirubin, mg/dL	0.60 ± 0.35	0.70 ± 0.45	>0.05

Table (6): Comparisons between (a) & (b) groups as regard pre & post bronchodilator FEV1 after salbutamol.

	pre bronchodilator		P value	post bronchodilator		P value
	HCV –ve (a) (n=17)	HCV +ve(b) (n=13)		HCV –ve(a) (n=17)	HCV +ve(b) (n=13)	
Baseline	2.119±.430	2.129±.493	P1 >0.05	2.577 ± .555	2.589±.597	P5 >0.05
1st year	2.092±.429	2.102±.495	P2 >0.05	2.492±. 506	2.501±.587	P6 >0.05
2nd year	2.060±.426	2.067±.497	P3 >0.05	2.535±.459	2.392±.632	P7 >0.05
3rd year	2.035±.426	2.047±.494	P4 >0.05	2.471±.499	2.345 ±.572*	P8 <0.05*
3rd yr Vs Baseline	P9 >0.05	p10 >0.05	-------	P11 >0.05	p12 <0.05*	---------

Table (7): Comparisons between (a) & (b) groups as regard increased FEV1 (ml) and Reversibility (%) after salbutamol in the study period.

	Increased FEV1(ml)		P value	Reversibility (%)		P value
	HCV –ve (a) (n=17)	HCV +ve(b) (n=13)		HCV –ve(a) (n=17)	HCV +ve(b) (n=13)	
Baseline	0.456 ±0.127	0.459 ±0.116	P1 >0.05	21±2.3	21.5±2.5	P5 >0.05
1st year	0.384 ±0.95	0.398 ±0.99	P2 >0.05	19.3±3.5	19±2	P6 >0.05
2nd year	0.427 ±0.115	0.349 ±0.123	P3 >0.05	20.5±2.1	16.8±2.4	P7 >0.05
3rd year	0.449 ±0.143	0.299 ±0.81	P4 <0.05*	22.1±1.8	14.7±1.2	P8 <0.001*
3rd yr Vs Baseline	P9 >0.05	p10 <0.05*	-------	P11 >0.05	p12 <0.001*	-------

Discussion

Several reports have documented the association of HCV infection with extrahepatic phenomenae, including mixed cryoglobulinaemia, glomerulonephritis, thyroiditis, sialadenitis, and lichen planus. Within the last decade, interstitial lung involvement has also been integrated into this list [9]. The possibility that chronic viral infection may increase the risk for development of accelerated lung destruction may have broad biological relevance to the pathogenesis of COPD [10]. To elucidate the association of HCV infection with interstitial pulmonary involvement, and to determine whether there is a decline in lung function in COPD and asthmatic patients with CHC, hundred patients were evaluated as previously illustrated.

In group I : Results of PFTs were abnormal as follows, FEV1% in 13.3%, FVC % in 16.7%, FEV1/FVC in 3.3% of cases, FEF 25-75 (<60%) in 16.7% and abnormal DLco in 9.1% of patients. This, more or less is near to results of a similar study[11]. While other authors[12-15] were reported higher values in their studies. Furthermore, total HRCT positive finding were found in 17(56.7%) of cases. Meanwhile in previous studies[11,12], they reported higher results. However Erturk et al., 2006[14], found abnormalities in HRCT in 40%, all of them were considered mild fibrosis. The difference between these studies may

be attributed to the degree of IPF, the number of study samples or the disease duration.

As regard the METAVIR scoring system, there was a significant positive correlation between liver fibrosis stage & HRCT score and inverse correlation with FEV1/ FVC % in the study group. This agrees with a previous study[11], and in a study done by Arase et al 2008 [6], they concluded that, the IPF development rate of HCV positive patients was significant with higher age & smoking index and in patients who had liver cirrhosis. On contrary to another study[12], they found no correlation between liver fibrosis stage and HRCT score because all the cases included in their study had mild degree of pulmonary fibrosis.

In the present study there was no significant correlation between activity grading (A) or viremia level with HRCT score, and PFTs. Moreover there was neither significant correlation between viremia level and liver fibrosis stage, nor between HRCT and PFTs. Actually viral load does not correlate with the severity of the hepatitis or with a poor prognosis[16], also there is a contradictory in results of literatures about the correlations between PFTs and HRCT score in patients with IPF. As normal PFTs cannot be assumed to exclude IPF in the presence of suggestive clinical or radiographic abnormalities[17]. There was no significant correlation between HRCT score and PFT which was explained by that, interstitial involvement was not diffuse and HRCT score was low as in a previous study[11]. In contradictory to other studies[12,18], there was a significant negative correlation between HRCT score with DLco and FVC in patients with CHC. In the present study the selected cases were clinically free from manifestations of IPF on contrary to their studies where cases had clinical and radiological manifestations of IPF.

In group II (COPD patients): Baseline values of FEV1 were significantly lower in HCV+ ve (non- and current smokers), compared to HCV-ve (non- and current smokers). While Δ FEV1 & DLco were significantly higher in group D than in groups B and C, which

in turn were higher than group A table (4). Moreover, there was significant negative & positive correlations between smoking index with (baseline FEV1 & DLco) and (Δ FEV1 & DLco) in subgroups B and D respectively. This findings agrees with another study[10], they suggested that, CHC may increase the risk of development of COPD.

The patients with liver diseases had a significantly high prevalence of COPD with airflow limitation, so the presence of liver disease might become a useful predictor for the early detection of COPD [19]. Mechanisms of declining PFTs in COPD patients with CHC are unclear, but several theories can be proposed. A morphometric analysis of lung biopsy from smokers have demonstrated a correlation between cytotoxic T cells and lung destruction [20]. Another study suggested that cigarette smoke induced lung inflammation was amplified in severe emphysema and that latent viral infection influenced this amplification process [21], thus CHC may be a cofactor in smoking induced decline in PFTs. The third possible mechanism is that chronic liver disease decreases glutathione synthesis in the liver, and an inadequate supply of glutathione in the lung would render the lung vulnerable to oxidative damage [22].

In the present study there was non significant correlation between baseline FEV1 with both liver profile tests and viremia level. This agrees with another study in which there was an uncertain relationship between HCV infection and pulmonary involvement [23].

In asthmatic patients (group III) : In comparison of each pre- and post-salbutamol FEV1 at baseline and for 3 years in both groups, There was significant decline for postbronchodilator FEV1, increased FEV1 (ml) & reversibility % after salbutamol at the 3rd yr for HCV+ve versus HCV-ve group and between baseline and 3rd yr measures for the same group. These results agrees with the study of Kanazawa and Yoshikawa, 2003 [24]. They concluded that, the relation between airway responses to bronchodilator in airway inflammation is poorly understood in asthma, and the airway

responses to ß2-adrenoceptor agonists are different in asthma and COPD. It was speculated that CD8+ T lymphocytes induced by chronic HCV infection causes asthma with COPD-like inflamma-tion that responds poorly to salbutamol, which is effective for typical asthma. These hypotheses would be strengthened if HCV-specific CD8+ T lymphocytes could be found in the lungs of these patients [24]. Although chronic airway inflammation is important in both asthma and COPD, in asthma the inflammation is due mainly to CD4+ T lymphocytes, whereas in COPD it is characterized by increased numbers of CD8+ T lymphocytes [25].

In conclusion: A growing pile of evidence suggests that pulmonary involvement is one of the extrahepatic manifestations of CHC. HCV infection may cause interstitial pulmonary abnormalities, which is related to liver fibrosis stage and HRCT is a good method for assessment of these alterations. It may accelerate decline of lung function in patients with COPD especially if cigarette smokers and in bronchial asthma with impairment to salbutamol response. Further studies to evaluate the prevalence of HCV in patients with IPF, COPD, and bronchial asthma are recomended. Moreover, studies to evaluate the relationship between liver fibrosis and annual decline of lung function & to document cases of interstitial abnormalities by transbronchial lung biopsy are also warranted.

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STUDY OF SOME PULMONARY ALTERATIONS IN PATIENTS WITH CHRONIC HEPATITIS C VIRUS INFECTION

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THE POTENTIAL BENEFICIAL EFFECTS OF MELATONIN ON HEPATIC FIBROSIS IN RATS

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Abstract

Background/Aim: *There is no standard treatment for liver fibrosis. Given a lack of effective treatment for many chronic liver diseases, this has been an active area. Liver fibrosis may regress following treatment with antifibrotic drugs. This study evaluate the antifibrotic effect of melatonin on rats with hepatic fibrosis.*

Methods: *Fibrosis was induced in rats by carbon tetrachloride (CCL4) administration for 6 weeks. Fibrotic rats were randomly assigned to one of three groups. Silymarin (50mg/Kg), melatonin (5mg/Kg) or melatonin (10mg/Kg), each given orally for 4 weeks starting 2 weeks after CCL4 injection. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total proteins, serum albumin and albumin globulin(A/G) ratio were performed by colorimetric methods. Hepatic tissue specimens were histopathologically evaluated according to Scheuer system by hematoxylin & eosin staining.*

Results: *There was significant decrease in fibrosis score in all treated groups Silymarin (50mg/Kg), melatonin (5mg/Kg) or melatonin (10mg/Kg) (p=0.000), no detectable differences between the silymarin and melatonin 5mg treated groups, but significant differences between the silymarin and melatonin 10mg and melatonin 5mg, melatonin 10mg treated groups(p=0.24, p=0.0004 and p=0.000) respectively, no detected focal hepatic steatosis in melatonin treated groups. Melatonin administration at a dose of 10mg more beneficial than melatonin at a dose of 5mg as regard the changes in biochemical parameters.*

Conclusion: *The results showed that melatonin exerted antifibrotic effect as well as improvement of hepatic steatosis in rats with*

CCL4 induced liver fibrosis , may be used as a therapeutic option against hepatic fibrosis.

Introduction

Hepatic fibrosis represents the consequences of a sustained wound healing in response to chronic liver injury from a variety of causes, including toxins (e.g. alcohol) chronic viral infection, cholestasis and metabolic disorders [1].

It can leads to cirrhosis and the liver architecture is diffusely abnormal and this interferes with liver blood flow and function. Such derangement produces the clinical features of portal hypertension and impaired liver cell function [2].

Liver cirrhosis shows a world wide distribution and affects all races, ages and both sexs. The triad of paenchymal cell necrosis, nodular liver cell regeneration and fibrosis constitutes the hallmark of any cirrhotic process [3].

If treated properly at fibrosis stage, cirrhosis can be prevented. However, no effective antifibrosis drugs are available at present. Several lines of evidence suggest that oxidative stress plays an important role in the etiopathogenesis of hepatic fibrosis [4].

Melatonin (N-acetyl-5 metyoxytryptamine), a secretory product of the pineal gland, is a powerful endogenous antioxidant, regulates circadian rhythms, sleep and immune system activity. It behaves as a free radical scavenger, eliminates oxygen free radicals and reactive intermediates [5].

Carbon tetrachloride had been investigated as a model of hepatic fibrosis in rats [6].

Both in vitro and in vivo experiments have shown that melatonin can protect cells, tissues, and organs against oxidative damage induced by a variety of free- radical-generating agents and processes, such as carbon tetrachloride (CCL4) [7,8].

In this study carbon tetrachloride was used as a pharmacological tool to induce hepatic fibrosis similar to chronic hepatitis. The hepatotoxic effect of carbon tetra-

chloride may be mediated by release of free radicals [7,8].

The aim of this work is to investigate the potential hepatoprotective effect of melatonin in 2 different doses (5mg/kg &10mg/kg) for 4 weeks on carbon tetrachloride induced hepatic fibrosis in male albino adult rats. Moreover the effect of melatonin was compared with a widely used hepatoprotective agent namely silymarin. This may help to find a novel therapeutic agent for treatment of hepatic fibrosis and to make a preliminary study to assign the proper dose for such purpose

Material and Methods

Animals :

Thirty male adult albino rats of locally breaded strain weighing between 150-250g at the beginning of the study were used. They have acclimatized for one week in groups (6/cage) in fully ventilated room at ordinary room temperature. Rats were allowed to ad libitum, access to water and balanced diet.

At the beginning of the experiment they were divided into 5 groups each contained 6 rats:

Group I : Control normal rats. They receive no drugs; they only receive drug vehicles(methyl cellulose & saline orally & olive oil subcutaneous and in volumes comparable to that of tested drugs.

Group II : was served to induce Chronic hepatic fibrosis by administration of 50% ccl4 (0.3mg/kg subcutaneously) twice weekly for 6 weeks (9).

Group III: was served to study the effect of silymarin on liver function in carbon tetrachloride induced hepatitis. Carbon tetrachloride was administrated for 2 weeks in the same dose as group II to induce hepatic fibrosis followed by carbontetrachloride + silymarin (50 mg/kg /day) four another for weeks to test the curative effect of silymarin

Group IV: was served to study the effect of low dose of melatonin (5 mg/kg) on liver function in carbon tetrachloride induced hepatitis. Carbon tetrachloride was administrated for 2 weeks in the same dose as group II to induce

hepatic fibrosis followed by carbontetrachloride + melatonin (5 mg/kg /day) for another four weeks to test the curative effect of silymarin.

Group V: was served to study the effect of higher dose of melatonin (10 mg /kg) on liver function in carbon tetrachloride induced hepatitis. Carbon tetrachloride was administrated for 2 weeks in the same dose as group II to induce hepatic fibrosis followed by carbontetrachloride + melatonin (10 mg/kg /day) for another four weeks to test the curative effect of silymarin.

Drugs:

- Carbon tetrachloride (ccl4) (Nour Esh'ShARK, Co. Egypt) it is supplied as colorless liquid.
- Melatonin: (crystals) (sigma. co. U.S.A).
- Silymarin: (powder) (sedico pharmaceutical.co).
- All drugs were dissolved in normal saline (0.9% NaCI) except
- Carbon tetrachloride was supplied as solution at concentration 50%
- It is diluted by olive oil to a working solution of 50%. Freshly prepared solution was used for each experimental session
- Melatonin which was dissolved in methyle cellulose. All drugs and chemicals were freshly prepared before each experiment.
- It should be mentioned that the doses of the drugs chosen in the present study, are based mainly on previous researches done by many investigator[6,9,12] and pilot experiment using the minimal and maximal human dose converted to its equivalent rat dose according to pilot experiments performed within therapeutic doses according to [13].

Melatonin and silymarin were administrated orally using a curved gag reaching up to the pharynx 2 weeks after induction of hepatic fibrosis by ccl4, for 4 weeks which was administrated subcutaneously in lower limb.

After the end of the experimental period (6 weeks), all animals were sacrificed by decapitation after making them lose consciousness by a blow on the back of the head. Blood samples were taken from each animal from decapitation wound and collected on hepa-

rinized tube. Serum was separated by centrifugation at 5000 round /minute for 5 minutes. Serum samples were kept at 4 C0

They were subjected to the following investigations within 24 hours:

- Serum alanine transaminase
- Serum aspartate transaminase
- Serum alkaline phosphatase
- Serum total protein
- Serum albumen
- Serum globulin by the calorimetric method of [10].

The ratio between albumen and globulin (A/G ratio) were determined for each animal spacemen. In addition, all animals were dissected for the liver which was rapidly washed with distilled water. They were dried with filter papter. A piece of liver tissue were cut and immediately kept on 10% formaldehyde solution till histopathological examination using H & E stain [11].

The degree of hepatic fibrosis was assessed using the simple scoring system of [14] was used for the evaluation of the different therapies used in the present study. In this scoring system stage 0: no fibrosis; stage 1: expansion of portal tracts without linkage; stage 2: portal expansion with portal to portal linkage; stage 3: expansive portal to portal and focal portal to central linkage; and stage 4: cirrhosis.

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

Administration of carbon tetrachloride in a dose of (0.3mg/kg subcutaneously) twice weekly for 6 weeks resulted in marked deterioration of liver function in tested animals evidenced by significant increase in serum alanine transaminase, aspartate transaminase and alkaline phosphatase amounted to 250%, 892%, 427% respectively. On the other hand, albumen and A/G ratio were significantly decreased amounted to -

33% and 30% respectively compared with significant control (table 1). This was associated with marked histopathological changes in the form of loss of hepatic architecture with marked perilobular fibrosis. The liver cells exhibited marked degeneration with dense lymphocytic infiltrate in dilated portal tract, dilated blood sinusoids and marked bridging necrosis (Fig. 1).

The administration of silymarin resulted in significant decrease in serum liver enzymes by -314% for ALT, 329% for AST and -65% for APL compared with non treated fibrosed rats. The above mentioned results were significantly more than normal values by 135%, 567%, 83% (table 2).

Low dose of melatonin (5 mg/kg /day for 4 weeks) significantly decreased respective serum values of liver enzymes in ccl4 induced liver fibrosis in rats by -41%, -41% and -69% compared with non treated fibrosed rats. Such values are more than normal values by 99%, 481% and 63% respectively. Serum albumen and A/G ratios were elevated by 23% and 38% compared with non treated fibrosed rats. Such values were within that of normal control animals. Fibrosis score was reduced by 23% below that of non treated fibrosed animals. (table 3).

On the other hand, the respective values of tested liver enzymes under the effect of high dose of melatonin (10mg/kg/day for 4 weeks) showed significant decrease by -54%, -63% and -69% compared with non treated ccl4 induced hepatic fibrosed rats. Compared with normal values, they were more than normal by significant % of 57, 263 and 57 respectively. Serum albumen and A/G ratio were significantly increased by significant values amounted to 39% and 63% respectively. Compared with non treated group. Fibrosis score was significantly decreased by -28% by treatment wtih high dose melatonin more than non treated group (table 4).

High dose of melatonin had more powerful effect than the lower dose of the same drug in lowering the tested liver enzymes by-

28% and -38% for ALT and AST respectively. Fibrosed score was decreased by significant % of 8% (table 7). Compared with silymarin, it was more depressant effect on ALT by -33% and -46%. Fibrotic score was decreased by-7%. All above values were statistically significant (table 6).

Regarding the effect of tested drugs on histopathological picture, there was marked improvement correlated with the depression of liver enzymes and elevation of serum albumin .

Ccl_4 induced hepatic fibrosis was improved in melatonion 5mg, melatonion 10mg and silymarin treated rats in comparison to the Ccl_4 induced hepatic finrosed untreated rats, as the hepatocyte degeneration as well as bridging necrosis were decreased, without detectable differences between the treated groups, but, silymarin treated group had whown focal hepatic steatosis that was not detected in other both treated groups (Fig. 2,3,4).

As regads fibrosis score,no detectable differences between the silymarin and melatonion 5mg treated groups, but significant differences between the silymarin, melatonin 10mg and melatonin 5mg, melatonin 10mg treated groups respectively (Fig. 5).

Table (1) : The effect of ccl4 induced hepatic fibrosis on liver function tests of control normal rats (n = 6).

Groups / Parameters	Control normal rats	Chronic hepatic fibrosed rats	% change	P-Value
ALT U/L	20.4 ± 1.9	70 ± 6.5*	250*	0.000*
AST U/L	18.15 ±1.8	180 ± 12.2*	892*	0.000*
ALP (U/L)	122.77 ± 6.8	647.3 ± 60.5*	427*	0.000*
Total protein (g/dl)	7.0 ± 0.46	6.1 ± 0.4*	-13*	0.0047*
Serum Albumin (g/dl)	4.0 ± 0.34	2.8 ± 0.21*	-30*	0.002*
A/G ratio	1.2 ± 0.05	0.8 ± 0.1*	-33*	0.005*

* Significant changes of chronic hepatic fibrosed rats compared with control normal rats.

Table (2): The effects of silymarin (50mg/kg/day orally) on liver function test in ccl4 induced hepatic fibrosed rats (n=6).

Groups / Parameters	Control normal rats	Untreated rats	Silymarin treated rats
ALT U/L % change compared to normal % change compared to untreated fibrosed rats	20.4 ± 1.9	70 ± 6.5 250*	48± 4.2 135* -314** (p=0.0004)
AST U/L % change compared to normal % change compared to untreated fibrosed rats	18.15 ±1.8	180 ± 12.2 892*	120.8 ± 7.2 567* -329**(p=0.0002)
ALP (U/L) % change compared to normal % change compared to untreated fibrosed rats	122.77 ± 6.8	647.3 ± 60.5 427*	224.1± 14.4 83* -65**(p=0.000)
Total protein (g/dl) % change compared to normal % change compared to untreated fibrosed rats	7.0 ± 0.46	6.1 ± 0.4 -13*	6.8±0.2 8 -2 11** (p=0.003)
Serum Albumin (g/dl) % change compared to normal % change compared to untreated fibrosed rats	4.0 ± 0.34	2.8 ± 0.21 -30*	3.43±0.12 -14 23**(p=0.0008)
A/G ratio % change compared to normal % change compared to untreated fibrosed rats	1.2 ± 0.05	0.8 ± 0.08 -33*	1.1±0.05 -8 38**(p=0.0001)
Fibrosis score % change compared to normal % change compared to untreated fibrosed rats		3.9±0.07	3.0±0.0 23**(p=0.000)

** Significant changes of silymarin treated rats compared with chronic hepatic fibrosed untreated rats.at $p<0.05$

* Significant compared with normal rats at $p<0.05$

Table (3): The effects of melatonin (5mg/kg/day orally) on liver function test in ccl4 induced hepatic fibrosed rats (n=6).

Parameters \ Groups	Control normal rats	Untreated rats	melatonin 5mg treated rats
ALT U/L % change compared to normal % change compared to untreated fibrosed rats	20.4 ± 1.9	70 ± 6.5 250*	41.5 ± 3.5 99* -41**(p=0.0003)
AST U/L % change compared to normal % change compared to untreated fibrosed rats	18.15 ±1.8	180 ± 12.2 892*	105.5 ± 10.2 481* -41**(p=0.0004)
ALP (U/L) % change compared to normal % change compared to untreated fibrosed rats	122.77 ± 6.8	647.3 ± 60.5 427*	200.5 ± 10.4 63* -69**(p=0.0007)
Total protein (g/dl) % change compared to normal % change compared to untreated fibrosed rats	7.0 ± 0.46	6.1 ± 0.4 -13*	6.8 ± 0.21 -3 11**(p=0.00035)
Serum Albumin (g/dl) % change compared to normal % change compared to untreated fibrosed rats	4.0 ± 0.34	2.8 ± 0.21 -30*	3.54 ± 0.2 -12 26**(p=0.0001)
A/G ratio % change compared to normal % change compared to untreated fibrosed rats	1.2 ± 0.05	0.8 ± 0.08 -33*	1.2 ± 0.11 0 50**(p=0.0003
Fibrosis score % change compared to normal % change compared to untreated fibrosed rats		3.9±0.07	3.05±0.07 -21**(p=0.000)

** Significant changes of melatonin 5mg treated rats compared with chronic hepatic fibrosed untreated rats at p<0.05.
* Significant compared with normal rats at p<0.05

Table (4): The effects of melatonin (10mg/kg/day orally) on liver function test in ccl4 induced hepatic fibrosed rats (n=6).

Parameters \ Groups	Control normal rats	Untreated rats	melatonin 10mg treated rats
ALT U/L % change compared to normal % change compared to untreated fibrosed rats	20.4 ± 1.9	70 ± 6.5 250*	32 ± 2.1 57* -54**(p=0.000)
AST U/L % change compared to normal % change compared to untreated fibrosed rats	18.15 ±1.8	180 ± 12.2 892*	65.8 ± 6.1 263* -63**(p=0.000)
ALP (U/L) % change compared to normal % change compared to untreated fibrosed rats	122.77 ± 6.8	647.3 ± 60.5 427*	193 ± 12.5 57* -70**(p=0.000)
Total protein (g/dl) % change compared to normal % change compared to untreated fibrosed rats	7.0 ± 0.46	6.1 ± 0.4 -13*	7.1 ± 0.2 2 16**(p=0.003)
Serum Albumin (g/dl) % change compared to normal % change compared to untreated fibrosed rats	4.0 ± 0.34	2.8 ± 0.21 -30*	3.89 ± 0.12 -2 39**(p=0.006)
A/G ratio % change compared to normal % change compared to untreated fibrosed rats	1.2 ± 0.05	0.8 ± 0.08 -33*	1.3 ± 0.1* 8 63**(p=0.0002)
Fibrosis score % change compared to normal % change compared to untreated fibrosed rats		3.9±0.07	2.8±0.06* ** -28**(p=0.000)

**Significant changes of melatonin 10mg treated rats compared with chronic hepatic fibrosed untreated ratsp<0.05.
* Significant compared with normal rats at p<0.05

Table (5): Comparison between the effects of silymarin and melatonin (5mg/kg/day orally) on liver function test in ccl4 induced hepatic fibrosed rats (n=6).

Groups / Parameters	Silymarin treated rats	melatonin 5mg treated rats	% change	P-Value
ALT U/L	48± 4.2	41.5 ± 3.5	-13*	0.015
AST U/L	120.8 ± 7.2	105.5 ± 10.2	-13*	0.013
ALP (U/L)	224.1± 14.4	200.5 ± 10.4	-11*	0.0008
Total protein (g/dl)	6.8±0.2	6.8 ± 0.21	0	1
Serum Albumin (g/dl)	3.43±0.12	3.54 ± 0.2	3	0.27
A/G ratio	1.1±0.05	1.2 ± 0.11	9	0.07
Fibrosis score	3.0±0.07	3.05±0.07	2	0.24

* Significant changes of melatonin 5mg treated rats compared with silymarin treated rats.

Table (6): Comparison between the effects of silymarin and melatonin (10mg/kg/day orally) on liver function test in ccl4 induced hepatic fibrosed rats (n=6).

Groups / Parameters	Silymarin treated rats	melatonin 5mg treated rats	% change	P-Value
ALT U/L	48± 4.2	32 ± 2.1	-33*	0.000
AST U/L	120.8 ± 7.2	65.8 ± 6.1	-46*	0.000
ALP (U/L)	224.1± 14.4	193 ± 12.5	-14*	0.002
Total protein (g/dl)	6.8±0.2	7.1 ± 0.2	4*	0.026
Serum Albumin (g/dl)	3.43±0.12	3.89 ± 0.12	13*	0.000
A/G ratio	1.1±0.05	1.3 ± 0.1	18*	0.0014
Fibrosis score	3.0±0.07	2.8±0.06	-7*	0.0004

*Significant changes of melatonin 10mg treated rats compared with silymarin treated rats

Table (7): Comparison between the effects of melatonin (5mg/kg/day orally) and melatonin (10mg/kg/day orally) on liver function test in ccl4 induced hepatic fibrosed rats (n=6).

Groups / Parameters	melatonin 5mg treated rats	melatonin 10mg treated rats	% change	P-Value
ALT U/L	41.5 ± 3.5	32 ± 2.1	-23*	0.0002
AST U/L	105.5 ± 10.2	65.8 ± 6.1	-38*	0.000
ALP (U/L)	200.5 ± 10.4	193 ± 12.5	-4	0.28
Total protein (g/dl)	6.8 ± 0.21	7.1 ± 0.2	4	0.29
Serum Albumin (g/dl)	3.54 ± 0.2	3.89 ± 0.12	10*	0.004
A/G ratio	1.2 ± 0.11	1.3 ± 0.1	8	0.13
Fibrosis score	3.05±0.07	2.8±0.06	-8*	0.000

* Significant changes of melatonin 10mg treated rats compared with melatonin 5mg treated rats

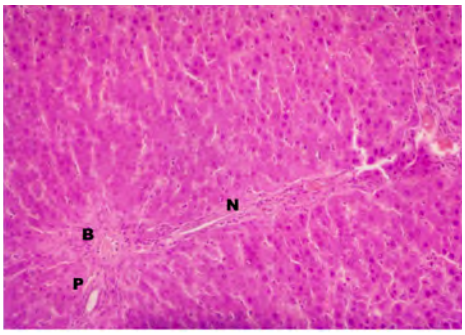

Fig. (1): H.E. section of hepatic tissues of ccl4 induced chronic hepatic fibrosed rats showing dense lymphocytic infiltrate in dilated portal tract (P), dilated blood sinusoids (B) and bridging necrosis (N) (H & E x 40).

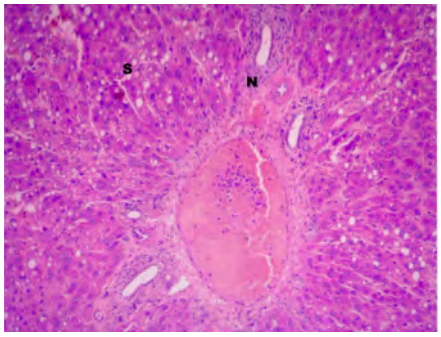

Fig. (2): H.E. section of hepatic tissues of ccl4 induced chronic hepatic fibrosed rats treated with silymarin shown moderate degenerative changes of hepatocytes with early bridging necrosis (N) and focal hepatic steatosis (S) (H & E x 40.

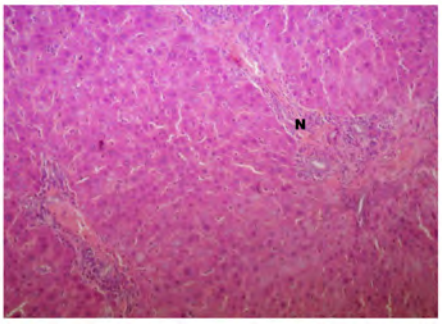

Fig. (3): H.E. section of hepatic tissues of ccl4 induced chronic hepatic fibrosed rats treated with melatonin 5mg (N) the hepatocyte degeneration as well as briding necrosis were decreased (H & E x40).

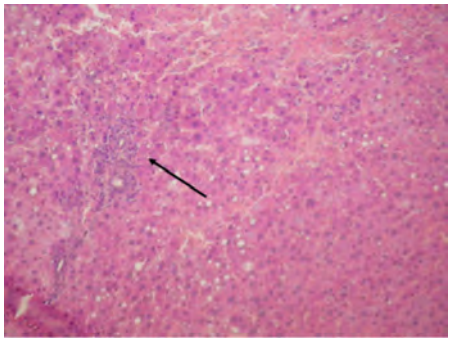

Fig. (4): H.E. section of hepatic tissues of ccl4 induced chronic hepatic fibrosed rats treated with melatonin 10 mg the hepatocyte degeneration, briding necrosis were markedly decreased, as well as focal hepatic steatosis (arrow) (H & E x 40).

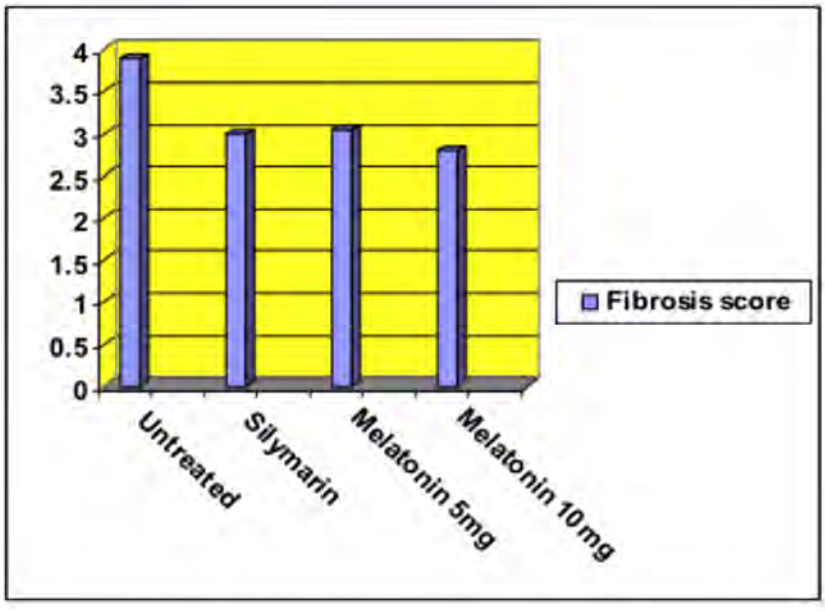

Fig. (5): Fibrosis score among the studied groups.

Discussion

Hepatic fibrosis is a common response to chronic liver injury, during which the normal liver architecture is distorted by scar tissue. The chronic liver injury may result from a number of causes including alcohol, persistent viral infection and metabolic disorders [15]. When advanced fibrosis is occurred causes portal hypertension, liver insufficiency and is a risk factor for developing hepatocellular carcinoma [1].

The data of the present work revealed that 6 weeks after carbon tetra chloride (ccl_4) administration, there was significant elevation in liver enzymes with significant decrease of total protein, serum albumin and A/G ratio. Histopathological examination revealed hepatic alteration in the form of developing thick fibrous septa connecting portal tracts. These results were in agreement with those of [16].

Hong et al. 2009[6] used the same model to investigate the protective effects of melatonin on ccl4 induced hepatic fibrosis in experimental rats.

The mechanisms involved in the development of hepatic fibrosis by (ccl4)were assessed by many investigators, as [16] Who had found that ccl4 is metabolized by cytochrome P450 to form a reactive trichloromethyl radical that triggers a chain of lipid peroxidation. These changes lead to cell injury, and chronic liver injury which leads to excessive deposition of collagen in liver, resulting in liver fibrosis.

In the present study, silymarin was used as a standard clinically hepatoprotective drug for comparing the efficacy of melatonin reducing the fibrogenic effect of ccl4 induced hepatic fibrosed rats. Chronic administration of silymarin had produced a significant improvement in both biochemical tests as well as liver histopathological pattern as proved by many investigators [17]& [18] .

The results of the present study were in agreement with [19] who studied silymarin effects on liver fibrosis model induced by (ccl4)in rats.

Another study by [20] and [21]

who studied the effects of silymarin on the resolution of liver fibrosis induced by carbon tetrachloride in rats, concluded that silmarin significantly decreased the elevation of aspartate aminotransferase (AST), alanine aminotransferase, and alkaline phosphatase in serum .

In the present study Ccl_4 induced hepatic fibroses was improved in both melatonin treated groups and silymarin treated group in comparison to the ccl_4 induced hepatic fibrosed untreated rats, as the hepatocyte degeneration as well as briding necrosis were decreased, without detectable differences between the treated groups, but, silymarin treated group had shown focal hepatic steatosis, that was not detected in other both treated groups. Further decrease in fibrosis score was detected in melatonin 10mg treated group in comparison with other treated groups.

It is well know that oxidative damage can induce hepatic fibrogenesis. Reactive oxygen species (ROS), such as H_2O_2, O_2, and OH, are implicated in the development and pathological progress of hepatic fibrosis [22]. Free radicals and biomolecular reaction products promote phagocytic and myofibroblastic activities. Lipid peroxidation accelerates collagen synthesis by stimulating stellate cells [23].

It has been shown that melatonin is an effective antioxidant and a free radical scavenger. Due to its small size and high lipophilicity, melatonin can crossbiological membranes easily and reach all compartments within the cell, thus protecting DNA, proteins, and biological membrane lipids from the deleterious effects of free radicals[24]. It has been found that melatonin has a higher antoxidant efficiency than vitamin E and L-carnitine [12].

The antioxidant properties of melatonin prevent acute liver injury induced by ischemia- reperfusion, irradiation, bile duct ligation, and toxins. Several lines of evidence suggest that melatonin plays an important role in regulation of collagen levels and inhibition of collagen accumulation.

Oxidative stress plays an important role in the formation of hepatic fibrosis via increasing stellate cell activation and collagen synthesis [23].

In a study by [6] he suggest that treatment with melatonin (5,10mg/kg) could decreases lipid peroxidation and plays an antioxidative role in hepatic fibrosis induced by CCl_4 in rats.

Melatonin is not only a direct antioxidant but also an indirect antioxidant through enhancement of antioxidant enzyme activities in liver. It was reported that melatonin can reduce free radical damage by elevating glutathione peroxidase activation [25 and 26].

Recent studies showed that melatonin exerts its cytoprotective effects in various experimental models of acute liver injury and reduces fibroblast proliferation and collagen synthesis [8] and [27], indicating that melatonin may have therapeutic effects on acute and chronic liver injury, through its antioxidant action[6].

In conclusion, melatonin may have beneficial effects on hepatic fibrosis induced by CCL_4 in rats. The protective effect of melationin on hepatic fibrosis may be related to its antioxidant activities as well as improvement of hepatic steatosis. Melatonin administration at a dose of 10mg more beneficial than melatonin at a dose of 5mg.

Further study was needed to detect any untoward effect of melatonin on other vital body function in animal models then on human being with hepatic impairment. Moreover, the effect possible combination of melatonin with other hepatoprotective and antiviral drugs commonly used in hepatic patients should be deleniated.

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THE POTENTIAL BENEFICIAL EFFECTS OF MELATONIN ON HEPATIC FIBROSIS IN RATS

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LONG TERM OUTCOME OF ISOLATED LIMB INFUSION IN MANAGEMENT OF LOCALLY ADVANCED EXTREMITY SOFT TISSUE SARCOMA.

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Abstract

Background: *We report our long term results of isolated limb infusion (ILI) in cases of locally advanced soft-tissue sarcoma (ASTS) of the extremities.*

Methods: *Forty cases of ASTS received ILI with doxorubicin. Preoperative external beam radiotherapy started within 3-7 days after ILI was administered. After 3-7 weeks, surgery was performed aiming at limb preservation. The long term outcome of these cases (Group I) was reported and compared to the outcome of comparable group of patients followed prospectively and treated by neoadjuvant systemic chemotherapy and external irradiation (Group II).*

Results: *The study included 40 cases in Group I, and 46 cases in Group II. Overall response to preoperative treatment was 85% in Group I versus 43% in Group II. Wide local excision was performed for 75% of Group I patients and in 34% of Group II cases. After a median follow up period of 76 months, local recurrence rate was 35% in Group I and 67% in Group II (P= 0.02). The overall survival rate was 60% in Group I and 35% in Group II (p= 0.008).Only initial response to ILI was associated with overall survival in Group I.*

Conclusion: *The outcome of ILI in management of ASTS is significantly better than systemic NACT in terms of disease free and overall survival.*

Key words: *Isolated limb infusion, extremity soft tissue sarcoma, long term survival.*

Introduction

The management of locally advanced extremity soft tissue sarcomas continues to be a challenge. The prognosis of patients with advanced soft-tissue sarcoma (ASTS) remains poor with a median survival of at best 12 months [1]. Current improvement in patients' quality of life and survival in STS have resulted from contribution of many advances in its management. There have been contributions from centralization of service, multidisciplinary management, improved surgical technique, multimodality therapy and the development of limb sparing surgery. However, controversies surround the main pillars of treatment such as the type of surgery (e.g. indications for limb salvage) and the neo-adjuvant and adjuvant treatment modalities, such as radiation therapy or chemotherapy [2].

Neoadjuvant chemotherapy in treatment of patients with STS is an area of controversies where small progress has been made over the past years. Sarcomas show variability in response to chemotherapy, which emphasizes the heterogeneity of this tumor entity. It is probably that the lack of distinction of sarcoma subtypes with regard to cytostatic therapies might be responsible for the conflicting data we have when patients are treated in a neoadjuvant setting [3].

Data on prognostic and predictive factors for outcome to neoadjuvant chemotherapy are not available from large series. Identification of such factors is essential for patient management and clinical trial design, in particular for STS given its heterogeneity. While tumor size and grade are well-established risk factors for local or systemic recurrence, other factors (e.g. tumor location, histologic type, margin status) have only been vaguely defined, which complicates the development of

evidence-based treatment algorithms [4].

Neoadjuvant isolated regional chemotherapy is an attractive treatment option because it allows much larger doses of chemotherapy to be delivered to the tumor with minimal systemic toxicity. Furthermore, where the tumor responds to the therapy, vital anatomical structures may be able to be preserved at the time of subsequent surgical excision, and previously unresectable tumors may be converted into resectable ones [5]. Isolated limb infusion (ILI) is a minimally invasive technique of delivering regional chemotherapy in ASTS patients. This technique was developed and implemented in the early 1990s by Thompson et al, at Sydney melanoma unit with the objective of obtaining the benefit of conventional isolated limb perfusion (ILP) without incurring its major disadvantages [6].

The authors present their initial results of isolated limb infusion (ILI) in patients with ASTS of the extremities in 2006 [7]. The goal of the present study is to discuss the results of ILI on ASTS cases on the long term (more than 5 years). We compared the survival of ILI cases with a comparable group of patients that received neoadjuvant systemic chemotherapy and external irradiation followed by limb salvage surgery. In addition, analysis for prognostic factors was performed aiming to establish independent predictive factors for best overall response (RR), disease -free survival (DFS) and overall survival (OS).

Patients and Methods

Patients:

The data of the 40 cases that underwent ILI since 2005 was collected and compared to a comparable group of cases that received NACT and external irradiation. Therefore, we retrospectively reviewed the medical records of 234 extremity STS patients treated at our institutions from 1998 to 2004. Inclusion criteria were the following: 1- high grade sarcoma, 2- large size > 5cm, 3- proximity to a neurovascular bundle and/or the bone, and 4- a minimum of 5 years duration since the initiation of treatment. Patients were allocated in one of two groups; isolated limb infusion (ILI) group and

neoadjuvant systemic chemotherapy (NACT) group. Patients with the chemosensitive subtypes Ewing sarcoma, rhabdomyosarcoma, and desmoplastic small round cell tumor were excluded from the study.

Treatment programs:

1- Preoperative isolated limb infusion (ILI) and external irradiation: ILI was performed using the same technique as had been described earlier by Thompson et al [6], and the investigators [7]. In brief, standard radiologic catheters were inserted percutaneously into the axial artery and vein of the disease-bearing limb via the contralateral groin. The catheter tips were positioned at the level of the major feeding vessels of the tumor. Then the contrast medium (urovidio) was injected through the catheter to evaluate the vasculature of the tumor region and to obtain angiographic run, determining the feeding vessel of the tumor (fig 1). When it was confirmed that the position of the angiographic catheter was satisfactory, a pneumatic tourniquet was inflated around the root of the limb to be treated and the cytotoxic agent (doxorubicin 0.7 and 1.4 mg/kg for the upper and the lower limbs, respectively) was infused into the isolated limb via the arterial catheter. For the duration of the ILI procedures (15-25 min), the infusate was then continually circulated by repeated aspiration from the venous catheter and reinjection into the arterial catheter by using a syringe attached to a three-way tap in the circuit. After 15-25 min the limb was flushed with 1 L of Hartman's solution via the arterial catheter. The limb tourniquet was then deflated to restore normal limb circulation and the catheters were removed. External beam radiotherapy (35 Gy in ten fractions) started within 3-7 days after ILI. After 3-7 weeks, limb sparing surgery was performed.

2- Neoadjuvant systemic chemotherapy (NACT) and external irradiation: The systemic chemotherapy of each cycle consisted of doxorubicin (adriamycin) 50 mg/m^2 on day 1, etoposide 125 mg/m2 on days 1 and 4, and ifosfamide 1250 mg/m^2 for 60 min on days 1-4. A total of 3-4 neoadjuvant courses were given

before assessment of the tumor response. External beam radiotherapy (35 Gy in ten fractions) started within 3-7 days after completion of the chemotherapy cycles. All cases were referred to surgery after finishing their neoadjuvant protocols.

Treatment evaluation and statistics :

After neoadjuvant protocols, systemic and limb toxicity and tumor response were assessed regularly. Systemic toxicities were graded according to the World Health Organization (WHO) grading scale. The scale proposed by Wieberdink et al [8] was used to assess limb toxicity. After 3 - 6 weeks, the tumor response was evaluated. Clinical response was assessed as follows: complete response (CR: complete disappearance of all measurable or evaluable tumor for a minimum of 4 weeks), partial response (PR, greater than 50% reduction of the tumor volume lasting at least 4 weeks), minor response (MR, reduction of the tumor volume by less than 50% for at least 4 weeks), stable disease (SD, less than 25% increase in the tumor volume for at least 4 weeks), and progressive disease (PD, an increase of greater than or equal to 25% of the tumor volume and/or occurrence of new lesion). In this study, response to chemotherapy was analyzed as a binary variable: responders were those who were reported as having achieved a complete, partial, or minimal radiologic response (according to the WHO criteria); all other patients were classified as non-responders.

By definition, patients were classes as NED (no evidence of disease) at the time of surgery in cases of R0 (negative margins on frozen section examination), R1 (positive margins on frozen section). Patients with R2 (residual disease), were considered non-NED.

Overall survivals (OS) were measured from the start of treatment until death from any cause or last follow up. Disease free survival (DFS) was defined as the time from start of treatment until radiologic documentation of disease recurrence or last follows up. For the patients not followed up in our institutions or missed a follow

up visit, the referring surgeon or the patient's family was contacted to determine the patient's long term follow up status.

All data were collected prospectively and collected in a computerized database.

Survival curves were calculated by the Kaplan Meier method. Comparison of survival curves were performed using the log rank test (Mantel-Cox). Age, sex, histologic type, grade, site of the tumor, type of surgery and margin status were investigated as potential prognostic factors for OS and DFS by means of a univariate log rank analysis. The independent significance of variables was assessed in a multivariate Cox regression analysis.

Results

Patients' characteristics

Eighty six patients were enrolled in this study; Group I included 40 patients who were treated by preoperative ILI and external irradiation, and Group II included 46 patients who were treated by neoadjuvant systemic chemotherapy and external irradiation. The mean ages (± SD) of the two groups at the time of diagnosis were 42 (±13). Men constituted 71 % of the patient population (n=65). The tumor site was upper extremity (at or beyond the shoulder) in 22% of the patients (n=19) and lower extremity (at or beyond the groin in 78% of patients (n=67). Tumor size was defined according to the pathologic report as the maximum diameter of the tumor. Tumors > 5cm were present in 95% of the patients. Of the 86 patients, 42% (n=36) had low grade tumors and 58% (n =50) were high grade (Table 1).

Response and Surgery results:

In Group I; 34 patients (85%) showed a response to ILI. Twelve cases (30%) showed partial response and 22 (55%) showed minimal response. All patients showed an increase in the extent of necrosis and extensive cystic degeneration of their tumors as detected in MRI. In Group II, overall response to neoadjuvant chemotherapy was 43% (n=20). Radiographic responses consisted of seven patients (15%) with partial response and 13 cases (28%) with minimal response. There was

no complete response in either group.

For the 34 responders in group I and 2 cases that showed stable disease, wide local excision was done in 30 cases (75%) and compartmental excision was needed for 6 cases only (15%). Four cases showed progressive disease; amputation was performed in three cases and the fourth case refused amputation and was referred for EBRT (external beam radiotherapy) and/or systemic chemotherapy. For cases who underwent limb salvage surgery (n= 36), R0 resection was achieved in 29 cases (72.5%) and R1 in 7 cases (17.5%). Only one case in this group needed reconstructive surgical procedure (arterial graft).

In Group II, 20 cases showed response and 17 patients showed stable diseases. These 37 cases underwent surgical excision of their primary tumors. Wide local excision was done in 20 cases (43%), compartmental excision in 15 cases (33%), and debulking in 2 case (4%). R0 resection could be performed in 18 cases (39%), R1 resection was achieved in 17 cases (37%), and R2 in the 2 debulking cases (4%). Seven cases in Group II needed reconstructive procedures (3 arterial grafts, 1 nerve graft, and 3 pedicled fasciocutaneous flaps). Nine cases showed progressive disease on systemic neoadjuvant systemic chemotherapy. Amputation was performed in 6 cases, and the other 3 cases developed systemic metastasis. The surgical outcome of the study groups are summarized in table (2).

Extensive histopathological examination for response after surgery was performed in all specimens. Histologic response was significantly better (P=0.002) in ILI cases (32 cases, 80 %), than in Group II cases (14 cases, 30%).

In group I, 34 cases completed adjuvant chemotherapy, and 6 cases didn't receive any postoperative treatment. In Group II, 42 cases completed adjuvant treatment.

Treatment related toxicity:

During neoadjuvant and adjuvant treatment, nonhaematological toxicity was usually mild

(WHO grade 1); severe side effects were not seen in our study population. The most frequent side effects were alopecia, which was observed for all patients, and nausea, which was seen in 36% of patients. Hematological toxicity mainly consisted of leucopenia and lesser extent thrombocytiopenia. In Group II cases, 44 patients (96%) experienced leucopenia, commonly WHO grade 3 (n= 26, 57%), and grade 4 (n= 10, 22%). The rate of systemic complications was significantly lower in Group I cases (P=0.002) where only 12 cases (30%) developed leucopenia mainly WHO grade 2.

Local morbidity to ILI developed in 12 patients (30%). These were graded using the scale proposed by Wieberdink et al [8]. Eight cases experienced grade 2 complications (slight edema and/or erythema) and four cases experienced grade 3 complications with only one case having slight motility impairment. No long term systemic or local toxicity was encountered in the two groups.

Relapse and survival:

After a median follow up period of 76 months (range: 13- 114 months), ILI cases showed 35% local recurrence rate with a median time of 29 months (range: 11-98). This result was significantly better (P=0.02) than Group II cases that showed a local recurrence rate of 67% with a median time of 13 months (range 9- 102). (Fig. 2).

Univariate analysis of DFS in the whole cohort of patients revealed significantly better survival with cases treated surgically by wide local excision (P= 0.032), and non metastatic cases (P= 0>002). In each group there was no significant difference in local recurrence rates according to age, sex, site of the tumor, size, stage, histologic type, grade, type of surgery or margin status. Only in group I, local recurrence rate was significantly affected by the tumor size after ILI (P=0.004).

Distant metastasis occurred in 40% of group I cases (median time 48 months, range: 14- 104), and in 63% in group II (median time 42 months, range: 7- 96). The difference just failed to show statistical significance (P=0.07). Margin status only was shown by logistic regression to be a prognostic fac-

tor related to metastasis in the whole population of patients (P=0.002). Metastasis was not influenced by any of the study parameters in each group.

At present, 60% of our ILI patients are alive with a median survival of 105 months (range 22-114). This result was significantly better (P=0.03) than Group II cases {35% alive with a median of 82 months (range: 13- 96)} (Fig. 3). In the whole population of patients, overall survival was significantly correlated with tumor size after treatment (P=0.001), type of surgery performed (WLE versus compartmental; P= 0.003) (Fig. 4), margin status (P=0.0059), presence of local recurrence (P=0.0007), and presence of metastasis (P=<0.0001). In group I overall survival was correlated with the tumor size after ILI (P=0.0005), and surgical margin (P=0.04). In group II, OS was correlated only with type of surgery (P=0.04).

The disease free and overall survival events in the study groups are summarized in table (3).

Table (1): Patients characteristics of the 3 groups:

	Group I ILI (n=40)	Group II Systemic CT (n= 46)	P value
Age (mean±SD)	42±10.3	41±14	0.95
Sex			
Male	33	38	0.33
Female	7	8	
Tumor Size (cm)			
Median	11.7	11.3	0.24
range	5-14	7-13	
Tumor site			
Lower limb	35	39	0.31
Upper limb	5	7	
Pathology			
MFH	16	13	0.12
Liposarcoma	18	21	
Fibrosarcoma	12	2	
Spindle cell sarcoma	0	2	
Synovial sarcoma	0	3	
Neurogenic sarcoma	4	5	
Tumor Grade			
Low	22	25	0.46
High	18	21	

Table (2): Surgical outcome of the study groups:

	Group I ILI (n=40)	Group II Systemic CT (n= 46)	P value
Surgery performed:			
Wide local excision	30 (785%)	20 (43%)	0.025
Compartmental excision	6 (15%)	15 (33%)	
Debulking	0	2 (4%)	
Amputation	3 (7.5%)	6 (13%)	
None	1 (2.5%)	3 (7%)	
Resection status in limb sparing surgeries :			
R0	29 (72.5%)	18 (39%)	0.037
R1	7 (17.5%)	17 (37%)	
R2	0 (0%)	2 (4%)	
Reconstruction			
Yes	1 (2.5%)	7 (15%)	0.014
No	39 (97.5%%)	39 (85%)	

Table (3): Disease free and overall survival events in the study groups

	Group I ILI (n=40)	Group II Systemic CT (n= 46)	P value
Local recurrence			
Number (%)	14 (35%)	31 (67%)	0.02
Treatment			
Re-excision	6	6	
CRT	4	2	
Metastasis			
Number (%)	16 (40%)	29 (63%)	0.07
Site			
Lung	10	17	
Skeletal	4	7	
Brain	2	5	
Overall survival			
Alive	24 (60%)	16 (35%)	0.03
Dead	16 (40%)	30(65%)	
Median survival (m)	105	82	

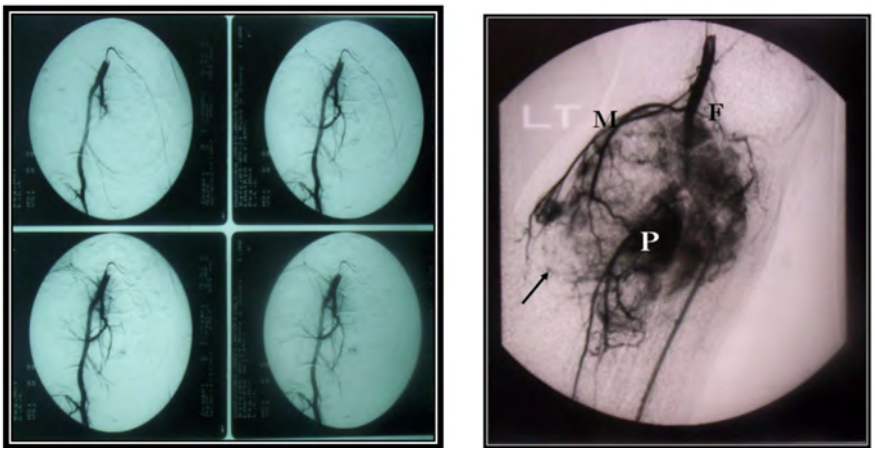

Fig (1) : A. ILI; the angiographic catheter was introduced over the guide wire for enough distance upward into the aorta till reaching bifurcation of the aorta. B. angiogram showing a hypertrophied feeding artery (F), marginal pathologic vessels (M), tumor plush (P), and intratumoral microaneurysms (arrow).

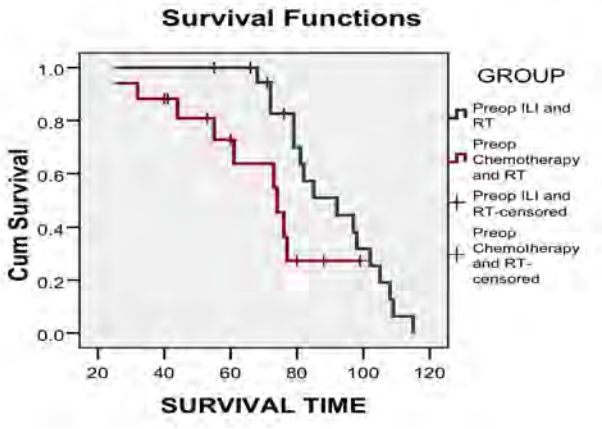

Fig (1) : Kaplan Meir estimate of disease free survival in the studied groups.

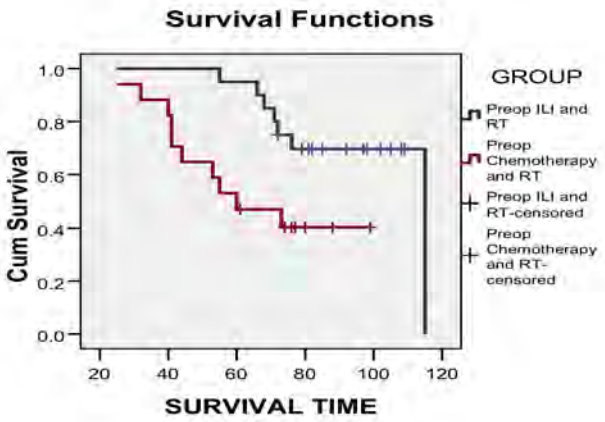

Fig (3) : Kaplan Meir estimate of overall survival in the studied groups.

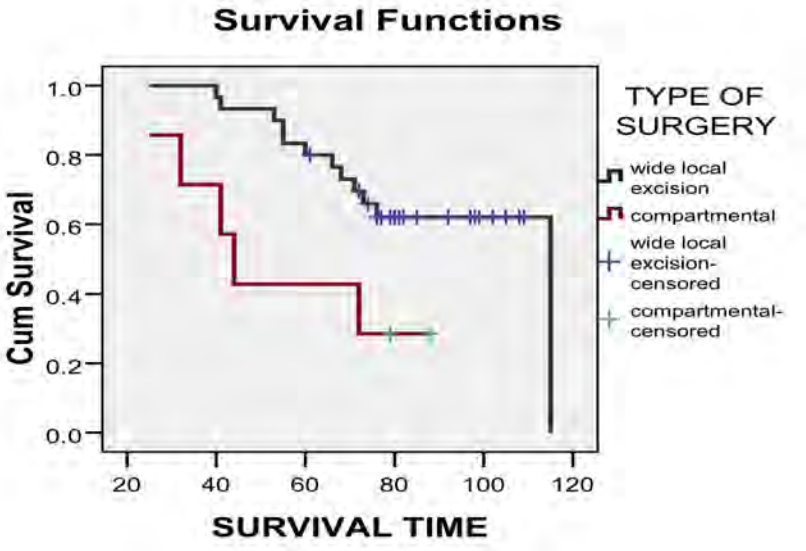

Fig (1) : Correlation between the type of surgery and overall survival in the whole patients' population.

Discussion

Regional chemotherapy is an attractive treatment option for patients with advanced extremity sarcoma. It has been traditionally carried out using a procedure called isolated limb perfusion (ILP), first developed in New Orleans in the mid-1950s [9]. The technique was based on the proposal that vascular isolation and perfusion of the extremity with chemotherapy would allow regional drug concentrations several orders of magnitude higher than could be attained with systemic administration. ILP is technically complex, however, and associated with significant morbidity and cost. Vascular isolation of the extremity for ILP requires surgical exposure and open cannulation of the artery and vein to the extremity. The extremity is then placed on bypass, requiring the presence of a perfusion team and a cardiopulmonary bypass machine in addition to surgical and anesthesia staff. ILI was developed at the Sydney Melanoma Unit (SMU) by Thompson and colleagues [6] as a simple alternative to ILP. Percutaneously placed catheters replace open surgical cannulation and the chemotherapy is recirculated manually so that no pump oxygenator is needed. Operating room time is approximately 1 h, compared with the 4 or 5 h needed to perform ILP. Most importantly, Thompson and colleagues reported that ILI has an efficacy similar to ILP. They reported significant clinical responses in 135 patients treated with ILI, with 41% CR and 44% PR [10].

The authors report their experience with ILI in a cohort of 40 ASTS patients and showed that they can effectively be treated when a combination of pre-operative ILI and radiotherapy is administered. All patients underwent surgical resection 3-7 weeks after the pre-operative treatment [7]. Survival rates were not mentioned in this study because we were convinced that the follow up period was too short to draw conclusions. After a median follow up of 76 months (range: 13-114 months), local recurrence occurred in 35% of cases with a median time of 29 months (range 11-98 months). Distant metastasis occurred in 40% of patients with a median time of 52 months. At

present, 60% of ILI cases are still alive with a median survival of 105 months (range: 22-114 months) (Fig 6). In a Sidney Melanoma Unit based study, the effect of ILI on STS was analyzed in 21 patients. Fourteen (67%) patients underwent ILI as neo-adjuvant therapy prior to surgery and seven patients underwent ILI to treat inoperable recurrences or for palliation. 57% of the patients had a CR and limb salvage was achieved in 76%. Local recurrence rate was 42% with a median recurrence free survival of 25 months. The overall disease specific survival was 62%. A lower stage of disease was a significantly associated with longer survival (P=0.008) [11].

A group from Switzerland reported their experience with ILP with TNF and melphalan for non resectable STS. After a mean follow up period of 38.9 months (4-159), 44% of their patients survived more than 5 years. Local recurrence occurred in 37% of cases and they explained this high rate on one hand by a longer follow up time (more than 3 years) allowing the recurrence registration and on the other hand by a higher proportion of high grade and advanced stage sarcomas. Systemic metastasis occurred in 68% of cases [12]. In 2003, Noorda et al reported 48% 5- years disease free survival, but with 20% grade I and 41% stage I and II tumors [13]. Other series did not mention 5-years survival rates because of a shorter follow up.

In order to document a survival advantage of ILI, we compared our results with a comparable group of ASTS cases that was treated with neoadjuvant systemic chemotherapy.

In the literature, conflicting data have been presented concerning the role of NACT in treatment of high risk extremity sarcomas. Merice et al[14], reported that only 12% of patients responded to chemotherapy enough to facilitate or simplify their tumor resection. Additionally 9% of patients required a larger surgery because of tumor progression despite of ongoing chemotherapy treatment. Menendez et al [15], in a retrospective review, determined that there was no statistical significance in recurrence free survival or overall survi-

val in a group of 82 patients who received three to four cycles of neoadjuvant doxorubicin, ifosfamide and cisplatin. In contrast, Eilber et al. [16] suggested that although the percentage of patients who respond is low, clinical benefit may be seen in a subset of patients. In the small group of patients (14%) that demonstrated a complete response to chemotherapy (95% necrosis), the 10-year local recurrence rate in that group was 11% as compared to 23%, and the 10-year survival rate was 71% as compared to 55%. The current clinical dilemma is that it is impossible to determine which patients will respond to chemotherapy, and unfortunately the percentage of patients that respond to treatment is near equivalent to the number of patients who progress on therapy and require larger resections [17]. In the only randomized clinical trial evaluating the effect of neoadjuvant chemotherapy upon disease-free and overall survival (EORTC 62874), 134 high-risk patients (with tumors of any grade >8 cm, grade II/III tumors <8 cm, or tumors either recurrent or residual from a prior operation) received three cycles of neoadjuvant AI (doxorubicin: 50 mg/m^2/cycle as an intravenous bolus; ifosfamide: 5 g/m^2/cycle as a 24-hr infusion) or surgery alone. The 5-year disease-free survival rate in the chemotherapy arm (56%) was slightly higher than in the control arm (52%) but was not powered for statistical significance [18]. In the present study, the results of systemic NACT are compatible with the previous negative reports. Overall response to treatment was present in 43% of cases. Although limb salvage surgery could be performed in most cases (35 cases, 74%), NACT did not facilitate a wider resection; R1 resection rate was similar to R0 resection (47%). Moreover the only R2 resection in the whole series of patients was among this group. Local recurrence rate was 67%, distant metastasis occurred in 53% of cases, and 35% of them are still alive with a median survival of 82 months.

Age, sex, tumor size, grade and localization are declared as prognostic factors on overall survival in the literature [19]. Positive margin, age and localization of recur-

rences are the prognostic factors for local recurrence [20]. According to this study, the statistically significant prognostic factors influencing the overall survival were: size of tumor after neoadjuvant treatment (reflecting tumor response), type of surgery performed (WLE versus compartmental excision), margin status, presence of local recurrence, and presence of metastasis. Age, sex and tumor location were found to have no impact on overall survival. The factors influencing local recurrence were the type of surgery and presence of metastasis. In Group I cases, the only factor that had a significant impact upon both overall survival and disease free survival was the tumor size after ILI reflecting tumor response. This is consistent with the results of ILP in treating melanoma, where CR was a statistically significant positive prognostic indicator after long term follow up which may reflect a more favorable tumor biology [21]. Moncreiff et al [11] reported that there was a trend for a CR to be associated with improved OS in their series of ASTS cases treated by ILI , but this just fails to reach statistical difference (P=0.07). Meanwhile radiologic tumor response did not affect local recurrence or overall survival. This is consistent with Issels et al [22] who treated 59 ASTS patients with neoadjuvant 4 EIA (etoposide, ifosfamide, and doxorubicin) cycles combined with regional hyperthermia to be followed by surgery and adjuvant treatment. The authors stated that responding patients appeared to have survival rates that were similar to those patients who did not respond to preoperative systemic chemotherapy. In the whole cohort of these patients complaining locally advanced STS, cases that underwent wide local excision had better local recurrence and OS rates. This may be attributed to the fact that the need for a more aggressive surgery to obtain a NED status reflects a less favorable response to preoperative treatment and more aggressive tumor biology.

Conclusion

In 2006, the authors inaugurated ILI as a simple method that provides a novel therapy in order to obtain local control and avoid amputation in cases of limb threatening soft tissue sarcoma.

In this study, assessing the long term outcome of ILI in the treatment of advanced-stage STS, a 12-year experience has been analyzed and presented. ILI cases experienced not only better tumor response rates but also significantly better local recurrence and overall survival rates than a comparable group of cases treated by preoperative systemic chemotherapy. Tumor response to ILI (and not systemic NACT) was associated with a better disease free and overall survival.

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REPRINT

BENHA MEDICAL JOURNAL

LONG TERM OUTCOME OF ISOLATED LIMB INFUSION IN MANAGEMENT OF LOCALLY ADVANCED EXTREMITY SOFT TISSUE SARCOMA

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CHARACTERIZATION OF PARANASAL SINUS MUCINS AND SINUS MUCIN GENE EXPRESSION

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Abstract

Paranasal sinus mucins were isolated and purified by sequential density gradient centrifugation in cesium chloride (CsCl). These mucins were characterized by gel chromatography, electrophoresis on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and their antigenic identity was identified by enzyme linked immune sorbent assay (ELISA). Antigenic identity studies showed that MUC2, MUC5AC and MUC5B were all expressed in the sinus mucus secretions. MUC5B was expressed in all the samples and most of samples showed an inverse relationship between MUC2 and MUC5AC expression i.e. samples with a high level of the MUC5AC mucins have a low level of MUC2 mucin and vice-versa. Polymeric sinus mucins rich in the MUC5AC mucin were largely excluded from Sepharose CL-2B. Mucin reduction and proteolysis (digestion) produced progressively smaller mucin species. On SDS-PAGE the MUC5AC and MUC2 rich sinus mucins behaved differently, in particular on proteolysis the MUC5AC rich mucins produced four distinct digestion products in the running gel. These results suggest two different disease processes in chronic sinusitis.

Key words: *sinusitis, mucin, expression, ELISA, gel chromatography, electrophoresis.*

Introduction

Mucus hypersecretion is one of the major criteria in chronic sinusitis[1,2]. The role of airways mucus is to protect the underlying mucosa preventing drying out, lubricating, insulating and binding micro-organisms. Bound micro-

organisms and particulates are removed by a mucociliary clearance mechanism [3,4].

Mucins are the major viscous components of mucus secretions. Mucins are highly glycosylated (50 - 80% carbohydrate) glycoprotein polymers maintained by disulphide bridges. All the mucins contain tandem repeats (VNTR) corresponding to the highly glycosylated, proteinase resistant regions found in mucins. The composition and sequence of amino acids in the tandem repeats differ markedly between all the secreted mucins [5,6].

The expression of protein backbone is controlled by various genes abbreviated as MUC followed by the number of the gene. To date 20 human mucin genes named MUC1, 2, 3A, 3B, 4, 5AC, 5B, 6-9, 11-13, 15-20 have been distinguished by cDNA cloning and all these genes with the exception of MUC9, 11, 16 and 17 have been shown to be expressed in the airways [7-9].

Mucin secretions of human sinuses are poorly understood, mainly due to the difficulty of obtaining mucus samples. Previous studies have focused on easily accessible nasal tissues such as inferior turbinates[10-13] and nasal polyps [12,14].

This study aims to study the character and antigenic identity of human paranasal sinus mucins. This is an important step to understand sinus physiology and pathology.

We isolated sinus mucins in an undegraded state and their protein composition was studies. The distribution of species was analysed by proteolytic reductive and proteolytic fragmentation. Specific antimucin antibodies were used to determine and quantify mucin expression in sinus mucins.

Methods

Patients and samples :

Human paranasal sinus mucus samples were collected from 9 patients undergoing functional endoscopic sinus surgery or polypectomy by suction through middle meatal antrostomy, antral washout or di-

rectly through the nasal cavity.

Mucin Purification :

Mucus samples were diluted 1 in 10 with 0.067 M sodium phosphate buffer pH 6.7, 4^0C containing proteinase inhibitors (5) and homogenised for 1 min in a Warring blender. Insoluble debris was removed by centrifugation 8000 x g for 1h at 4^0C and the soluble mucin separated from the mucus components by CsCl density gradient centrifugation[15]. The distribution of the mucin rich fractions was initially assessed using a slot blot method[16]. The absolute glycoprotein content was measured using the Periodic acid-Schiff assay (PAS) using papain digested Sigma bovine gastric mucin as standard.

Glycoprotein-rich fractions were pooled (density range 1.44 - 1.53 g/ml) and further purified in a second CsCl density gradient from which the glycoprotein-rich fractions were pooled, dialysed against distilled H_2O, freeze-dried and used as polymeric sinus mucin. The integrity of this preparation was determined by SDS-PAGE.

Immunoassay :

An ELISA was used to identify antigenic identity of sinus mucins. Three antibodies, were used: Anti-human gastric monoclonal antibody NCL- HGM-45 M1 (recognises MUC5AC mucin), LUM2-3 (recognises MUC2 mucin) and TEPA-2 (recognises MUC5B mucin).

Purified mucin samples were blotted onto nitrocellulose sheets (0.45μm) and incubated overnight at 4^0C in phosphate buffer saline (PBS) containing 2% w/v bovine serum albumin (BSA). NCL-HGM-45M1, LUM 2-3 and TEPA-2 were used in dilutions of 1:50, 1:1000 and 1:5000 respectively. Anti-mouse and anti-rabbit perioxidase conjugated IgG were used as secondary antibodies with H_2O_2 as substrate and 3'3' diaminobenzidine for colour development. Antibody binding was quantified using Shimadzu CS930 dual wavelength scanning densitometer at 595 nm.

Polymeric bovine gastric mucin, human colonic mucin and human middle ear mucin were used as standards for MUC5AC, MUC2 and MUC5B respectively.

Mucin fragmentation :

Purified polymeric sinus mucin was fragmented by reduction with dithiothreitol (DTT) or by proteolytic digestion with trypsin.

Reduction: 2mg of polymeric mucin was incubated with 10mM DTT, 20mM tris/HCl, 6M Guanidinium chloride, pH8, 37^0C for 5h. Iodoacetamide 25mM was added to block free-SH groups and incubation continued overnight at room temperature in the dark.

Proteolysis: 2mg of mucin was incubated with bovine trypsin 0.5µg/mg of mucin in 100mM ammonium hydrogen carbonate buffer pH8.0 at 37^0C for 24h,

After extensive dialysis, polymeric and fragmented mucins (2mg/ml) were fractionated by gel filtration chromatography using a Sepharose CL-2B column (125 x 1.5cm), eluted by upward flow with 0.2M NaCl, 0.02% (w/v) NaN_3. Two ml fractions were assayed by PAS assay for glycoprotein. The Kav values were calculated for glycoprotein rich peaks.

SDS-PAGE :

This was performed on polymeric and fragmented mucins before and after gel chromatography. Mucin rich peaks were pooled and dialysed against distilled H_2O for 48h and freeze-dried. Mucin aliquots, 1µl of 5mg/ml in non-reducing loading buffer, were applied to 4-15% polyacrylamide gradient gels. Gels were then stained with PAS and scanned at 555nm using a Shimadzu dual wavelength Chromato scanner.

Results

Over 90% of mucus was solubilised by homogenisation and the insoluble material was not studied further. Mucins contributed 33.6±11.5% of the total non-dialyzable material in sinus mucus. Sinus mucins banded in the buoyant density range 1.44-1.53 g/ml in CsCl density gradients. After two density gradients the mucin fraction was shown to be free of contaminating proteins by SDS-PAGE.

Antigenic identity :

All the antibodies reacted with their respective standards and

with sinus mucins in a concentration dependent manner. MUC5B mucin was expressed in all the samples (Table 1) with a strong expression in 3/9 samples and very low reactivity with 1 sample. The relative reactivity ranged between 13% and 118%. MUC2 was expressed in 7/9 with a wide variation in reactivity ranging from 0-150%. MUC5AC mucin was expressed in 8/9 of samples. The reactivity ranged from 0-260% with a strong expression in 4/9 samples.

An inverse relationship of MUC2 and 5AC expression was noted as MUC5AC expression predominated in samples 1,3,4 and 8 and MUC2 reactivity predominated in samples 2,5,6 and 9 (r = -6). Extreme situation was found in samples 1 and 3 where high levels of MUC5AC expression were associated with no MUC2. Samples 5 and 9 had MUC2 expression 3.8, and 7 times greater than MUC5AC.

Gel Chromatography-size distribution of mucins :

Gel filtration was performed on two of the purified sinus mucin samples; S1 and S3, both of which were rich in the MUC5AC gene product. The elution profiles were identical and shown in Figure 1. It was not possible to chromatograph the other sinus mucin samples as there was not enough remaining after the other analyses.

On Sepharose CL-2B chromatography (Figure 1), 75% of the polymeric sinus mucin was excluded (Kav 0) and the remaining 25% spread into the partially included volume as a trailing edge (fig 1). On reduction with DTT sinus mucin now eluted as two peaks, the first representing 20% of the glycoprotein was excluded. The other 80% of the glycoprotein eluted as a partially included broad second peak (Kav 0.28). Trypsin digestion of the whole mucin produced smaller molecular species than reduction. Trypsin digested mucin eluted as two included peaks. The major one, making up 80% of the glycoprotein loaded had a Kav of 0.47 and the minor peak constituting a shoulder of smaller size material (20% of the glycoprotein, Kav 0.69).

SDS PAGE :

Both MUC2 and 5AC rich sinus mucin samples gave similar profiles, with the majority of the PAS staining remaining at the point of application in the stacking gel with a small amount of PAS staining material migrating to the interface between the stacking and running gels. On reduction the two mucin groups behaved differently. MUC5AC rich mucins showed a small amount, 24% of the PAS staining material remaining at the loading point, with the remaining glycoprotein migrating towards and into the running gel. 44% of the glycoprotein was located between the origin and the interface between the stacking and running gel, the remaining 31% spread from the interface into the running gel. This indicates a change to smaller hydrodynamic size mucin units produced by disulphide bridge cleavage. MUC2 rich samples showed little evidence of any material in the running gel with 84% of the PAS positive material remaining at the origin and 16% migrating into the stacking gel. Trypsin digestion of the MUC5AC rich samples produced four bands at 5.5, 8.5, 10.5 and 13.5mm making up 54, 24, 13 and 9% of the total PAS staining respectively. Trypsin digestion of the MUC2 rich samples did not give distinct running gel species.

Table 1 : Expression of MUC2, MUC5AC and MUC5B in sinus mucins.

Samples	S1	S2	S3	S4	S5	S6	S7	S8	S9
MUC2	0	70%	0	20%	150%	130%	30%	90%	70%
MUC5AC	250%	00%	200%	180%	40%	00%	60%	260%	10%
MUC5B	50%	14%	62%	118%	107%	27%	24%	103%	13%

Sinus mucus samples were collected from 9 chronic sinusitis patients (S1-9). The values quoted are calculated relative to the standard mucin expression being considered as 100%.

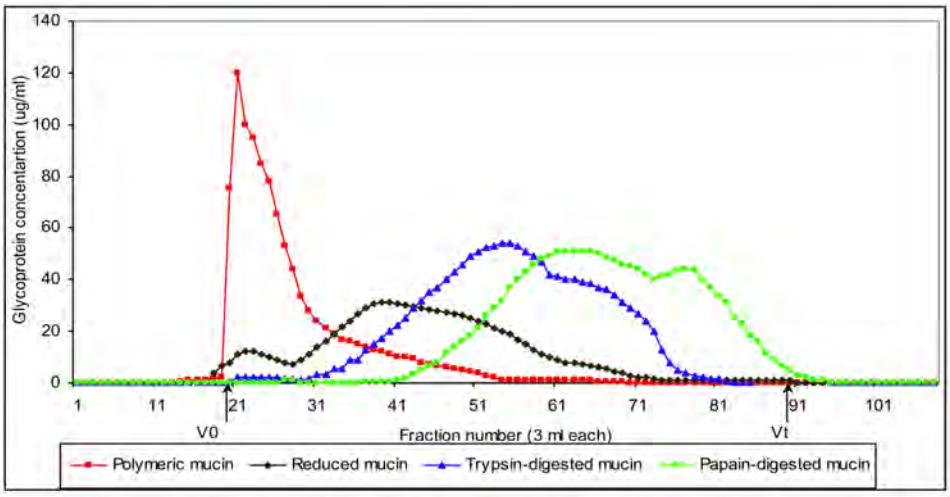

Figure 1. Chromatographic profile of the purified fractionated sinus mucins.

Sepharose CL-2B gel column (125 x 2.5 cm) was eluted by upward flow with 0.2 M sodium chloride containing 0.02 % (w/v) sodium azide and flow rate 18 ml/h. Calibration was firstly performed using 1% (w/v) dextran blue solution containing 0.05 % (w/v) methyl orange. The glycoprotein content was estimated by the PAS solution assay. Vo and Vt are the void and total volumes respectively.

Discussion

Airway secretions including nasal and paranasal sinus mucus consist of mucus and serous components. Mucins are the main component of mucous secretion responsible for mucus viscelastic properties. Studies on the polymeric structure, composition and antigenic identity of sinus mucins have not been performed in detail before. This study describes the isolation and characterization of mucins from the paranasal sinuses in chronic sinusitis. Sinus mucus was solubilised by mild homogenization conditions which solubilised over 90% of the PAS positive material. This method has previously been shown to solubilise ~95% and ~70% of middle ear effusions and colonic mucus scrapings respectively[17-19]. It is unlikely that a significant mucin population present in sinus mucus has been missed. All the sinus mucus samples contained mucins of buoyant densities in the range 1.44-1.53 g/ml and this is characteristic of other mucins purified

under these conditions [19].

ELISA was employed in this study to investigate final mucin glycoprotein expression. Studies measuring mucin expression at the level of m-RNA may not give the correct compositional picture of actual mucin secretion. For example, MUC2 has been reported as a prominent mucin in nasal turbinates[10], nasal polyps[14], tracheal tissue in cystic fibrosis [20] and bronchus[21], based on m-RNA transcription products, yet MUC2 protein could not be detected in respiratory secretions[22].

In this study, sinus mucins from all patients contained MUC5B. Two out of nine samples contained no MUC2 mucin and 3/9 showed a strong expression ~100% or above. Three of the sinus mucin samples showed strong MUC5AC Mucin expression 200% or above compared to standard mucin. The reasons for this greater than 100% reactivity can be explained as follows: 1) Standard mucin antibody might have cross reactivity with other mucins and not be exclusively reactive with one mucin. 2) The difference between bovine and human mucins. Bovine standard mucin has 76% homology with human MUC5AC and thus maybe less reactive. 3) Our studies have shown that only 30-35% of purified bovine gastric mucin is reactive with antihuman gastric antiserum on a weight for weight basis. This highlights the difficulty in obtaining a single purified mucin to use as a standard. However, these standard mucins allow a relative comparison of various mucins expressed although they are not perfect.

The inverse relationship between MUC2 and MUC5AC expression noted in this study suggests the existence of two different inflammatory processes controlling sinus mucin expression: one up regulates MUC2 and down regulates MUC5AC and the other working in the opposite direction. Furthermore, as both MUC2 and 5AC are secretory, gel forming mucins, mucin expression in nasal and sinus mucus might be regulated to a finite pool so that when one mucin is upregulated the other one is downregulated to maintain a biologically stable mucin secretory mucin level. This dif-

ferential expression would explain the conflicting results with m-RNA studies of sinus and polyp mucosa where some studies show MUC5 predominates[14] and others showed MUC2 to be predominant[23] in 3/6 polyps and the other 3/6 polyps had much higher MUC5 than MUC2.

Fragmentation of human sinus mucins followed similar patterns to other mucins[24], polymeric mucins were essentially excluded on Sepharose CL-2B. Gel electrophoresis of whole sinus mucins confirmed the gel filtration studies, in that the MUC5AC rich and the MUC2 rich mucin samples were of large molecular size with the majority of the sample remaining at the point of application. Reduction produced a size change consistent with a subunit structure based on disulphide bridges. Sinus mucin samples subjected to fragmentation and analysis on gel filtration were S1 and S3 which only contained MUC5AC and 5B and no MUC2, both these gene products isolated from respiratory mucus have been shown to have a polymeric structure based on disulphide bridges[25,26]. No reduction data was available on Sepharose CL-2B for the MUC2 rich mucins but SDS-PAGE results were and previous studies have shown that distribution of mucins and mucin glycopeptides on 4-15% gradient gels mirrors that on Sepharose CL-2B gel filtration[27,28]. This is confirmed here with 76% of the PAS positive material migrating from the point of application and 24% remaining as compared to 80% included and 20% excluded on gel filtration of the reduced MUC5AC rich mucins. Therefore the MUC2 rich samples would be expected to give a gel filtration profile, based on the SDS-PAGE results, with the majority of the PAS positive material being excluded, as 84% remains at the point of application. This would be the expected profile for the MUC2 gene product[19]. Trypsin digestion gave two included peaks of smaller hydrodynamic size than those produced by reduction. The antigenic identity of these peaks was not determined but these peaks could reflect the reported different sized glycosylated domains of MUC5B[29]. However there are large amounts of MUC5AC in these samples so it

could be that the MUC5AC gene product also contains different sized glycosylated domains. Evidence for two populations of MUC5AC mucins has been reported in airway secretions[25]. Another interpretation could be that one peak represents the MUC5AC breakdown product and one the MUC5B breakdown product. Proteolysis of sinus mucins gave some very interesting results on SDS-PAGE. This distinct pattern is not a product of MUC2 as there was no MUC2 in these samples and it is very unlikely to be from the MUC5B gene product as both the MUC2 and the MUC5AC rich samples contained MUC5B. This data may provide evidence that the MUC5AC gene product in the sinus has more than one glycosylated units of different sizes or charges or both. However the presence of other mucins in MUC5AC rich mucin pool which could also be deficient in MUC2 rich pool cannot be ruled out.

Conclusion

The results presented here demonstrate that physical characters and fragmentation patterns of sinus mucins are similar to other airway and digestive mucins. At least three mucin gene products are expressed in paranasal sinus mucus and there is an inverse relationship between MUC5AC and MUC2 expression. MUC2 is a major secreted mucin in paranasal sinus mucus. This may well reflect two distinct pathophysiological disease processes in chronic sinusitis.

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CHARACTERIZATION OF PARANASAL SINUS MUCINS AND SINUS MUCIN GENE EXPRESSION

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FACIAL NERVE DECOMPRESSION FOR TRAUMATIC FACIAL NERVE PALSY, A CASE REPORT

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Abstract

Introduction: *Facial nerve palsy is common association with temporal bone fractures. Immediate complete traumatic facial nerve palsy is a bad prognosticator for facial nerve recovery.*

Aim of the work: *In this study we aimed to present and show feasibility of facial nerve decompression in cases of immediate complete post traumatic palsy.*

Case Study: *A 14 years old male patient with immediate complete post ttraumatic facial nerve palsy planned for tympanic and mastoid segments decompression.*

Conclusion: *We'll continue to offer this technique of facial nerve decompression through facial recess approach for patients with complete traumatic facial nereve palsy.*

Key Words: *Facial palsy; facial nerve decompression; facial recess.*

Introduction

Facial paralysis is a devastating condition. Lack of facial expression is an aesthetic and profound functional and psychological disability. Therefore, maintaining or restitution of facial nerve function is a great concern to both patients and surgeons.

Facial nerve may be traumatized as an association with head trauma where it accounts for 2 to 3% in general or 7 to 10% when temporal bone trauma is considered [1-5].

Methods for exploration of the Facial nerve have been developing

progressively and probably the most important advance in improving the outcome of facial nerve exploration and repair is the development of the operating microscope, surgical micro-drills, needle and suture materials, and improved surgical technique[6].

Restitution of facial nerve function could be through direct primary repair of the facial nerve, decompression, graft repair, free nerve transplantation (interposition graft), or neurotization techniques [7].

Case Report

A 14 years old male patient presented to the neurosurgery Department, Mansoura Emergency Centre with blunt head trauma resulting in immediate complete right facial nerve palsy. Temporal bone CT revealed longitudinal temporal bone fracture and electrophysiological studies, done two weeks later revealed complete denervation picture.

We planned tympanic and mastoid segments decompression via classic mastoidectomy and facial recess approach.

The incision is along postauricular sulcus, figure (1). Subcutaneous dissection to identify boney landmarks, figure (2). Classic mastoid drilling resulting in identification of mastoid antrum, lateral semicircular canal, digastric ridge, facial nerve, and chorda tympani, figure (3).

Opening the facial recess between facial nerve, chorda tympani leading to middle ear cavity, figure (4).

Drilling of the fallopian canal to expose the facial nerve in tympanic and finally mastoid segments.

Opening facial nerve sheath and exploration of the nerve. No nerve cut was observed, but diffuse nerve swelling, figure (5).

Closure in layers and patient is discharged on the second day and instructed on physiotherapy and nerve tonics with appropriate eye protection measures.

Follow up at week one for stitches removal and checking

middle ear, then at two months interval.

The patient started to regain his facial muscles tone at month four postoperative and achieved House-Brackmann GIII recovery at month six.

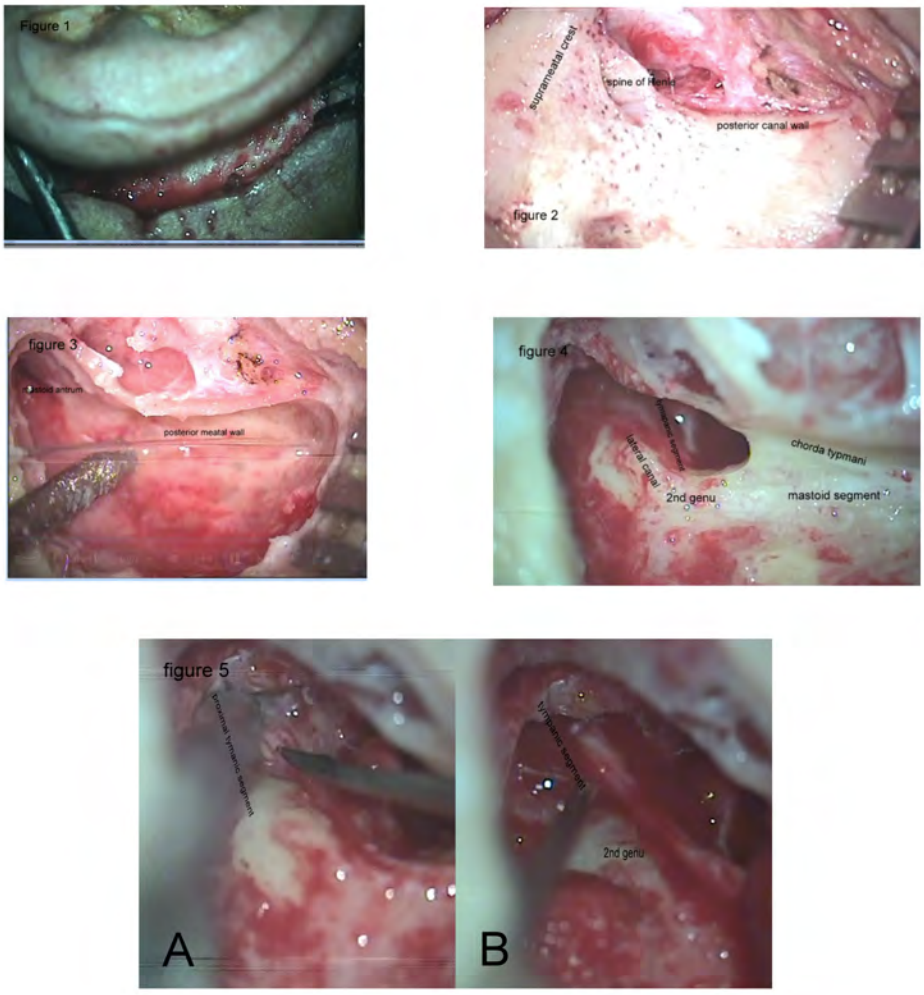

Fig. 1 : Postauricular skin incision, Figure (2) subcutaneous dissection, Figure (3) mastoidectomy, Figure (4) opening facial recess, Figure (5) a, opening facial nerve sheath, b, after decompression revealing diffuse edema.

Discussion

Post-traumatic facial palsy being complete at any time is considered a bad prognosticator if occur at any time throughout the course of recovery. It is stated that in the acute setting it is impossible to tell which patients will fully recover or require operative intervention[8].

Clinically, immediate onset of the facial paralysis and imaging of disruption of the bone canals through which the facial nerve travels or of bone fragments displaced into these canals often is an indication for surgery[9,10].

Several surgical approaches are described for facial decompression surgeries. Based on hearing status, if intact, combined approaches are described; temporal fossa approach to decompress the labyrinthine ± tympanic segment and trans-mastoid extra-labyrinthine approach to decompress the mastoid segment.

In cases of hearing loss, transmastoid trans- labyrinthine approach may decompress all the three segments[11-13].

The whole three segments decompression is advocated based on that the narrowest regions of the facial canal are the meatal foramen and the part of the second genu near the stapes, which is grossly termed the perigeniculate region[14].

Our policy was to explore the fracture line based on the preoperative radiology and to decompress the tympanic and mastoid segments. We tried to deal with the proposed site of injury, the policy which is supported by other series [9,15]. In addition we are usually able to decompress the distal perigeniculate region and preserve the labyrinth. The argument that this approach are narrow to deal with nerve cut, is no longer supported as fibrin glue will enable strong repair with equal results compared to sutures[16].

Conclusion

We could successfully achieve decompression of tympanic and mastoid facial nerve segments through a limited transmastoid approach. The patient achieved accepted recovery at relatively short follow-up period.

We believe that there is much needed to be done in the future in relation to more targeted approach in patient selection to come up with standard indications for exploration.

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INFLAMMATORY MARKERS OF CARDIOVASCULAR RISK AMONG DIABETIC PATIENTS WITH NEUROPATHIC FOOT ULCERATION

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Abstract

Cardiovascular disease (CVD) had been reported in patients with neuropathic foot ulceration (NFU) without clear explanation .Inflammation is thought to be one of the major processes contributing to atherosclerosis. High-sensitivity CRP (hsCRP) had been shown to predict future CVD in a variety of clinical settings regardless of traditional cardiovascular risk factors. The aim of this work was to study serum levels of hsCRP and determine the clinical variables that could be associated with elevated levels in diabetic patients with NFU. The study included three groups of patients: the neuropathic group (forty-one diabetic patients with NFU; 52.1+5.4 yrs; M/F 20/21), the diabetic group (twenty diabetic patients without clinical evidence of peripheral nerve dysfunction; 49+6.6 yrs; M/F 9/11) and the control group (twenty healthy subjects of matched age and sex; 49.6+5.2 yrs; M/F 10/10). Subjects with PAD, smoking, obvious inflammatory or infectious conditions were excluded. Serum hsCRP concentrations were measured using immunoenzymometric assay (Monobind Inc., Lake forest, CA 92630, USA). Subjects with serum CRP levels greater than10 µg/ml were excluded from the analysis in order to exclude possible exogenous acute-phase stimuli. Results: serum hs CRP levels were significantly higher in the neuropathic group (5.1+2.4 µg/ml) in comparison to the diabetic or

the control group (3.9+1.6 and 2.4+1.6 µg/ml respectively). hsCRP correlated with known duration of diabetes (r=0.466, P 0.002) and body mass index (r=0.362, P 0.02) but not with age, ulcer size or duration, systolic or diastolic blood pressure. Nearly two-thirds of patients with NFU (65.9%) were considered as high risk for CVD using 3 µg/ml cut-off values of hsCRP. Patients with hsCRP >3 µg/ml were more obese (BMI 39.9+7.5) with female predominance (F/M 18/9) in comparison to the rest of the patients (BMI 33.2+5.4 and F/M 3/11). It is concluded that hsCRP is elevated in diabetic patients with uncomplicated NFU especially in obese women with long duration of diabetes. About two-thirds of diabetic patients with NFU had elevated hsCRP levels to the degree that will categorize them as high risk for CVD.

Introduction

Previous studies revealed increased cardiovascular morbidity and mortality in diabetic patients with ischemic foot ulcerations[1]. The widespread nature of the atherosclerotic arterial disease can simply explain the cardiac affection in patients with ischemic ulcers. However, it seems that the same hold true for the neuropathic foot ulceration as well. Pinto and his associates (2008) reported high mortality among patients with neuropathic ulcers[2]. Also, high mortality was demonstrated in patients with chronic Charcot foot [3,4]. Van Baal (2010), additionally, spotted the light on increased mortality allied to acute Charcot foot[5]. Furthermore, adopting an aggressive cardiovascular risk management improve survival of all types of diabetic foot ulcer afterwards[6]. Peripheral neuropathy could be the culprit for high mortality in Charcot arthropathy and uncomplicated neuropathic ulcer. However, the underlying mechanism is not yet clear. Subclinical inflammation had been recently acknowledged as a principle mechanism contributing to atherogenesis development. hsCRP had been shown to predict future CVD in a variety of clinical settings regardless of traditional cardiovascular risk factors[10]. It is therefore conceivable for inflammation-related

molecules such as CRP to be advocated as markers of atherogenesis and its complications.

This prospective case control study aimed to determine:

1- Wether serum levels of hsCRP will be different in diabetic patients with NFU from diabetic patients without PN dysfunction or healthy subjects.

2- To establish some of the potential determinants of raised CRP concentrations in diabetic patients.

Subjects and Methods

The study comprised of 81 patients among those attending the outpatient clinic of Diabetic Foot Unit, Specialized Hospital for Medicine, Mansoura University, Egypt in the period from January 2009 till January 2010. Patients were belonging to three distinct groups: a diabetic neuropathy group with neuropathic foot ulcer (41 patients), a diabetic group (20 patients without clinical evidence of peripheral nerve dysfunction) and a control group of healthy subjects of matched age and sex (20 persons). All subjects included in the study were subjected to examination by Neurothesiometer (Bailey Instruments Ltd, UK) for assessing and determining vibration sensitivity thresholds. Patients in the neuropathic group should have vibration perception threshold (VPT) >25 V at the apex of the hallux, while subjects in the diabetic and control group had VPT < 25 V. All subjects were tested by vascular Doppler (Hadeco Bidop ES-100V3, Kawasaki, Japan) for peripheral blood flow and should have Ankle brachial pressure Index > 0.9 and Toe brachial index > 0.75 to verify peripheral arterial diseases before inclusion in the study. Serum hsCRP concentrations were measured in all subjects using immunoenzymometric assay (Monobind Inc., Lake forest, CA 92630, USA).

Exclusion criteria in this study included patients with pre-diagnosed peripheral arterial disease, smokers, those with inflammatory conditions including infection, CRP> 10 µg/ml (in order to exclude possible exogenous acute-phase stimuli), and those who were on acetyl salicylates or statin

treatment within the previous 30 days.

Statistical analysis

Data analysis was performed using SPSS version 13. Results were expressed in the form of mean ± SD and percentages. The chi-squared test was used to compare proportions. Student's t test and one-way analysis of variance (ANOVA) were used to compare means, as appropriate. Statistical significance was set at $p < 0.05$.

Results

Table (1) for baseline clinical characteristics showed that all subjects in different groups were matched for age, sex and BMI. Both systolic and diastolic blood pressures were significantly higher in the neuropathic and non neuropathic groups. Duration of diabetes was significantly longer in the neuropathic group in comparison to the diabetic group. All patients in the neuropathic groups were on insulin while 13 out of the 20 patients in the diabetic group were maintained on insulin.

In this study, we adopted the classification of risk groups based on hs-CRP serum levels proposed by the American Heart Association (AHA) and the Centre for Disease Control (CDC) 2003 which identified 3 groups 11.

1. Low risk group with CRP concentrations less than 1 mg/l
2. Moderate risk group with CRP between 1 and 3 mg/l
3. High risk group when CRP is higher than 3 mg/l.

Based on CRP risk categorization, subjects in the control group were classified as low or moderate risk. All subjects in the neuropathic and the diabetic groups were belonging to both moderate and high risk categories and the majority of patients in the neuropathic group belonged to the high risk category. About two-thirds of diabetic patients with neuropathic foot ulcers (65.9%) had high levels of hs-CRP, to the degree that will categorize them as high risk for cardiovascular disease. There was a significant increase of hs-CRP in diabetic patients with neuropathic foot ulceration (5.1± 2.4 ug/ml) in comparison to either diabetic non neuropathic patients or the con-

trol group (3.9± 1.6 and 2.4 ±1.6 ug/ml respectively).

Table (3) showed the association between hs-CRP risk categories and clinical variables studied to establish some of the potential determinants of raised CRP concentrations in diabetic patients with NFU. Patients with hsCRP >3 µg/ml were more obese (BMI 39.9 ±7.5) with female predominance (F/M= 18/9) in comparison to the rest of the patients. There was a significant correlation with the duration of diabetes, gender and body mass index. Non significant correlation was found with age, ulcer size, and ulcer duration, systolic or diastolic blood pressure. Patients with CRP levels more than 3µg/ml had longer duration of diabetes in comparison to patients with moderate risk.

Table (1) Characteristics of the study and control groups

	Neuropathic group	Non neuropathic Group	Control Group	p	LSD
Number (n)	41	20	20	NS	
Age (years)	52.1 (±5.4)	49 (±6.6)	49.6 (±5.2)	NS	
Sex (Male/Female)	20/21	9/11	10/10	NS	
BMI	37.6 (±7.5)	37.5 (±9.3)	34.1 (±6.3)	NS	
Systolic BP (mmHg)	143.7(±24.6)	142.8(±23.3)	124 (±12.3)	0.004	18.75
Diastolic BP (mmHg)	88.1 (±12.5)	87.5(±12.1)	80.5(±6.3)	0.043	7.55
Duration of DM (yrs)	15.7 (±6.6)	7.6 (±5.2)	-	0.000	
Treatment (Ins/OHA)	41/0	13/7	-	0.000	

Table (2) DFU patients with CRP >3 µg/ml versus those with <3 µg/ml

	hsCRP < 3 µg/ml	**hsCRP > 3 µg/ml**
Number	14	27
Age (yrs)	52.6 (3.7)	51.8 (6.1)
Sex (M/F)	11/3	9/18*
Duration of Diabetes (yrs)	13.1 (6.4)	17.1 (6.4)
BMI	33.2 (5.4)	39.9 (7.5)**
Ulcer area (mm^2)	198.9 (266.1)	408.1 (624.9)
Ulcer Duration (months)	29.1 (35.7)	24 (28.9)
Systolic BP (mmHg)	143.6 (24.1)	143.7 (25.3)
Diastolic BP (mmHg)	88.6 (13.5)	87.8 (12.2)

* p= 0.009 ** p= 0.002

Table (3) Correlation of hsCRP with clinical variables in the neuropathic group

	R	**P**
Age	0.122	NS
Sex	0.390	0.012
Duration of diabetes	0.451	0.003
Ulcer duration	0.148	NS
Ulcer Area	0.038	NS
Systolic BP	0.195	NS
Diastolic BP	0.111	NS
BMI	0.437	0.004

Discussion

The hs-CRP is a golden inflammatory marker that has been proposed to be a more sensitive predictor of coronary heart disease than LDL itself. It is a proxy marker of subclinical inflammation which represents a chronic low grade inflammation in the arterial wall [12,13]. To our knowledge, this prospective case control study was the first report about the possible relation between hs-CRP and diabetic patients with neuropathic foot ulcer (NFU).

In this study, hs-CRP was significantly increased in diabetic patients with neuropathic foot ulceration. Furthermore, the hs-CRP response was also positively correlated with duration of diabetes which comes in agreement with previous studies [14,15]. This strong correlation between the concentration of hs-CRP and duration of diabetes is possibly a result of the chronic metabolic derangements of diabetes on CRP production.

The present study reported that women had higher serum levels of CRP in comparison to men and the effect of gender on CRP was only noticed in the neuropathic group. Nevertheless, the small number of subjects in the diabetic and control group (n= 20) made it difficult to come to a definite conclusion about the effect of gender. The gender differences might be related to the body mass index (BMI) which was high in our study groups (34.1-37.6). This study showed a strong correlation between hs-CRP and BMI. This was similar to previous studies which revealed higher serum levels of CRP in obese women with uncontrolled blood glucose [16]. This correlation could be explained by the fact that increased BMI is usually associated with insulin resistance and metabolic syndrome with underlying subclinical inflammation. Also increased abdominal adiposity associated with increased BMI is the other major determinant of hepatic CRP synthesis via production of proinflammatory cytokines (interleukin [IL]-6, IL-1, and tumor necrosis factor-μ [17]. On the other hand, Levinson (2005) believed that hs-CRP is a non specific inflammatory marker as its level could be affected by smoking, age, obesity, hormone replacement therapy (HRT) [18]. Ford et al.

(2004) found that 40% of women between ages 30–39 have hs-CRP concentrations >3.3μ/L, and this high percentage was similar in normal women and those treated with HRT [19].

We are aware of the shortcomings of this pilot trial. The study comprised of a relatively small number of patients especially in the diabetic group. We should have measured A1c, urinary albumin excretion and traditional risk factors for CHD that can represent an important confounding variables. Some of the data are obtained by self-report e.g. the duration of diabetes, use of anti-inflammatory medications, and smoking status. We have no data about oral contraceptive treatment and menopausal status. All these important variables should be included in a bigger cohort study in the future.

Overall, from these preliminary data, it can be concluded that hsCRP is elevated in diabetic patients with uncomplicated NFU. About two-thirds of diabetic patients with NFU had elevated hsCRP levels to the degree that will categorize them as high risk for CVD. Long duration of diabetes and female sex were the main determinants of raised CRP concentrations in diabetic patients with NFU. We suggest that identification of nontraditional risk factors for cardiovascular diseases such as high hs-CRP should be an integral part of the multidisciplinary foot care in order to save not only limbs but also lives.

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INFLAMMATORY MARKERS OF CARDIOVASCULAR RISK AMONG DIABETIC PATIENTS WITH NEUROPATHIC FOOT ULCERATION

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PREDICTIVE VALUE OF ADIPONECTIN TO LEPTIN RATIO FOR DIAGNOSIS OF STEATOHEPATITIS IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

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Abstract

Background: *Discrimination between non-alcoholic steatohepatitis (NASH) and simple steatosis (SS) is critical for proper management. Adiponectin to leptin ratio (A/L) might be of value for the diagnosis of NASH.*

Aim: *to assess the value of serum levels of adiponectin, leptin and their ratio (A/L) for diagnosis of steatohepatitis in patients with non-alcoholic fatty liver disease.*

Material and methods: *A total of 46 patients with biopsy-proven non-alcoholic fatty liver disease (25 with NASH and 21 with SS) and 22 control subjects with age, sex and body mass index were included. In all subjects fasting serum insulin, glucose, liver transaminases, triglycerides, cholesterol, leptin, adiponectin and Homeostasis Model Assessment Method (HOMA) were determined.*

Results: *Leptin levels were significantly higher in patients with NASH and SS compared to controls (P1<0.0001). Adiponectin levels were significantly lower in NASH compared to controls (P1<0.001). No significant difference in adiponectin was found between patients with NASH vs SS (P>0.05) and no difference between those with SS compared to controls (P>0.05). The mean A/L ratio was significantly lower in NASH compared to SS and controls (P<0.001 and P<0.0001 respec-*

tively). The AUROC curve for A/L ratio to distinguish between NASH and SS was 0.85. At a cut-off value of 0.71, the sensitivity was 80%, specificity 77%, PPV 78%, NPV 79% and accuracy 79%. In multivariate analysis, both A/L ratio <0.71 and HOMA index >3 were independently associated with NASH. Leptin levels were positively correlated with HOMA index (r= 0.51, P<0.003) and BMI (r=0.32 and p<0.02). The A/L ratio was negatively correlated with HOMA index (r= -0.6, P<0.001) and BMI (r=-0.4 and p<0.002). In Univariate analysis, fibrosis was significantly associated with age, BMI, hyperglycemia and serum leptin levels. In multivariate analysis, age and BMI were independent factors associated with fibrosis.

Conclusion: *The A/L ratio has a good predictive value for diagnosis of NASH and could be utilized as a non-invasive test for assessment of liver injury in patients with NAFLD.*

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome.[1,2] NAFLD is the most common cause of liver test abnormalities among adults[3-5] with an estimated prevalence of 10-24% in the general population. The estimated prevalence of non-alcoholic steatohepatitis (NASH) is between 3-5%.[3,6] Excessive hepatic deposition of free fatty acids and triglycerides in the hepatic parenchyma is the patholo-gical hallmark in patients with NAFLD.[6,7] The pathological spectrum of NAFLD ranges from simple steatosis (SS) on the benign end of the spectrum with progression to NASH, an intermediate stage of the disease, and increa-sed risk for progression to cirrhosis, need for transplant and/or hepatocellular carcinoma in a minority of patients with NAFLD.[8,9]

For proper management, distinction between SS and NASH is critical.[4,8] Currently the only definitive diagnostic test for the progressive forms of NAFLD is liver biopsy.[10,11] However, due to the increased cost, possible risk, and health-care resource utilization, an invasive liver biopsy is poorly suited as a diagnostic test for such a prevalent condition.

Furthermore, the histological lesions of NASH are unevenly distributed throughout the liver parenchyma; therefore, sampling error of liver biopsy can result in substantial stratification and staging inaccuracies. [12]

White adipose tissue-secreted adipokines were found to have a pathogenic role in the progression of NASH.[13,14] Among these adipokines, leptin has been shown to exert pro-inflammatory effects while adiponectin has been shown to exert anti-inflammatory effects at the liver level.[15] Leptin has been suggested to promote fibrogenesis[16] while adiponectin has antifibrotic and antisteatotic effects in the liver.[17,18] It has been suggested that serum levels of leptin and adiponectin could predict the severity of liver injury in patients with NAFLD. A relationship between leptin and SS has been reported[19,20] while a relation between leptin and NASH has not been well documented.[21]

Several studies have reported hypoadiponectemia in patients with SS compared to controls[22-27] Hui et al.[28] found a decreased level of adiponectin in patients with NASH compared to patients with SS. However, this finding was not confirmed by subsequent studies. [23,29,30]

Because leptin and adiponectin exert opposite effects on liver fibrogenesis and inflammation, their ratio has been suggested to be a more sensitive marker than their individual values for the diagnosis of NASH compared with SS. [31]

This study was conducted to determine the value of adiponectin, leptin and their ratio (A/L) in patients with NASH and SS compared to a control group and their relation to the underlying histopathological changes.

Patients and Methods

This study was prospectively conducted on 46 patients with NAFLD (28 males and 18 females) and 22 control subjects (15 males and 7 females), matched for age, sex and body mass index. Patients were recruited from Mansoura University Hospital from March 2009 to April 2011. The control group was healthy blood donors,

free from hepatic, neoplastic and endocrine diseases, had no history of alcohol consumption and normal ultrasonographic hepatic echopattern. Diagnosis of NAFLD was based on the following criteria : (1) Chronic elevation of transaminases (> 1.5 times the upper normal value for more than 3 months. (2) No alcohol consumption. (3) No history of intake of hepatotoxic drugs. (4) Exclusion of chronic liver diseases with the appropriate tests: viral hepatitis B and C, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, biliary obstruction, Wilson's disease, haemochromatosis and alpha-1-antitrypsin deficiency. (5) Bright liver at ultrasound scanning. In all patients, diagnosis of NAFLD was confirmed by liver biopsy. All patients and controls gave written informed consent.

All patients were on unrestricted dietary regimen and had no history of intake of lipid lowering medications or metformin. The following data were recorded at the time of liver biopsy: age, sex, BMI, history of diabetes mellitus, hypertension or hyperlipidemia.

After an over night fast, a venous blood sample for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), fasting blood glucose, insulin, total cholesterol, low density lipoprotein (was calculated according to Friedwold formula, 1973) (LDL), high density lipoprotein (HDL), triglycerides, adiponectin and leptin were collected at 08.00-09.00. Serum immediately separated and an aliquot was kept frozen at -80C until analysis of insulin, leptin and adiponectin. The liver enzymes and lipid profile markers were measured by automated enzymatic methods, glucose levels were measured by glucose-oxidase based kits using Hitachi 911 analyser, Roche, Germany). Insulin and leptin were measured by enzyme amplified sensitivity immunoassay kits (Biosource, Belgium) according to the manufacturer's instructions.

Insulin resistance was assessed by the Homeostasis Model Assessment Method (HOMA) as follows: Fasting insulin (mIU/ml x Fasting plasma glucose (mmol/L)/

22.5.[32] HOMA > 5 was taken as an indicator of insulin resistance.

Pathology:

Liver biopsy specimens were fixed in formalin, stained with haematoxylin & eosin and Masson trichome. The degree of steatosis was assessed and graded according to Brunt et al[33] as follows: 0= none, 1= <5%, 2=6-33%, 3= 34-66% of hepatocytes affected. Pattern of steatosis, whether, macro or microvesicular as well as presence of zonal distribution were recorded. Fibrosis was graded as follows: F0, absent; F1, perisinusoidal/pericellular fibrosis; F2, periportal fibrosis; F3, bridging fibrosis; F4, cirrhosis. Lobular necrosis was graded as follows: 0= absent; 1, less than one necrosis injury per lobule; 2, one or more necrosis injury per lobule; 3, more than 2 necrosis injuries per lobule. Hepatocyte ballooning was graded 0-2 where 0= absent, 1= ballooned hepatocytes in less the 50% of lobules, 2= ballooned hepatocytes in more than 50% of lobules. Presence of polymorph nuclear inflammatory cells in intimate relation to steatotic hepatocytes was the minimum criterion to diagnose steatohepatitis. Steatohepatitis was graded according to Kleiner et al.[34] NAFLD activity score (0-8): score 5 or >5 (NASH), <3 (not NASH), 3&4 (probable Steatohepatitis).

Statistical analysis:

The statistical analysis of data was done by using **SPSS** (SPSS, Inc, Chicago, IL) program statistical package for social science version 10. To test the normality of data distribution K-S (Kolmogorov-Smirnov) test was done. N.B: all tested data revealed to be parametric. The description of the data was done in form of mean (+/-) SD for quantitative data and frequency & 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 for comparison between two groups. Chi square test was used for qualitative data. Correlation coefficiency was done to detect association between variables. P is significant if < or = 0.05 at confidence interval 95%.

Results

Clinical and demographic data

are summarized in table (1). The prevalence of diabetes mellitus, hypertension, hyperlipidemia and BMI were significantly higher in patients with NASH compared to patients with simple steatosis and controls. Serum ALT, AST, total cholesterol, LDL-C, TG, fasting blood sugar, insulin and HOMA index were significantly higher in patients with NASH compared to patients with simple steatosis and controls. Moreover, serum ALT, AST, albumin, total cholesterol, TG, insulin and HOMA index were significantly higher in patients with simple steatosis compared to controls (Table 2).

Serum leptin levels were significantly higher in patients with NASH compared to controls (P1<0.0001). Also, leptin levels were significantly higher in patients with SS compared to controls (P1<0.0001). No significant difference in leptin levels were found between patients with NASH and those with SS (P2>0.05). Adiponectin levels were significantly lower in patients with NASH compared to controls (P1<0.001), but there were no significant difference between patients with NASH and those with SS (P2>0.05) and no difference between those with SS compared to controls (P>0.05) (Table 3).

The mean A/L ratio was significantly lower in patients with NASH compared to patients with simple steatosis and controls (P2<0.001and P1<0.0001 respectively). Also, the mean A/L ratio was significantly lower in patients with simple steatosis compared to controls (P<0.0001) (Table 3). Fig.1 showed the mean A/L ratio of the studied groups. The mean A/L ratio was negatively correlated with HOMA index (r= -0.6, P<0.001) and BMI (r=-0.4 and p<0.002). Serum leptin levels were positively correlated with HOMA index (r= 0.51, P<0.003) and BMI (r=0.32 and p<0.02). Serum adiponectin levels were negatively correlated with HOMA index (r= -0.29, P<0.03) and BMI (r= -0.36 and p<0.04).

The serum ALT levels were not correlated with serum leptin, adiponectin and A/L ratio. The AUROC curve for A/L ratio to distinguish between NASH and simple steatosis were 0.85. At a cut-off

value of 0.71, the sensitivity was 80%, specificity 77%, PPV 78%, NPV 79% and accuracy 79% (Fig. 2). In multivariate analysis, both A/L ratio < 0.71 and HOMA index >3 were independently associated with NASH.

Among the 25 patients with NASH, no or mild fibrosis was found in 18(72%) patients, 7 (28%) had significant fibrosis (stage 2 or more) including one patient with established cirrhosis. The degree of steatosis was significantly higher in patients with NASH compared to patients with simple steatosis (severe steatosis: 44% vs 9.5%). (Table 4) In univariate analysis, fibrosis was significantly associated with age, BMI, hyperglycemia and serum leptin levels. In multivariate analysis, age and BMI were independent factors associated with fibrosis. (Table 5)

Table 1: Clinical characteristics of studied groups.

	Contol (n=22)	SS (n=21)	NASH (n= 25)	P	P1	P2
Age (years)	40±9	39±13	42±11	0.76	0.5	0.4
Sex M/F	15/10	13/8	15/10	0.89	1	0.89
BMI (kg/m2)	24.8±6.5	27.7±4.7	31.2±5.4	0.1	<0.001	0.02
Diabetes mellitus (%)	0	4(19%)	12(48%)	0.03	<0.001	0.04
Hypertension (%)	0	3(14%)	9 (36%)	0.06	<0.001	0.04
Hyperlipidemia (%)	0	4(19%)	10(40%)	0.03	<0.001	0.02

P: SS vs control. P1: NASH vs control. P2: NASH vs SS.

Table 2: Laboratory investigations of the studied groups.

	Control (n=22)	SS (n=21)	NASH (n=25)	P	P1	P2
ALT (IU/L	27±12	59±21	98±55	<0.001	<0.001	<0.001
AST (IU/L)	25±14	51±17	85±38	<0.001	<0.001	<0.001
Albumin (gm%)	4.5±0.3	4.2±0.5	3.8±1.6	0.02	0.049	0.27
Bilirubin (mg%)	0.8±0.2	0.8±0.5	0.7±0.4	0.99	0.29	0.45
Cholesterol (mg%)	165±35	170±35	195±45	0.64	<0.001	<0.01
HDL-C (mg%)	48±9.4	40.3±6	38±10	<0.001	<0.001	0.35
LDL-C (mg%)	97±20	100±28	125±30	0.86	<0.001	<0.001
TG (mg%)	100±21	149±29	165±30	<0.001	<0.001	0.07
Glucose (mg%)	86.4±9	100.8±18	113.4±21.6	<0.001	<0.0001	<0.01
Insulin (uIU/ml)	3.1±2.2	13.8±3.2	20.8±8.2	<0.001	<0.001	<0.001
HOMA	0.6±0.2	3.4±1.5	5.8±2.3	<0.0001	<0.0001	<0.001

P: SS vs control. P1: NASH vs control. P2: NASH vs SS.

Table 3: Adipokine levels of the studied groups.

	Leptin (ng/ml)	Adiponectin (ug/ml)	A/L ratio (10^3)
Control (22)	6.7±3.5	15.1±5.1	2.3±0.1
SS (21)	15.6±3.0	12.3±5.8	0.8±0.3
P	<0.0001	>0.05	<0.0001
NASH (25)	17.5±5.0	10.3±4.2	0.59±0.2
P1	<0.0001	<0.001	<0.0001
P2	>0.05	>0.05	<0.001

P: SS vs control. P1: NASH vs control. P2: NASH vs SS.

Table 4: Pathological features of patients with NAFLD.

	Simple steatosis (n=21)	NASH (n=25)
Steatosis grade		
1	14 (66.7%)	6 (24%)
2	5 (23.8%)	8 (32%)
3	2 (9.5%)	11 (44%)
Fibrosis grade		
0	21 (100%)	11 (48%)
1		6 (24%)
2		4 (16%)
3		2 (8%)
4		1 (4%)

Table 5: Univariate and multivariate analysis of predictive factors of fibrosis:

	No or mild fibrosis no. 18	Moderate to severe fibrosis no, 7	Univariate analysis P*	Multivariate analysis P#
Age (years)	39±5	44±8	0.02	0.04
BMI (kg/m^2)	28.2±3.5	33±5.2	0.002	0.03
S glucose (mg%)	100.4±9.4	128±32.3	0.05	NS
Leptin (ng/ml)	14.3±8,6	18.4±10.7	NS	NS
Adiponectin (ug/ml)	11.2±6.1	9.7±3.5	NS	NS

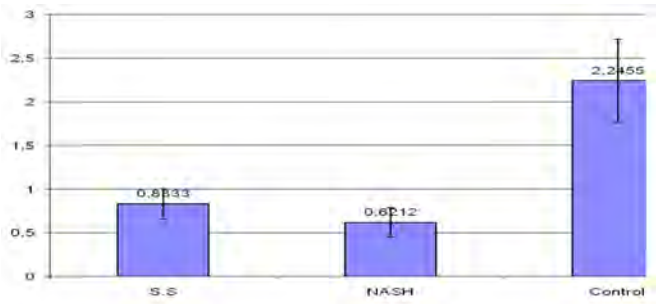

Fig. 1: Adiponectin/leptin (A/L) ratio in patients with simple steatosis, nonalcoholic steatohepatitis and controls

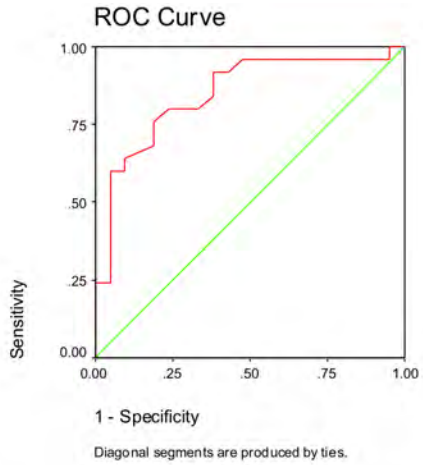

Fig. 2: ROC curve of the adiponectin to leptin (A/L) ratio for the diagnosis of nonalcoholic steatohepatitis.

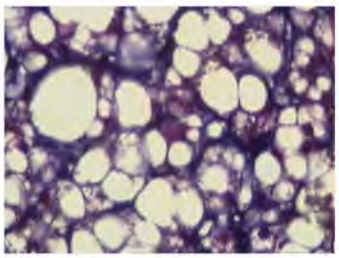

Fig 1: Evident macrovesicular steatosis, pericellular fibrosis 'F2' (masson trichrome stain)

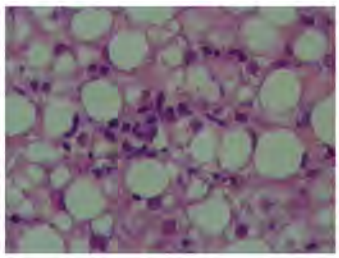

Fig 2: Massive steatosis with steatohepatitis (H & E stain)

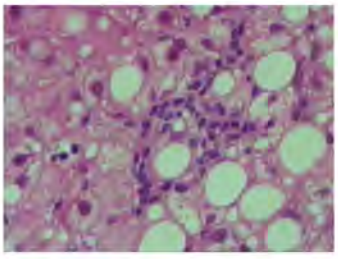

Fig 3: Moderate steatosis with steatohepatitis (H & E stain)

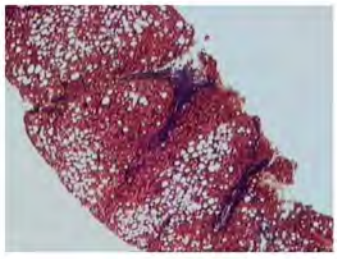

Fig 4: Moderate macrovesicular steatosis with early bridging fibrosis 'F2' (masson trichrome stain)

Discussion

This study was conducted to determine whether adipokine levels could predict the degree of liver injury in patients with NAFLD and whether their determination could be used as a noninvasive approach to discriminate NASH and simple steatosis. Among these adipokines, serum leptin and adiponectin seem to play an important role in the development of NASH.[15-18] Evidence has been provided that adiponectin is associated with inhibition of hepatic fibrogenesis and hepatic protection. Adiponectin through its receptors (AdipoR1/2) activates AMP activated protein kinase, which in turn inhibits lipogenesis through sterol regulatory element- binding protein-1 and acetyl CoA carboxylase.[35,36] By contrast, leptin is associated with enhanced fibrosis in various chronic liver diseases including NASH. [37]

In this study, we assessed serum levels of leptin and adiponectin in patients with NAFLD who underwent liver biopsy because of elevated transaminase levels. Higher serum levels of leptin and lower levels of adiponectin were found in patients with NAFLD compared with control group and no significant difference in leptin or adiponectin between patients with NASH and those with simple steatosis. Because adiponectin and leptin exhibit opposite variations, we determined the A/L ratio in order to sensitize the adipokine changes. The A/L ratio was evaluated and found to be correlated with carotid intima-media thickness, pulse wave velocity than either leptin or adiponectin alone. [38] We found lower A/L ratio in patients with NAFLD compared with controls. Most importantly, this ratio was significantly lower in patients with NASH compared to those with simple steatosis. A/L ratio less than 0.71was able to discriminate between NASH and simple steatosis. The AUROC curve for A/L ratio to distinguish between NASH and simple steatosis was 0.85. At a cut-off value of 0.71, the sensitivity was 80%, specificity 77%, PPV 78%, NPV 79% and accuracy 79%. In multivariate analysis, the A/L ratio was an independent factor associated with NASH independent from BMI. Lemoine et al [31] has reported that the com-

bination of HOMA index and A/L ratio showed an AUROC curve of 0.82 for discrimination between patients with NASH from those with simple steatosis.

Also, the use of acute phase proteins, including high sensitivity C-reactive protein[39] and pentraxin 3[40] were also suggested to be useful non-invasive markers for discrimination between NASH and SS with AUROC curve of 0.88 and 0.755 respectively.

In this study, adiponectin levels were significantly lower in obese NAFLD patients with BMI more than 30 than those with BMI < 30 and adiponectin were significantly negatively correlated with BMI. Our findings agree with those of previous studies.[28,41,42] Obesity, especially visceral obesity, is frequently associated with NAFLD and their coexistence in the same individual increases the likelihood of having more advanced forms of liver disease. NAFLD occurs in 60-95% of people with obesity. [43]

Also, serum adiponectin was negatively correlated with BMI and with the level of insulin resistance as measured by HOMA index. Serum leptin was positively correlated with BMI and HOMA. These results are in agreement with a number of previous studies.[27,44,45]

We also found that insulin resistance was higher in patients with NAFLD compared with controls thus confirming the previous link between steatosis and insulin resistance that has been demonstrated in previous studies.[15] Most importantly, the HOMA index was found to be significantly higher in patients with NASH compared to those with simple steatosis. The good predictive value of HOMA for diagnosis of NASH has been previously reported in several studies confirming the well established association between NASH and insulin resistance.[46,47]

We assessed the predictive value of leptin, adiponectin and the A/L ratio as markers of fibrosis. The level of these adipokines was not predictive of fibrosis and these results are consistent with previous studies.[28,31] Age

and body mass index were independently associated with fibrosis in multivariate analysis. Age and BMI have already been identified as predictive factors of fibrosis. [48,49]

In summary, the use of A/L ratio either alone or in combination with other serological tests could be used as a non-invasive approach for diagnosis of NASH and limit liver biopsy to those with suspected liver injury.

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PREDICTIVE VALUE OF ADIPONECTIN TO LEPTIN RATIO FOR DIAGNOSIS OF STEATOHEPATITIS IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

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THE IMPACT OF ACUTE ADMINISTRATION OF ORAL SILDENAFIL ON SEMEN PARAMETERS IN INFERTILE MEN

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Abstract

Objectives: *Nowadays, phosphodiestrase type5 (PDE5) inhibitors are the most prescribed oral agents in medical treatment of erectile dysfunction in men. They are sought to affect human spermatozoa, since 2 isoforms of phosphodiestrase (PDE1, PDE4) are present in human sperm cells and there is cross-reaction between phosphodiestrase family. So in this study we are going to evaluate the acute effect of sildenafil administration on semen parameters in a group of infertile men .*

Material and Methods: *A prospective randomized study was conducted on 60 male patients attending the Urology Department of Benha University Hospitals, Benha Faculty of Medicine, for infertility counseling with male infertility factor. After clinical examination, laboratory studies and basal semen analysis according to WHO protocol, all patients were asked not to ejaculate for 3-5 days, then they were given on empty stomach 50mg sildenafil orally and then they were asked to give semen sample after one hour. We compare the changes in semen parameters before and after sildenafil administration.*

Results: *The mean age of the study group is 37 ± 3.4ys with range between 30-46 years. The mean duration of infertility was 4.2 ± 2.7ys, with 36 men having primary infertility (60%) and 24 men having secondary infertility (40%), 11 patients (18.3%) reported side effects after sildenafil administration, but all were minor and self limited. Regarding semen analysis parameters, there was significant increase in percentage of total sperm motility after sildenafil administration (28.6% ± 5.4 versus 40.5% ± 7.2) and also highly significant increase in sperm rapid progressive motility i.e. grade 3 (13.1% ± 2.3 versus 20.4% ± 3.6) after sildenafil administration. However, no significant differences were ob-*

served in other semen parameters.

Conclusion: *The acute oral administration of sildenafil 50mg significantly affect increased percentage of total sperm motility and rapid progressive motility in infertile men, and we recommended its use one hour before any assisted reproductive technology procedures as intrauterine insemination (IUI) and intracytoplasmic sperm injection(ICSI), even if men have normal erectile function to enhance the fertilizing potential of poor quality spermatozoa as well as to alleviate the stress experienced by men in semen collection room.*

Abbreviations

PDE = Phospho diesterase.
IVF = In vitro fertilization.
INSL3 = Insulin like factor 3.
ROS = Reactive oxygen species.
IUI = Intra uterine insemination.
WHO = World health organization.
LCSF = Leydig cell secretory function.
ART = Assisted reproductive technology.
ICSI = Intra cytoplasmic sperm injection.
C-AMP = Cyclic Adenosine mono phosphate.
C-GMP = Cyclic guanesine mono phosphate.

Introduction

Phosphodiestrase (PDE) forms a complex group of structurally related enzymes consisting of about 15 identified families[1]. Since its first introduction, nowadays phosphodiestrase type5 (PDE5) inhibitors are the most commonly prescribed oral agents in medical treatment of erectile dysfunction in males[2]. Phosphodiestrase inhibitors are sought to affect human spermatozoa, where 40% are type 1 (PDE1) mainly in sperm head and 30% are type 4 (PDE4) mainly in sperm mid- piece and sperm flagella. It has been demonstrated in vitro that semen samples with abnormal parameters treated with PDE4 inhibitor had significantly increased motility compared to the control after 2 hours of incubation, while PDE1 inhibitor treated semen samples

were not significantly different from the control[3]. Regarding the non-selective PDE inhibitors as pentoxifylline, it has been considered to stimulate flagellar motility by increasing sperm intracellular cyclic adenosine monophosphate (c-AMP), as well as reducing DNA damaging reactive oxygen species (ROS), in particular, it appears to affect sperm motility characteristics and not increased number of spermatozoa, and these characteristics are mainly the curvilinear velocity and hyperactivation[4].

Since, it has been emphasized that there is cross reaction between PDE family types hence, PDE5 inhibitors were sought to affect human spermatozoa, and the first in vitro study was conducted by Cauadra et al., who demonstrated a dose dependent effect of PDE5 inhibitor sildenafil on sperm motility parameters with increased progressively motile spermatozoa. Also they demonstrated stimulated sperm acrosome reaction by almost 50% above the control which suggests a possible role of PDE5 inhibitors in preventing premature acrosome reaction which is associated with failed fertilization[5]. After that, many investigators have demonstrated the effects of PDE5 inhibitors in vivo on semen parameters in healthy men, and only few studies were applied on infertile men[6],[7],[8]. So in this study we aimed to evaluate the acute effect of PDE5 inhibitor sildenafil on semen parameters in a group of infertile men.

Patients and Methods

A prospective randomized study was conducted on 60 male patients attending the Urology Department Benha University Hospitals, Faculty of Medicine, for infertility counseling with male infertility factor. After clinical examination, laboratory studies including reproductive hormones assay (serum FSH, LH, Prolactin, and Testosterone), and basal semen analysis according to WHO for all the patients[9]. They were asked not to ejaculate for 3-5 days, then asked to give a semen sample after one hour of ingestion of sildenafil tab 50mg on empty stomach. We compare the changes in semen parameters before and after sildenafil administration. Study pro-

cedures received institutional review board approval and all patients gave written informed consent.

Semen collection, processing and examination

The sample was ejaculated into a clean, wide mouthed plastic container, allowed to liquefy at 37°C and then analyzed according to WHO protocol. During microscopic examination of the sample, concentration, motility, sperm abnormal forms and agglutination were assessed. Motility was graded into 3 grades: as 1 (non-progressive motility), 2 (slow progressive motility), 3 Rapid progressive motility).

Statistical methods

Continuous variables were presented as means and standard deviation and the differences between semen parameters before and after sildenafil administration were evaluated by Student Newman Keuls test (t-test) and $P < 0.05$ was considered statistically significant (SPSS ver 11.0 for Windows).

Results

The study involved 60 infertile males, aged between 30-46 years, with mean age 37 ± 3.4ys. The mean duration of infertility was 4.2 ± 2.7y, with 36 patients having primary infertility (60%) and 24 patients having secondary infertility (40%). Smokers represented 30% of patients (18 patients). Eleven patients (18.3%) reported side effects after sildenafil administration, most frequently were flushing and headache, but all were self limited. Hematology, serum chemistry and reproductive hormones were all in the normal range as shown in table (1).

Regarding semen analysis parameters, there was significant increase in total sperm motility after sildenafil administration compared to baseline motility (28.6% ± 5.4 versus 40.5% ± 7.2) and also there was highly significant increase in sperm rapid progressive motility (Grade 3) after sildenafil administration (13.1% ± 2.3 versus 20.4% ± 3.6). Significant increase in semen volume has been

also noticed after sildenafil administration (2.5ml ± 0.41 versus 3.4ml ± 0.65). However, no significant differences were observed for other semen parameters as sperm concentration and sperm abnormal forms, as presented in table (2).

Table (1): Serum reproductive hormonal profile results

Serum hormone	Mean value + S.D
FSH	6.24 (mIU/ ml ± 3.1
LH	3.72 (mIU/ ml) ± 2.8
Prolactin	180.74 (µIU/ml) ± 38.7
Testoterone	13.74 (noml/L) ± 4.6

Table (2): Semen parameters results before and after sildenafil administration

Semen parameter	Before sildenafil	After sildenfil	P-value
Volum (ML)	2.4 ± 0.42	3.1 ± 0.63	< 0.05*
Sperm count (million/ml)	25.2 ± 4.7	27.5 ± 5.9	< 0.5
Total sperm motility (%)	28.6 ± 5.4	40.5 ± 7.2	< 0.01*
Rapid progressive motility (%)	13.1 ± 2.3	20.4 ± 3.6	< 0.003**
Sperm abnormal forms (%)	86.3 ± 6.1	85.2 ± 3.8	< 0.5

* Significant ** Highly significant

Discussion

Sildenafil is a potent and specific inhibitor of cyclic guanesine monophosphate (c-GMP) specific PDE5, which is the most common PDE in the corpus cavernosum, this leads to its value in preventing c-GMP breakdown, thereby leading to cavernous smooth muscles relaxation and increasing intracavernousal pressure and penile erection[10]. Sperm function has also been shown to be altered by PDE5 and other PDE inhibitors, namely PDE1, which degrades c-AMP and c-GMP, and PDE4 which is c-AMP specific suggesting that this is not a response specific to only one of the PDE family[11]. There are two effects of this PDE inhibitor family on sperm function, first is a sustained enhancement of sperm motility, mainly the progressively motile spermatozoa which is mediated via PDE4 inhibitors, secondly is the activation of sperm acrosome reaction which is mediated via PDE1 inhibitors.

Our results in this study show a positive correlation between administration of sildenafil and increase of total sperm motility, as well as the rapid progressive motility in infertile men. The explanation for that is PDE inhibitors can hydrolyze both c-AMP and c-GMP, however c-GMP concentrations in spermatozoa are very low and remain unchanged, while c-AMP concentrations are high and increased more during sperm capacitation. With the use of sildenafil in absence of PDE5 in spermatozoa, it can inhibit other types of PDE present in spermatozoa mainly PDE1, and PDE4, that are known to have high affinity for c-AMP leading to increase intracellular levels of their substrate which has been clearly demonstrated by Pomara et al., to play a central role in the signaling pathway to modulate sperm motility by direct action on sperm mitochondria [12]. These effects on motility are mainly on curvilinear velocity and hyperactivation through sperm flagella. Also high intracellular level of c-AMP causing activation of phosphokinase and associated tyrosine phosphorylation of important fibrous sheath protein leading to activation of acrosome reaction and sperm capacitation [1].

Many consistent data have been published about sildenafil since its introduction, in which various reports have addressed the in vitro and in vivo effect of sildenafil on semen parameters as in our study. In the study of Kanakas and his colleagues who collected 3 semen samples from each of 13 oligozoospermic infertile men without and after sildenafil treatment, and evaluated the different semen parameters, in addition to evaluation of each sample for α- glucosidase (marker of epididymal function), fructose (marker of seminal vesicle function), and citrate (marker of prostatic secretory function) they found that the percentage of motile spermatozoa and seminal plasma citrate levels were significantly larger in semen sample collected after sildenafil administration compared to that collected prior to its administration. However they found no significant differences in the markers of epididymal or seminal vesicle function, so they concluded that sildenafil treatment promoted prostatic secretory functions through greater sexual stimulation prior to and during ejaculation and this could provide a resonable explanation for the enhanced sperm motility by the increased concentrations of prostatic markers, mainly citrate that will provide an ideal environment for sperm motility and transport, and could also explain the significant increase in semen volume as in our study [7]. Also in a prospective double blind placebo controlled study conducted by du Plessis et al. on 20 healthy males who randomly taken single dose of 50mg sildenafil or placebo, and they found statistical significant differences in sperm straight line velocity and rapidly moving spermatozoa, between sildenafil group and placebo. Also they observed increase in outcome of sperm oocyte binding assay after sildenafil administration which could be explained by the findings of more spermatozoa become rapidly motile, thus increasing the chances for them to bind with oocyte which will result in increasing fertilization rate [8]. That findings could be applied before any assisted reproductive technology (ART) procedures to increase the chance of pregnancy in IVF centers as in the study of Jannini et al. who recommended the administration of sil-

denafil prior to semen collection and performance of ART procedures as intrauterine insemination (IUI) and intracytoplasmic sperm injection (ICSI)[13].

More recent studies have also demonstrated these positive effects of sildenafil on sperm motility as the study of Pomara et al. who reported a significant increase in sperm progressive motility in semen samples collected after sildenafil administration as in our study[12]. Also it has been demonstrated in the study of Ali and his Collegues that the administration of 50mg sildenafil to diabetic neuropathy patients has resulted in increase in sperm motility and semen volume, while sperm morphology and sperm count remained unaffected[14].

Our results are compatable with that of Dimitriadis et al. who found that after treatment with 5o mg. sildenafil, the mean values of sperm count, percentage of motile spermatozoa and the percentage of morphologically normal spermatozoa were significantly greater than before treatment, postulated that sildenafil enhance Leydig cell secretory function (LCSF) in oligoasthenospermic infertile men, evident by the significantly larger serum insulin like factor 3 (INSL3) profiles after its administration. INSL3 is a peptide with a role in the first phase of testicular descent, and is a marker of LCSF [15],[16]. Optimal LCSF is of paramount importance for maintaining normal spermatogenesis, also, intra epididymal testosterone profiles that are important for the epididymal sperm maturation and development depend on LCSF.

The increase in LCSF after sildenafil administration might stimulates sertoli cell secretory function which is androgen dependent, this in turn provide a more optimal biochemical environment within the seminiferous tubules stimulating spermatogenesis in oligoasthenospermic men[17]. Consistent with our results, are the results of Jannini et al., who found that PDE5 inhibitors has been suggested to enhance prostatic secretory function and improve sperm motility and sildenafil treatment reduces the overall stress levels then inducing a more

complete ejaculation and subsequently more good quality spermatozoa in the semen.

Regarding the in vitro effects of sildenafil, a concentration dependent stimulatory effect of sildenafil on sperm motility was demonstrated by Mostafa when 85 semen specimens from athenozoospermic patients were exposed to different five concentrations of sildenafil incubated for 3 hours[18]. Also, Glenn and his group have clearly demonstrated the positive effect of sildenafil incubated with semen samples of 57 athenozoospermic patients with a concentration equivalent to plasma concentration of sildenafil one hour after its ingestion of 50mg, they found increased percentage for number and velocity of progressively motile spermatozoa by 46% and 52% respectively after 2 hours of incubation. They also noticed that sildenafil caused a significant increase in the proportion of acrosome reacted spermatozoa, that could be explained by the increased levels of c-GMP in spermatozoa as a result of inhibitory effect of sildenafil that would affect calcium transport into spermatozoa, in which altered levels of intracellular calcium may potentially affect sperm motility and an energy dependent influx of calcium into the sperm cells that may be responsible for the initiation of acrosome reaction and capacitation[11].

In contrast to our study is the study of Aversa et al. who found no changes in semen parameters regarding sperm concentration, sperm motility and sperm abnormal forms before and after administration of 100mg sildenafil orally to 20 sexually healthy men with semen profiles done 1 hour after its administration[19]. Also, the study of Purvis et al. on 16 sexually healthy male volunteers who examined the effect of 100mg sildenafil versus placebo on sperm motility, and reported lack of effects of sildenafil on sperm motility in both the percentage of total sperm motility and in percentage of rapidly progressive sperm motility[20]. However, the high dose of sildenafil (100mg) in these studies may have toxic effects on spermatozoa. Hence the optimum dose for positive effect of sildenafil is only 50mg as in our study, as well as in the study of

Andrade and his Colleagues who demonstrated the in-vitro effects of low and high concentration of sildenafil on sperm motility[21]. Regarding other PDE5 inhibitors as tadalafil 20mg and vardenafil 10mg, few studies have demonstrated the same effect on sperm motility, as the study of Montsori et al. for tadalafil[22] and the study of Grammeniatis et al. for vardenafil[23].

In conclusion, our study confirms the positive effects on total sperm motility and sperm progressive motility in infertile men after acute administration of 50mg sildenafil citrate. These findings could have important implications for the use of PDE5 inhibitors as sildenafil in ART procedures in IVF centers to enhance the fertilizing potential of inherently poor quality spermatozoa, in addition to its role in alleviating the stress experienced by men in semen collection room especially if they have erectile dysfunction. So, we recommended the use of sildenafil one hour before any ART procedures as IUI or ICSI, even if the males have normal erectile function.

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THE IMPACT OF ACUTE ADMINISTRATION OF ORAL SILDENAFIL ON SEMEN PARAMETERS IN INFERTILE MEN

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SUBCAPSULAR (SUBFACIAL) PAROTIDECTOMY: A SIMPLE AND EFFECTIVE SURGICAL TECHNIQUE TO PREVENT FREY'S SYNDROME

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Abstract

Objective: *To assess the efficacy of subcapsular subfacial parotidectomy technique in preventing the development of gustatory sweating (Frey's syndrome).*

Design: *Case series, surgical technique description with data collection.*

Setting: *Otolaryngology department in a university hospital.*

Method: *Subcapsular (subfacial) superficial parotidectomy was performed on 21 patients for benign parotid tumours between January 2006 and January 2010. Surgical procedures involved superficial parotidectomy with inclusion of parotid capsule (facia) in the elevated skin flap. Patients were followed-up for 12 months and finally at 22-53 months (mean 37.5 months) for subjective and objective gustatory sweating.*

Results: *None of the patients experienced FS symptoms. At final follow objective gustatory sweating was found in 3 patients but no patient had subjective gustatory sweating.*

Conclusion: *Subcapsular parotidectomy for benign parotid tumours is an effective technique to prevent the development of Frey's syndrome during the follow up period.*

Introduction

Gustatory sweating (Frey's syndrome, FS) is a well recognised complication of parotidectomy. Gustatory stimuli clinically result in unilateral facial sweating, flushing, and paraesthesia in the skin area innervated by the auriculotemporal nerve. It can be very disabling and socially embarrass-

ing. This occurs due to misguided parasympathetic re-innervation of previously denervated subcutaneous sweat glands and blood vessels normally innervated by sympathetic cholinergic fibres. The incidence of FS varies considerably from 1.7% to 97.6% [1,2] and can take as long as 8 years to develop [3].

Traditionally, subjective assessment of Frey syndrome is documented when patients experience gustatory sweating, even if they were not perturbed by the symptom. FS is objectively diagnosed by Minor's iodine starch test (MIST). A positive MIST in absence of symptoms indicates asymptomatic (subclinical) FS which has a much higher incidence than symptomatic FS [1,4].

Medical treatment of FS including systemic or topical anticholinergic agents (scopolamine, glycopyrrolate, diphemnanil-methylsulfate) and the use of stellate ganglion blockade has been unsuccessful. Local injection of botulinum toxins, type A [5], type B [6], type F [7] is a well recognised treatment modality of FS. Surgical treatment includes cervical sympathectomy, tympanic neurectomy and division of subcutaneous nerves with interposition of soft tissue between parotid skin and parotid gland remnants [8].

More efforts have been devoted to the preventive measures to avoid the development of FS. These include intra operative use of synthetic or semi synthetic grafts [e.g. dermis-fat graft[9], acellular dermal matrix[10], and oxidised regenerated cellulose[11] to form a barrier between parotid bed and overlying skin. A wide spectrum of local soft tissue flaps has also been utilised for the same purpose including temporo-parietal facial flap[12], sterno-mastoid muscle flap, combined platysma muscle-cervical fascia-sternomastoid flap[13] and superficial musculoaponeurotic system (SMAS)[14,15]. The multitude of these methods indicates that none of these is ideal for all patients. Furthermore, these treatments are not always effective and often have unwanted risks and adverse effects.

In this article, a simple tech-

nique to prevent the development of FS, which seems to be effective during follow up period of up to 53 months, is presented.

Anatomical background :

At the anterior border of sternomastoid muscle, the superficial layer of deep cervical fascia splits into two layers to enclose the parotid gland. These form the enclosing parotid facia (PF). The superficial sheet is thick and condensed covering the external aspect of the parotid gland. The thin deep sheet surrounds the deep aspect of the gland. At the anterior border of the parotid gland, the two sheets fuse and cover the masseter muscle forming masseteric fascia which attaches to the zygomatic arch. The outer layer of the OPF is covered by extending part of the platysma. The lax space between the parotid gland and OPGF makes it possible to elevate the superficial layer of PF without much difficulty except the lobule septum.

The great auricular nerve (GAN) is a superficial branch of the cervical Plexus (C2, C3). It leaves the cervical plexus at the midpoint of posterior border of the sternomastoid muscle and courses anteriorly over the lateral surface of the muscle dividing into anterior, posterior auricular and lobular branches. The posterior branch supplies the skin over the mastoid process and lower external ear, and the anterior branch supplies the skin overlying the parotid gland and lower pre auricular region. Te anterior branch sends a small twig (or several small twigs) which penetrate the parotid fascia and distribute into the substance of the parotid gland. The terminal trunk and branches of the GAN run within the OPF.

In this article, subcapsular (subfacial) superficial parotidectomy (SCSP) means parotid dissection with preservation of the outer layer of parotid capsule (facia, OPF). For the purpose of simplicity, parotid facia (PF) is used in this article to indicate the OPF as the surgical technique described in this article deals only with the outer layer of parotid facia not the inner layer.

Methods

Study design: Case series and

surgical technique description.

Setting: Otolaryngology department in a university hospital.

Patients and Methods

The study included 21 patients diagnosed, on clinical and cytological criteria as suggested by preoperative fine needle aspiration cytology, with benign parotid tumours (pleomorphic adenoma and Warthin's tumour). Patients with suspected malignancy or recurrent benign disease were excluded. Institutional Review Board approval was obtained. Informed consent was obtained and patients informed of potential benefits and complications of surgery including post operative depression as there was no plan to correct it. Surgery was done between January 2006 and January 2010.

Operative technique: A standard parotidectomy (Blair's facio-cervical) incision starting in the pre auricular skin crease, curves around the ear lobe and then curves again backward and downward in a neck skin crease. In the facial part, the incision carefully deepened to includes the platysma down to the parotid facia by identifying the parotid tissue with care not to cut through the parotid tumour capsule. Dissection of the parotid facia continues superiorly toward the upper end of incision till reaching the upper pole of the gland. In the cervical part, the skin and platysma flap is elevated till identifying the anterior border of sternomastoid muscle which is then incised with care taken to identify and fully expose the great auricular nerve (GAN) crossing the muscle and running in the parotid facia (PF). Sharp dissection of the nerve and its branched (anterior auricular, posterior auricular and lobular branches) was performed. Glandular branches of the GAN are divided whereas are auricular branches preserved. In the cases when it was not possible to preserve all the greater auricular nerve branches, posterior auricular branch, including the lobular branch, was preserved. The parotid facia was then incised inferiorly and posteriorly with incision extending upward to join the facial part. The parotid facia is carefully dissected off the parotid tissue and raised included in the skin flap. Care is taking that no parotid

tissue is included with the facia if at all possible. Dissection then proceeds anteriorly under the parotid facia toward the anterior border of the gland but stopping close to it on achieving adequate exposure of the tumour. This is to avoid injury to facial nerve branches at the anterior border of the parotid gland.

Care was taken to avoid rupture of tumour capsule if it was close to the parotid facia. Flap raising was facilitated by extending the dissection beyond the limits of parotid facia both superiorly and inferiorly till a satisfactory exposure of the parotid gland is achieved.

The rest of surgical procedure was then performed with identification and dissection of facial nerve and its branches as in standard superficial parotidectomy and superficial lobe of parotid gland is removed leaving the facial nerve and its branches over the deep lobe of parotid gland. Facial nerve integrity monitoring is routinely employed. Bleeding was controlled by judicious bipolar diathermy of fine bleeding points and ligation of bigger vessels. Suction drain was secured with care taken that the drain was not adjacent to the facial nerve. The incision was then closed in 2 layers, one for the parotid facia and second one for the skin suing 4/0 proline for cervical skin and 6/0 for facial skin. No external dressing was used. The drain was removed after 1-2 days and patients were discharged afterward and stitches removed after 1 week.

Patients were followed up after 1, 3, 6 and 12 months for early and late postoperative complications particularly the symptoms of FS. Patients were then invited for final check (average 37.5 months post-operative) to assess their general impression on the operation, any residual facial weakness, FS symptoms and MIST performed with un-operated side used as a control.

Results

Twenty one patients, (8 males, 13 females; mean age 50 years; range 37 to 63 years) diagnosed with benign parotid gland tumours (pleomorphic or Warthin's adenomas) were treated by SCSP.

Their benign status was determined by history, clinical criteria and fine needle aspiration cytology. The author operated on all cases. In all patients, the tumour was located entirely within the superficial lobe of the parotid gland as suspected clinically and found operatively. Postoperative pathology confirmed the benign nature of all excised tumours including 19 pleomorphic adenomas and 2 Warthin's tumours (Table 1).

Patients were followed up at 1, 3, 6 and 12 monthly intervals. At 12 months' review, all patients were assessed for clinical outcome with main emphasis on facial nerve functions and symptoms of FS (facial sweating, flushing, or moisture with eating). Four patients (19%) had transient facial nerve weakness but all recovered fully by the end of 12 moths' follow up. Three patients experienced transient numbness in the lobule and in the infra-auricular region. All cases recovered their normal sensation within 6 months after the operation. No cases of postoperative hematoma, wound infection, salivary fistula, or hypertrophic scar formation were noted. None of the patients complained of symptoms of gustatory sweating, facial flushing or hyperaemia.

At the final visit (22-53 months post operatively, with average follow up 37.5 months), none of the patients had symptoms of FS or local tumour recurrence. MIST was performed on all patients and was positive only in 3 cases at the retro mandibular skin. None of the 3 patients has complained of FS symptoms.

Gender	Number	age range	Mean age	Pleomorphic adenoma	Warthin's tumour
Male	8	46-67	53	8	0
Female	13	37-63	48	9	2

Discussion

When FS is diagnosed and patient requires treatment, botulinum toxin injection would be the recommended treatment modality. It can, however, cause temporary perioral muscle paresis[16] or mouth dryness[17] and its effect is temporary and secondary treatment will be required. Furthermore, botulinum toxin injection may have no effect on facial skin flushing[18]. However, prevention of the development of FS would be preferred.

Several surgical techniques have been adopted to reduce the risk of developing FS after parotidectomy for benign tumours. Reducing the excised amount of parotid tissue was tried as a simple way to reduce the risk of developing FS as well as facial nerve injury. Controlled tumour excision has thus been practised in 2 forms. The first is local extra capsular dissection of parotid tumour without complete classical superficial parotidectomy and without facial nerve dissection[19,29]. However, the problems associated with this technique include tumour puncture and spillage leading to tumour recurrence [21], incidental malignant tumour being found and the potential risk to a branch of the facial nerve deeper than the tumour. The second technique is partial superficial parotidectomy (PSP) [22]. However, this doesn't always reduce FS development[23]. With these 2 techniques, remaining parotid tissue with its connecting ducts transacted could result in salivary fistula formation.

Other surgical techniques used various local tissues as physical barriers between parotid bed and skin flap to reduce the risk of FS. These include the sternomastoid SF[24]. However, the evidence that this reduces the development of FS was inconclusive[25,26]. SF raising includes more soft tissue dissection, puts the spinal accessory and greater auricular nerves at risk, and creates greater dead space and potential for haematoma formation[15]. It can also create a donor site hollow deformity or asymmetry of the upper neck, especially in slim patients.

Although the superficial musculoaponeurotic system (SMAS) flap was advocated as a surgical

modality to prevent FS development by some[15,27], conflicting results have been reported. Some authors have reported that SMAS flap may delay the onset and reduces the intensity of FS, but does not prevent the occurrence on the long term[28], others found no significant reduction of FS development following SMAS flap [2,29].

Acellular dermis appears to reduce post-parotidectomy gustatory sweating; however, its complication rate (seromas and wound infections) was higher[10]. Synthetic implants have also been tried to prevent the development of FS with cited advantage. However, material costs, risk of foreign body reaction, masked tumour recurrence and predictable difficulty in re- exploration of the tumour bed are valid arguments against their use. These are not recommend in patients with a high risk of tumour recurrence, e.g. patients who have undergone revision surgery for benign tumours or patients who have a malignant neoplasm or intra operative tumour spillage [30].

A posterior pedicled parotid gland fascia flap was raised without separating the great auricular nerve branches to reduce FS[31,32]. However, this technique did not prevent subjective FS development in all cases due possible to compromised parotid facial flap vasculature when it is raised as a separate flap and this might allow aberrant regenerating parasympathetic nerves to penetrate the facial layer to the overlying skin blood vessels and sweat glands.

The technique described in this paper involves raising the intact PF with the overlying skin and platysma flap preserving most of its vascular supply. This is likely to maintain the integrity of parotid facia which functions as a normal biological and physical barrier against the regenerating parasympathetic nerves and thus preventing the development of FS. Positive MIST at the retro mandibular region could be due to torn PF during great auricular nerve dissection. Positive MIST at the retro mandibular region could be due to torn PF during great auricular nerve dissection.

In the current study classical Blair's incision was employed for adequate access of the parotid gland tumour. Other incisions such as modified rhytedectomy face lift incision[32] have been employed to avoid a neck scar. However, this might restrict the surgeon's access to parotid tail tumours or large size tumours.

During great auricular nerve dissection, the torn PF was repaired and stitched to the sternomastoid facia. However, the positive MIST at the retro mandibular region suggests the development of PF defects due possibly to failed repair or compromised vasculature. This however, was not significant as none of the 3 MIST positive patients had complained of gustatory sweating. Similar to the aforementioned techniques, the described technique is not suitable for cases of malignant parotid tumour that approaches the PF, large benign tumour where the elevation of PF might result in rupture of tumour capsule and if the tumour capsule is adhered to the PF. In these cases, preserving PF may compromise complete tumour resection.

The techniques described in this paper should not be confused with the extra capsular parotidectomy in which the parotid tumour is removed from the parotid gland alone with no parotid tissue and no facial nerve dissection. In the subcapsular technique, the standard superficial parotidectomy is followed with facial nerve dissection and monitoring.

Subjective perception of gustatory sweating is widely variable and this is probably why it is has much lower incidence than subclinical (objective) FS (positive MIST) which can be found in almost all cases [2,33]. In this study, MIST was not routinely done at the 12 month visit as none of the patients had FS symptoms. However, for the purpose of this study, patients were invited for clinical and objective assessment of FS and MIST was done to diagnose subclinical FS which was found in 3 individuals, none of whom had symptoms of FS.

This technique preserves the outer layer of PF which was draped on the parotid bed. This would facilitate further surgical

intervention in revision cases. It is not known however, weather dissected facial nerve branches adhere to the parotid facia. This would be clarified in the future in cases which require parotid re-exploration for tumour recurrence.

Weakness of the study: The results of this study suggest that subcapsular (subfacial) parotidectomy with preservation of the parotid facia is effective in preventing, at least, subjective (clinical) FS. However, the number of patient is small and follow up is not long enough. Further studies with large patients' number and long enough follow up are required to evaluate the long term clinical effectiveness of this approach.

Conclusion: Subcapsular (subfacial) parotidectomy seems to be a satisfactory approach of superficial parotidectomy to prevent the development of clinical Frey's syndrome in patients with benign parotid tumours although it might not prevent subjective (subclinical) FS. It appears to be a safe and straight forward technique in trained hands.

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gram (s)	g	minute (s)	min
milligram (s)	mg	second (s)	S
microgram (s)	ug	centimeter (s)	cm
nanogram (s)	ng	cubic millimeter	cmm
micrometer	um	millilitre (s)	ml
millicurie(s)	mCi	milliequivalent	mEq
molar	mol/l	millimole	mmol

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Measures of location (e.g. mean, median) should be accompanied by an appropriate measure of variability, e.g. standard deviation (SD) or standard error of the mean (SEM). Methods of statistical analysis should be indicated, but details are not required unless relevant to the discussion. In multivariate analysis, an indication of the goodness of fit of the model should be given (e.g.multiple correlation coefficient). When a result is claimed to be statistically significant the test used and the level of probability should be specified (e.g. paired t-test $p<0.01$) When only one type of statistical test is used in the paper, it should be stated in the scction for methods to avoid repetition. Tables should be self explanatory .

References

In the text, use the name of the author(s) followed by their date of publication: Where there are more than two authors use Robinson et al. (1979).

Arrange the list of authors quoted at the end of the text in alphabetical order as follows:

Name (s), and initials of all author (s) year of publication (in brackets), title of the article, name of the journal (abbreviated according to Index Medicus), voulme number, first page number. In the case of books the order is: Name(s) and initials of author(s), year of publication (in brackets), full title, edition, publisher and place of publisher, page numbers. Where appropriate, the chapter title and the names of the editors should be given.

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Examples of the style to be used are given below:

Trudiner B.J., Lewis p.j. & petit, B. (2000); fetal breathing in intrauterine growth retardation. Br J Obstet Gynaecol, 86:432.

Hytten, F.F & Leitch I. (2000): The physiology of Human pragnancy 2nd edn, Blackwell Scientific publications, Oxford, p.26.

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