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BRAIN PROTECTIVE EFFECT OF PROPOFOL-FENTANYL VERSUS SEVOFLURANE-FENTANYL IN PEDIATRIC OPEN HEART SURGERY: TRANSCRANIAL DOPPLER EVALUATION STUDY

Gamal Z. El-Morsy MD, Nabil A. Mageed MD, Mostafa M. Said MD, Hani I. Mohamed MD and Mohamed Adel F. Elgamal MD*

Departments of Anesthesia and Surgical Intensive Care
*and *Congenital Pediatric Cardiac Surgery, Cardiothoracic Surgery,*
Faculty of Medicine, Mansoura University., Egypt

Abstract

Objectives: *Neurological injury after cardiopulmonary bypass in pediatric cardiac surgery is a major complication. The aim of this study was to compare the neuroprotective effect of propofol with that of sevoflurane in pediatric open cardiac surgery.*

Methods: *After obtaining the research ethics board (REB) approval and written informed parents consent 40 patients of either sex aged 1-3 years, ASA II & III submitted for elective correction of simple congenital heart diseases using cardiopulmonary bypass. Patients were double blinded randomly located into two groups, propofol group (n=20) received propofol as inducing and maintenance agent for anesthesia and sevoflurane group. (n=20) received sevoflurane as inducing and maintenance agent for anesthesia Neurological examination, CT scan and transcranial doppler evaluation to monitor the velocities of flow and pulsatile index in middle cerebral artery were recorded to compare the neuroprotective effect of both agents.*

Results: *Patients in sevoflurane group showed significant elevation in mean cerebral blood flow velocity, pulsatile index and embolic events than propofol group. Also the heart rate and mean arterial blood pressure were significantly higher in sevoflurane group than propofol group. There were no differences between the 2 groups as regard postoperative neurological examination, CT study and postoperative complications.*

Conclusion: *propofol is as effective as sevoflurane in brain neuroprotection and preconditioning after using cardiopulmonary bypass in open pediatric cardiac surgery.*

Keywords: *Neuroprotection, Cardiopulmonary bypass, Sevoflurane, Propofol, Cerebral flow velocity, Pulsatile index.*

Introduction

Brain injury after cardiac surgery still occurs despite improvements in surgical techniques over the years and the implementation of effective neuroprotective strategies. Maintenance of sufficient cerebral blood flow is important for oxygenation and function of the brain. During cardiac surgery, physiologic variables should be preserved within the normal range to maintain adequate cerebral oxygen supply.[1,2]

Neuroprotection can be achived by reducing O_2 demand, enhanced O_2 delivery, or attenuation of pathologic processes that contribute to cellular injury or death.[3]

Propofol causes inhibition of glutamate release, and the positive modulation of GABA-A receptor function. Also it causes a dose-dependent reduction in cerebral blood flow (CBF) and cerebral metabolic rate oxygen consumption ($CMRO_2$). The in vivo cerebral vasoconstrictor effect of propofol is possibly related to the intact CBF-CMR coupling and thus are typical of the ideal neuroprotective drug.[4,5]

Sevoflurane increases the velocity of cerebral blood flow (CBFV) and decreases the cerebrovascular resistance (CVR) in a dose-dependent manner which cause an impairment of cerebrovascular autoregulation mechanisms but different transient hyperemic response tests, carried out on adults and children, showed that cerebral autoregulation is well preserved during anesthesia, with up to 2.0 MAC sevoflurane.[6,7]

Adequate monitoring is essential to detect potentially harmful conditions early enough that permit initiation of effective interventions before irreversible injury occurred. In neonates and children, hypoxic-ischemic mechanisms are

responsible for the majority of preventable injury.[8]

Transcranial Doppler ultrasound (TCD) is a sensitive, real-time monitor of embolic events and velocity of cerebral blood flow (CBFV) during congenital heart surgery. It can measures the middle cerebral artery (MCA) cerebral blood flow velocity (CBFV), which relates to three important parameters: blood flow, vascular cross-sectional area, and blood viscosity. TCD technique is the only unique standard method that detects the passing of emboli through the brain vessels in real-time and allows quantification of embolic signals during monitoring. Correlating the amount and timing of emboli with the clinical and neurophysiological findings may provide an explanation of brain injury after CPB.[9,10]

In this study we tried to evaluate the brain protective effect of TIVA using propofol-fentanyl versus sevoflurane-fentanyl by using transcranial Doppler, neurological examination, and CT study. Our hypotheses was that the use of propofol as an induction and maintenance agent in pediatrics undergoing open heart surgery for congenital heart diseases has the same brain protective effect as the use of sevoflurane. We also tried to find a correlation between using TCD, neurological examination and CT study as indicator for cerebral injury.

Patients and methods

This double - blind randomized study was conducted over a period of 12 months. 40 patients of either sex aged 1-3 years who were planned to undergo elective correction of simple congenital heart diseases using cardiopulmonary bypass at Mansoura University children hospital and after obtaining an informed written consent from the parents. Patients with previous open heart surgery, endocarditis, neurological, renal or pulmonary disease, heart failure, presence of preoperative coagulation disorders were excluded from the study. All surgical procedures were done by a well-trained team of pediatric cardiothoracic surgeons.

All patients were subjected to preoperative clinical examination

for assessment of cardiovascular function. Laboratory investigations include complete blood count (CBC), electrolyte, arterial blood gas and urine analysis, coagulation survey, blood glucose level, liver and renal function tests. Neurological and radiological examination (including brain CT), were fulfilled. Patients received a premedication in the form of intramuscular 0.07 mg/Kg midazolam and 0.02 mg/Kg atropine sulphates respectively 15 min before induction. Patients were blind randomly assigned into two groups : propofol group (Propofol- Fentanyl group) and sevoflurane group (sevoflurane-Fentanyl group).

Heart rate, mean value of invasive arterial blood pressure, central venous pressure, end tidal CO_2, oxygen saturation, nasopharyngeal temperatures were monitored (using Datex, Helsinki, Finland AS).

Anesthesia induced by I.V. administration of fentanyl 5 μg/Kg, propofol 2- 2.5 mg/Kg (in propofol group) or sevoflurane 2-3MAC (in sevoflurane group) and 0.9 mg/kg rocuronium I.V. to provide muscle relaxation. With loss of consciousness, patients were mechanically ventilated by positive pressure ventilation via face mask at a rate of 20-28 breathes per minute with 50% O_2 and when the ETT inserted the end-tidal CO_2 was monitored by side-stream capnograph and maintained between 30-35 mmHg. Anesthesia was maintained with Propofol infusion at a rate of 100-150 μg/Kg/min (in propofol group) or sevoflurane 2 MAC (in sevoflurane group) and fentanyl 0.05 μg/Kg/min, to maintain blood pressure within 20-25% of its basal value and entropy value between 45-55 with infusion requirements for rocuronium 0.3 mg/Kg/minute to maintain muscle relaxation.

Arterial blood gases were recorded before starting of general anesthesia [basal], after induction of general anesthesia, before initiation of cardiopulmonary bypass, after termination of CPB, at the closure of skin, basal in ICU, 2, 4, 8, 12 hours in ICU. Aortic cross clamping time (minutes), cardiopulmonary bypass time (minutes), spontaneous

regaining of the heart function, need for DC shocks and the duration of inotropic and / or vasopressor requirements to wean the heart from CPB were recorded in all patients.

Transcranial doppler was used to monitor the velocities of flow, at this time intervals: before the surgery, before CPB, during CPB (after establishment of full flow including detection of embolic events), after CPB, and after the surgery in the ICU (after 2 days). Neurological examination and CT scan were done before and after surgery (after 2 days). Extubation time, the need for reintubation and postoperative complications were noticed and recorded. The depth of anesthesia was monitored by Entropy monitoring. Entropy was collected with an M-ENTROPYTM module of the S/5TM Anesthesia Monitor (Datex Ohmeda).

Statistical analysis was done by using statistical package for social scientists (SPSS) program version. Data was expressed as percentage and mean ± SD as appropriate. The changes in quantitave data was done by Shapiro-Wilk's W test. Changes in values between two groups was compared by unpaired t-test. Chi-square test were used for qualitative data. Spearman test was used for correlation between data. P <0.05 was considered significant.

Results

All patients completed the study successfully with no drop-outs. No considerable differences were observed between the two groups as regard demographic data. (table 1).

Velocity maximum was significantly high in the after bypass and 2 days in ICU periods in sevoflurane group than propofol group. Velocity minimum showed no difference between the two groups Velocity mean was significantly high in the after induction and after bypass periods in sevoflurane group when compared with propofol group. Pulsatile index was significantly high in the after induction, during CPB and after bypass periods in sevoflurane group when compared to propofol group. (Table 2 & figure 3).

Duration of inotropic support, spontaneous recovery of conscious level, need for DC shock, vomiting, respiratory depression, need for reintubation, were insignificant between both groups. (Table 3)

The incidence of air embolic events was lower in propofol group relative to sevoflurane group (figure 4). Pre and postoperative neurological examination, CT study were of no significance between the two groups. (Table 3) There was no correlation between velocity mean measured 2 days in ICU and the neurological examination done on the same time interval.

The heart rate changes showed higher values after induction and after CPB in sevoflurane group when compared with propofol group. On the other hand the mean arterial pressure showed higher value in sevoflurane group when compared with propofol group after induction. (Figure 1, 2 respectively).

Table (1) : Demographic data of the studied groups: Propofol Group (n=20); Sevoflurane Group (n=20);. Data are in number %, mean ± SD

	Propofol Group	**Sevoflurane Group**
Age (months)	18.85 ± 10.56	19.20 ± 8.1
Sex Male	65% (13)	55% (11)
Female	35% (7)	45% (9)
Weight (kilogram)	10.50 ± 4.03	11.25 ± 3.47
Diagnosis ASD	40% (8)	45% (9)
VSD	40% (8)	40% (8)
TOF		5% (1)
TGA	15%(3)	
Single ventricle		5% (1)
Total anomalies pulmonary venous return	5% (1)	5% (1)

Table (2) : Velocity Maximum, Velocity Minimum, Mean Velocity, and Pulsatile Index in the studied groups. Propofol Group (n=20); Sevoflurane Group (n=20). Data are in mean ± SD. Bpm; beat per minute, CPB; cardiopulmonary bypass.

		Propofol Group	Sevoflurane Group
Velocity Maximum	Basal	106.1±11.01	113.2±20.7
	After induction	103.7±15..8	93.3±21.6
	During CPB	73.45±21.46	83.15±15.7
	After Bypass	105.6±26.8	140.2±16 *
	2Days in ICU	99.2±22.5	126.86±9.9*
Velocity Minimum	Basal	35.9±11.4	34.4±10.4
	After induction	21.8±2.3	26.9±1.7
	During CPB	13.85±2.96	17.8±2.86
	After Bypass	25.9±3.3	48.0±5.2
	2Days in ICU	40.7±3.6	43.6±6.4
Velocity Mean	Basal	48.6±12.5	45.6±17.7
	After induction	33.0±13.8	49.3±5.2 *
	During CPB	27.24±8	27.26±5.5
	After Bypass	51.4±2.7	61.7±7.0*
	2Days in ICU	58.1±2.7	59.14±5.6
Pulsatile Index	Basal	1.16±0.18	1.2±0.24
	After induction	1.13±0.20	1.38±0.73*
	During CPB	0.92±0.15	1.10±0.05*
	After Bypass	0.95±0.15	1.05±0.07*
	2Days in ICU	0.92±0.23	0.84±0.38

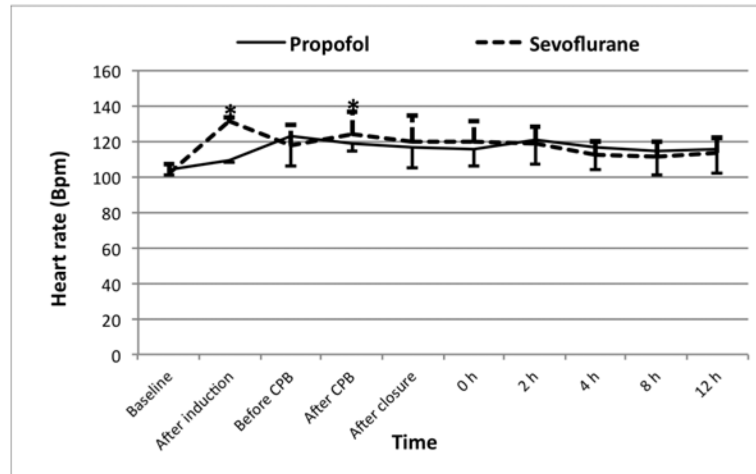
*P <0.05significant when compared with the propofol group.

Table (3) : Duration of intropic support, Spontaneous recovery of conscious level, Need for DC shock, vomiting, Respiratory depression, the need for reintubation, postoperative neurological examination, CT study and incidence of air embolisation in the studied groups:
Propofol Group (n=20); Sevoflurane Group (n=20). Data are in number %, mean ± SD

	Propofol Group	Sevoflurane Group
Duration of entropic support (hours)	21.7 ± 2.12	20.95 ± 1.79
Spontaneous recovery of conscious level	(20)100%	(20)100%
Need for DC shock	0%	5%
Vomiting	0%	(1) 5%
Respiratory depression	0%	5%
Need for reintubation	0%	5%
Normal neurological examination	(20) 100%	(20) 100%
Normal CT study	(20) 100%	(20) 100%
Incidence of embolization	(1) 5%	(3) 15% *

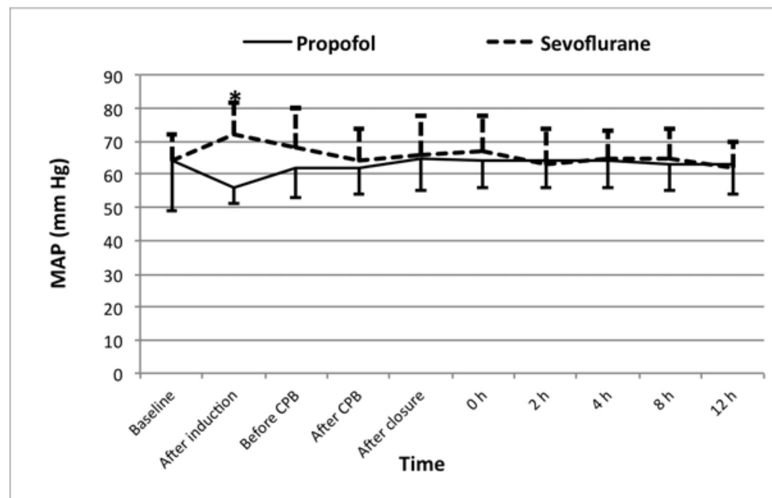
*P <0.05significant when compared with the propofol group.

Fig. (1): Perioperative heart rate (Bpm) changes in the Propofol (n=20) and Sevoflurane Groups (n=20).Data are presented as mean ± SD.



* P <0.05significant when compared with the propofol group.

Fig. (2) : Arterial blood pressure (MAP) (mm Hg) changes in the Propofol (n=20) and Sevoflurane Groups (n=20). Data are presented as mean ± SD. Bpm; beat per minute, CPB; cardiopulmonary bypass.



*P <0.05significant when compared with the propofol group.

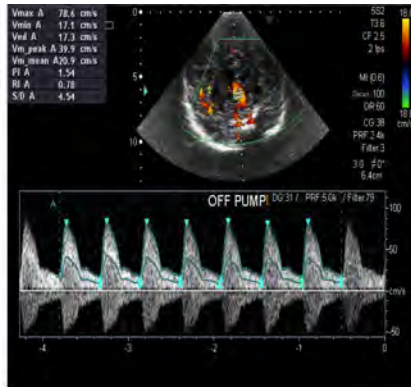


Fig. (3) : Propofol decreased CBFV after CPB

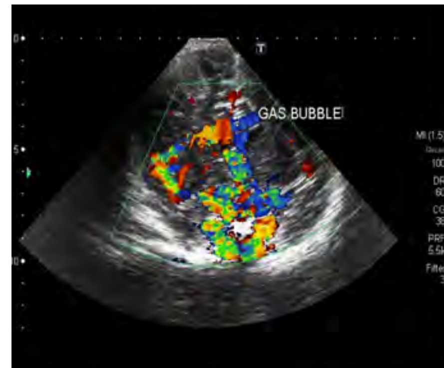


Fig. (4) : Incidence of air embolisation after establishment of full flow CPB

Discussion

The principal finding of the study is that cerebral blood flow velocity was decreased in propofol anesthetized patients group as compared to sevoflurane anesthetized patients group during the surgery.

This is accordance with previous studies, who found that propofol cause reduction in cerebral blood flow velocity.[11,12,13,14,15]

Basil and kuroda , et al in their studies proved that sevoflurane has cerebral vasodilator effect and increases CBFV which matches with the present study[16,17]. However, in contrast to our study, Cho, et al in their study states that sevoflurane decreased cerebral blood flow velocity this may be due to different MAC used.[16,17,18]

The cerebral blood flow velocity reduction by propofol within the middle cerebral artery can be explained by several mechanisms: such as vasoconstricting effect of propofol on the small resistance arterioles,[19] decreasing the cerebral metabolic rate of oxygen, and suppressing effect on endothelium-dependent relaxation that might reduce the steady-state CBF velocity, in that order.[20] On the other hand all volatile anesthetic agents are known to cause

direct cerebral vasodilatation and it has been suggested that desflurane and sevoflurane may be a more potent cerebral vasodilator than the other volatile agents.[21]

Kaisti, et al in their study comparing effects of sevoflurane, propofol, and adjunct nitrous oxide on regional cerebral blood flow, oxygen consumption, and blood volume in humans by using positron emission tomography tracers reported that propofol reduced rCBF and $rCMRO_2$ comparably at a BIS value of 40. Propofol reduced rCBF more than sevoflurane but rCMRO2 to an extent similar to sevoflurane.[14]

In another study by Engelhard, found that propofol caused cerebral vasoconstriction, intact cerebrovascular autoregulation and maintain CO_2 reactivity. On the other hand sevoflurane caused cerebral vasodilatation, decreased CPP, impaired cerebrovascular autoregulation and maintain CO_2 reactivity that support our findings.[22]

Also Mostafa, et al comparing neuroprotection of propofol versus sevoflurane using protein S100 as indicator of cerebral injury during CPB concluded that propofol appears to offer no advantage over sevoflurane for brain protection during CPB which matches our results.[23]

In contrast to our study, neurological outcome showed no significant differences between propofol and sevoflurane groups although propofol decreased CBF more than sevoflurane. This may be attributed to the facts that (1) propofol did not impair cerebrovascular autoregulation regardless the concentration used,[24] (2) propofol reduces cerebral metabolism (with a parallel reduction in $CMRO_2$ and EEG activity) and mitigates intracranial pressure (ICP),[25,26,27] (3) propofol has been proposed to attenuate glutamate-mediated excitotoxic mechanisms by either decreasing NMDA receptor activation, reducing glutamate release, or recovering the function of transporters responsible for glutamate uptake into neuronal and glial cells (4) propofol potentiates GABAergic neuronal activity, mainly due to its counteracting effects on excitatory neurotoxici-

ty,[28] (5) propofol has antioxidant activity against free radicals generated during ischemia.[29]

On the other hand sevoflurane exhibit its neuroprotective effect by a dose dependant reduction in cerebral requirements, or related to decreased apoptotic cell death in the post-ischemic period.[30,31]

Another important finding in the present work is that the incidence of embolic events is lower in propofol group than sevoflurane group. This fact may be explained by the vasoconstrictor effect of propofol on small arterioles and the reduction in CBF and CMRO2, indicates a potential of propofol to reduce cerebral exposure to the embolic load during CPB. This in accordance with previous investigators.[32,33] They found that propofol decreased cerebral embolisation.

The present study showed that the heart rate is lower after induction in propofol group when compared to sevoflurane group. This may be due to the fact that propofol caused a resetting of baroreceptors to allow a slower HR despite decreased arterial blood pressures. Cullen, et al, and Alka Shan, et al. in their studies, support this finding.[34,35]

In contrast to the present results, Ozkose et al. reported bradycardia during the use of sevoflurane and propofol in patients undergoing neurosurgical procedures.[36]

Another finding in this study is that the MAP is lower in propofol group than sevoflurane group. Propofol is known to decrease MAP while sevoflurane maintain it. This may be explained by the fact that it causes decrease in SVR, negative intropic effect, and resitting of baroreceptors.[34,37]

This finding is reported by previous studies.[38,39,40,41,42]

Although propofol decreased CBFV and incidence of embolic events as recorded by TCD when compared to sevoflurane. The neurological outcome was the same as revealed by CT study and neurological examination between both groups. So propofol proved to have

the same neuroprotective effect as sevoflurane. The present study could not prove the presence of direct correlation between TCD and the adverse neurological outcome diagnosed by neurological examination and CT study. This may be contributed to alteration of body temperature during cooling on CBP that decreased the CMRO2, changes in Paco2 that may affect CBF and autoregulation mechanisms and small size of microemboli (less than 200 micron) that was not large enough to occlude the small arterioles.[43]

Conclusion

Although propofol decreases the mean arterial blood pressure, decreases cerebral blood flow velocity and incidence of emboli as monitored by transcranial Doppler it proved to have the same neuroprotective effect as sevoflurane on postoperative neurological outcome.

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SURGICAL TREATMENT OF TYPE 2 DIABETES MELLITUS IN PATIENTS WITH BMI BETWEEN 30 TO 35 AGAINST STANDARD MEDICAL CARE; EXPERIENCE IN MULTICENTERIC RANDOMINZED CONTROL TRIAL

Hosam Elghadban MD, El-Laithy R. MD, Scopinaro N. MD*, Dawoud I. MD and Ameen M. MD

Department of Surgery, Faculty of Medicine, Mansoura University, Egypt

**Department of Surgery, School of Medicine, Genoa University, Italy*

Abstract

Hypothesis: *Resolution or improvement of type 2 diabetes have been obtained so far in the range of overweight above the BMI level of 35 kg/m2, which comprises less than 10 per cent of the type 2 diabetic population. Therefore, the principal aim of this study is to verify the effect of GBP and BPD, the two operations which have shown specific actions on glucose homeostasis control, on type 2 diabetic patients with BMI between 30 and 35, that is mild obesity, and to compare this effect with control patients receiving the standard of medical care.*

Methods: *During my fellowship at San Martino Hospital data were collected from the multicenteric study. A group of type 2 diabetic patients were randomized into either surgical arm (BPD or GBP) or standard treatment care. Variables included weight, BMI, FPG, HbA1c, Cholesterol, triglycerides and HOMA for insulin sensitivity.*

Results: *Significant improvement of HbA1c and FPG occurred in the surgical group compared with no improvement or even worsening of those levels in standard treatment care arm, this even occurred without marked weight loss. Improvement of insulin sensitivity as measured by HOMA was observed in the surgical group and maintained throughout the first year.*

Conclusions: *Surgical option beneficially affects type 2 diabetic patients with BMI 30 to 35, without causing any excessive weight loss. BPD is more effective in controlling type 2 DM than GBP. The main*

difference between the response to surgery of morbidly obese and low BMI type 2 diabetic patients seems to be mainly due to different diabetes biologic severity.

Introduction

Diabetes mellitus represents an expanding pandemic that contributes markedly to worldwide morbidity and mortality. The world prevalence of diabetes among adults (aged 20-79 years) was 6.4%, affecting 285 million adults, in 2010, and will increase to 7.7%, and 439 million adults by 2030[1]. There is a strong relationship between obesity and type 2 diabetes mellitus (T2DM)[2]. The primary risk factor for type 2 diabetes mellitus is obesity, and 90% of all patients with type 2 diabetes are overweight or obese [3-4].

ß-cell dysfunction and insulin resistance are the main pathophysiological defects responsible for the development of hyperglycaemia[5]. It is generally held that ß-cell function is irreversibly lost already at the time the disease manifests itself and thereafter continues to decline linearly with time. Several studies, however, have documented the possibility that ß-cell function may be restored, at least partially, in type 2 diabetes [6-10].

Of major relationship to the issue of ß-cell recovery in diabetes are the following findings: (a) Bariatric surgery in morbidly obese patients with type 2 diabetes can restore euglycaemia, the acute insulin response to glucose[11-14] and insulin sensitivity[15,16]; (b) Studies have reported that diabetic subjects return to euglycaemia and normal insulin levels within days after surgery, long before a significant weight loss has occurred[17]; and (c) Whereas RYGB improves insulin sensitivity in proportion to weight loss, BPD improves insulin action out of proportion to weight loss, i.e., it normalises it at a time when patients are still markedly obese [18].

The results in type 2 diabetes improvements or resolution have been obtained so far in the range of overweight above the BMI level of 35 kg/m^2, which comprises less than 10 per cent of the type 2 diabetic populations. Therefore,

the principal aim of this study is to verify the effect on T2DM of GBP and BPD, the two operations which have shown specific actions on glucose homeostasis control, in the type 2 diabetic patients with BMI between 30 and 35, that is mild obesity, and to compare this effect with control patients receiving the standard of medical care.

Patients and Methods

Throughout Fourteen month of clinical and research fellowship at the San Martino Hospital, Genoa University (principal investigator center), data had been collected and analyzed multicenteric randomized control trial that compare the effect of Gastric bypass and Biliopancreatic diversion on uncontrolled type 2 diabetic patients with BMI between 30 to 35.

Study organization :

The Pilot Center Principal Investigator asked the hospital Ethical Committee to authorize a multicentric study.

Subjects :

Patients with type 2 diabetes (ADA definition, either sex, BMI ≥30 and ≤34.9 $kg.m^{-2}$) meeting the following;

Inclusion criteria:

- Age between 35 and 70 years
- Duration of diabetes ≥ 5 years
- Poor glycemic control (i.e., HbA1c ≥ 8%) in spite of hypoglycemic therapy in accordance with good clinical practice (GCP)
- Presence of significant comorbidities or complications [such as dyslypidemia, arterial hypertension, impaired renal function (i.e., an estimated GFR <60 ml.min-1.1.73m^{-2}), neuropathy, retinopathy, CVD (myocardial infaction, stroke, or TIA, having occurred more than 12 months before the recruitement)].

Exclusion criteria:

- Specific contraindication to obesity surgery or GBP or BPD (depending on the center operation choice).
- Pregnancy.
- Medical conditions requiring acute hospitalization.
- Severe diabetes complica-

tions or associated medical conditions [such as blindness, end-stage renal failure (i.e. serum creatinine >2 mg/dl), liver cirrhosis, malignancy, chronic congestive heart failure (NYHA class III and IV).

- Recent (within preceding 12 months) MI, stroke or TIA.
- Unstable angina pectoris.
- Psychological conditions which may hamper patient's cooperation
- Geographic inaccessibility
- Any condition which, in the judgement of the Investigator, may make risky the participation in the study or bias the results.

Control subjects

For each operated patient, one T2DM control patient, of the same gender and as close as possible for age, BMI, diabetes duration and presence of complications, meeting the same inclusion and exclusion criteria, selected from the list of the patients followed by the local diabetology center. Those patients continued to stay on medical therapy and not asked to undergo any examination besides those related to their clinical care, constitute a group of individuals followed-up to explore the effect of surgery versus standard care as far as long term morbidity and mortality is concerned.

Prerandomization screening tests :

- determination of serum autoantibodies anti-pancreas islet
- determination of serum C-peptide
- usual preoperative assessment for major abdominal surgery
- usual preoperative evaluations for suitability to each of the two surgical procedure.

Study design :

The study is a multicentric prospective 2-arm randomized controlled trial. Each center participating in the study performed only one type of surgical procedure (GBP or BPD), depending on which one it is more familiar with. The randomization was centralized in the organizating center.

Patients randomly assigned in each clinical center with a 2 to 1 ratio to receive either immediate bariatric surgery or standard anti-diabetic care. Patients assigned to bariatric surgery will undergo GBP or BPD, depending on each participating center. Recruitment continued, independently of the number of recruited patients per center.

Each center was responsible for selecting one diabetic subject for each operated patient, matched as closely as possible with the patients assigned to surgical therapy, to be picked up from the local population in medical treatment. These patients served as controls for long term mortality and morbidity.

Endpoints :

Primary endpoints :

Diabetes full remission (HbA1c 6% or below, on free diet and with no antidiabetic medical therapy); diabetes control (HbA1c between 7% and 6.1%, under the same conditions); diabetes improvement (stable reduction of preoperative HbA1c by at least 1%, with less antidiabetic therapy).

Secondary endpoints:

- BMI between 22 and 30.
- Acceptable surgical mortality and morbidity rate
- Reduction or disappearance of the other major components of the metabolic syndrome.

These endpoints evaluated longitudinally within subjects and cross-sectionally between arms over a 1 year period.

Study procedures :

Fully eligible patients were asked to provide the formal informed consent to the study. After the informed consent had been signed, each eligible patient was assigned at random to bariatric surgery (GBP or BPD depending on each center), or STC.

Randomization :

Patients had been assigned to bariatric surgery or standard care by means of web-based randomization procedure Computer-generated random lists will be used, with a 2:1 randomization ratio (2 patients assigned to bariatric surgery for each patient assigned to standard care),

balanced in blocks of varying size in random sequence, and stratified according to the randomizing center.

Surgical procedures :

Biliopancreatic diversion (BPD) Figure: A distal two-third gastrectomy had been carried out aiming at leaving an about 400 ml gastric remnant. The gastrointestinal continuity had been re-established by sectioning the small bowel 300 cm proximal to the ileocecal valve, closing the intestinal stumps, and joining the proximal one end-to-side to the distal ileum at 50 cm from the ileocecal valve. The distal stump of the transacted bowel had been anastomosed to the left corner of the gastric stump, preferably in a totally hand-sewn fashion. However, any gastroenterostomy technique was allowed, according to the surgeons preference. Cholecystectomy (prophylactic or therapeutic) had been considered part of the operation.

Gastric bypass (GBP) Figure: A subcardial gastric pouch with a 30±10 ml capacity had been created on a naso-gastric 36F calibrating tube by sectioning the stomach with a linear stapler 6 cm horizontally for 3-4 cm on the lesser curve, 4 cm distal to the esophagogastric junction, and then vertically until attainment of the angle of Hiss. After identification of the Treitz ligament, the jejunum is transected at 100 cm from the ligament of Treitz and the two stumps are closed. The distal stump is anastomosed to the distal end of the gastric pouch. The preferred gastro-jejunal anastomosis is done by hand-sewing one (but using any other technique the surgeon is more familiar with it, is accepted). Finally, the proximal stump of the transacted bowel anastomosed end-to-side to the jejunum 150 cm distal to the gastroenterostomy. Cholecystectomy (prophylactic or therapeutic) was considered part of the operation.

Both these procedures entail the risk of all early (within 30 days after operation) complications of major abdominal surgery, that is general complications such as pneumonia or pulmonary embolism, and surgical complications, such as wound infection, wound

dehiscence, stenosis of the gastroenterostomy, intraperitoneal or intraluminal bleeding, anastomotic leak (with possible localized or diffuse peritonitis) sepsis and lesions of the biliary tree while doing the cholecystectomy.

Each center performed only the procedure that is most familiar with it. Similarly, both laparotomic and laparoscopic approaches were accepted.

Follow-up antidiabetic medical care :

In principle, basing on the results of the pilot studies, patients were discharged with no antidiuabetic medication. However, the same pilot study had demonstrated that some patients, especially during the first postoperative month, may have occasionally high serum glucose levels.

Patients in the study asked to measure, with the frequency decided by the local diabetologist, serum glucose level in fasting conditions, before meal (s) and two hours after meal(s). In order to minimize beta-cell glucotoxicity, excessively high serum glucose levels had been then corrected with antidiabetic medications, at discretion of the diabetologist, until this will be proven not to be anymore necessary. The suggested cut-off serum glucose level is 200 mg/dl.

Statistical Considerations :

Primary analyses; The primary aim of the study was to assess the efficacy of bariatric surgery in inducing clinical remission of type 2 diabetes mellitus in mildly obese patients (BMI 30-35). To this purpose, all randomized patients, at 1 month, 6 months, 1 year were classified as follows:

1. Diabetes full remission (HbA1c 6% or below, on free diet and with no antidiabetic medical therapy).
2. Diabetes control (HbA1c between 7% and 6.1%, under the same conditions).
3. Diabetes improvement (stable reduction of preoperative HbA1c by at least 1%, with less antidiabetic therapy)
4. Failure (none of the above).

In the primary analyses of the study, the percentages of surgery

patients achieving a Diabetes full remission, and that of those achieving Diabetes control or Diabetes full remission were compared with the percentages of STC patients achieving a Diabetes full remission, and that of those achieving Diabetes control or Diabetes full remission by means of the standard chi-square test for the comparison of percentages.

Results

This figure shows significant weight loss in surgical group after one year,with slight weight gain in the standard medical care group.

This figure shows significant reduction of fasting plasma glucose level in surgical group with no or even slight worsening in standard medical care arm.

Significant reduction of HbA1c level in the surgical group more in the BPD subjects than GBP subjects with no or even worsening of HbA1c level in the standard medical care arm.

Significant improvement was detected in surgical group, more in BPD operated patient.

	preop	1 mt	4 mts	8 mts	1 yr
medical history	X	X	X	X	X
alimentary interview	X	X	X	X	X
complete physical examination	X	X	X	X	X
Body weight	X	X	X	X	X
Fasting plasma glucose	X	X	X	X	X
HBA1c	X	X	X	X	X
Plasma insulin	X	X	X	X	X
lipid profile	X	X	X	X	X
liver enzymes	X	X	X	X	X
Plasma creatinine	X	X	X	X	X
Serum protein and albumin concentration	X	X	X	X	X

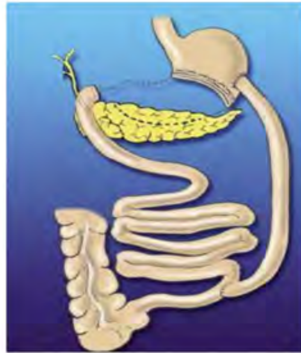
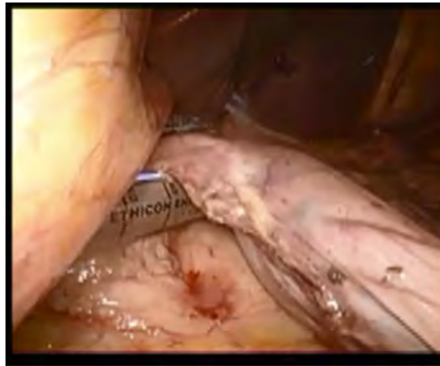
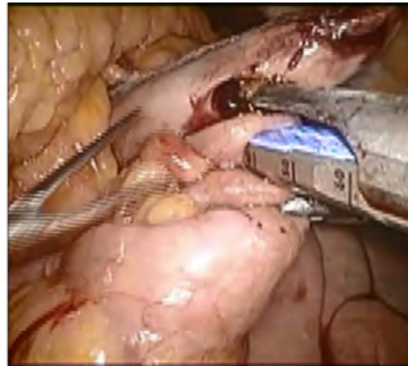


Fig. 1 : Biliopancreatic Diversion (BPD)

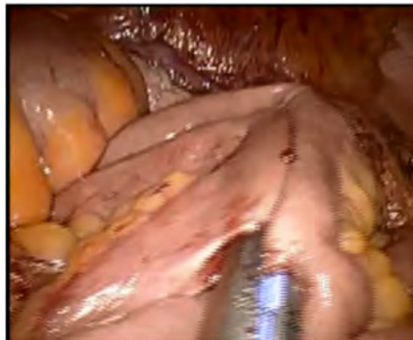
Fig. 2 : Gastric Bypass (GBP)



Duodenal Transection



Gastroenterostomy



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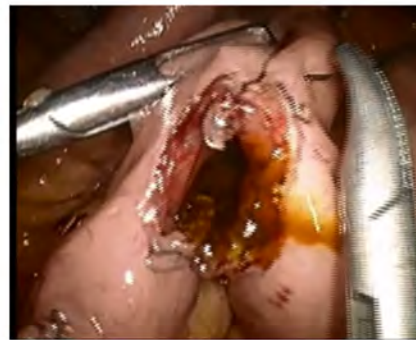


Gastroenterostomy

Fig. (3) : Changes in weight throughout one year in standard treatment care arm and surgical arm.

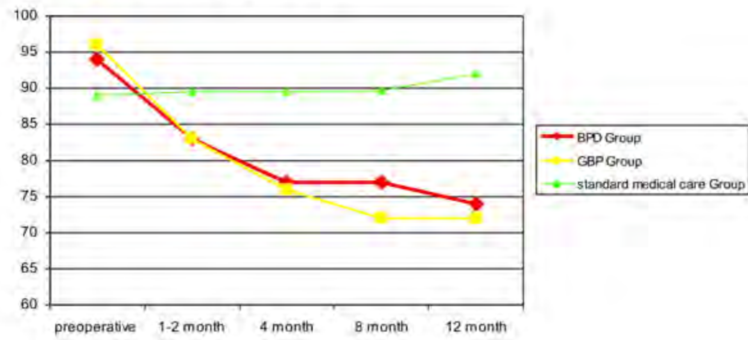


Fig (4) : Changes in fasting plasma glucose throughout one year in standard treatment care arm and surgical arm.

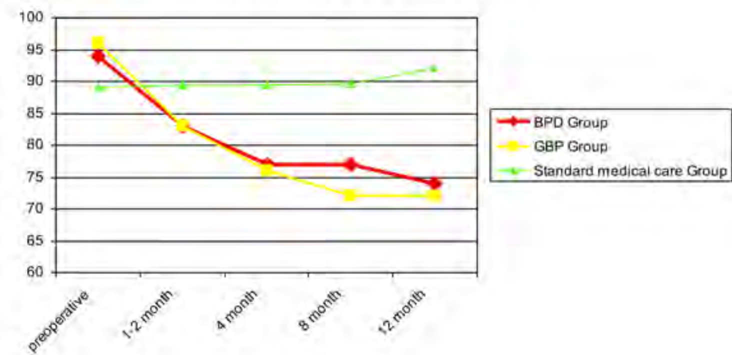


Fig (5) : Changes in HbA1c throughout one year in both Standard treament care arm and surgical arm.

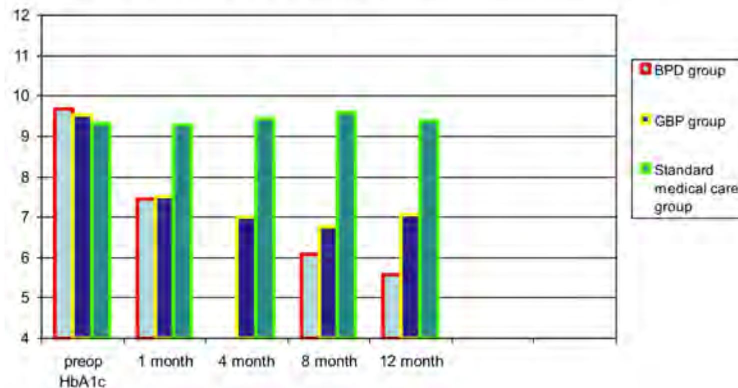
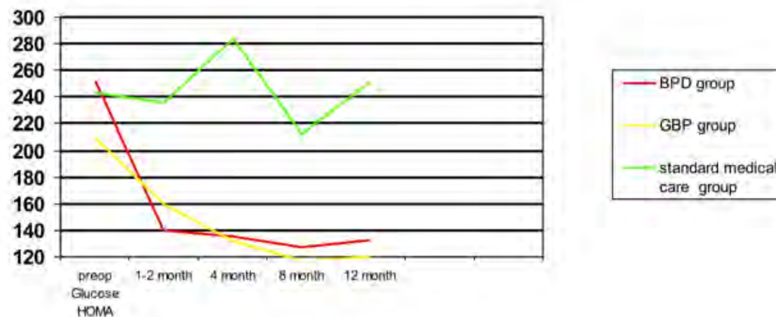


Fig (6) : Changes in HOMA-IR throughout one year in both standard treatment care arm and surgical arm.



Discussion

Weight loss achieved by life-style interventions has been shown to be effective in preventing and treating T2DM[19-21]. However, conventional treatment such as lifestyle modification, and pharmacotherapy has produced small improvements in weight[21-24]. By contrast, bariatric surgery has been shown to effectively provide durable weight loss[25].

Currently, bariatric surgery is now considered appropriate for T2DM patient with BMI > 35 kg/m2. Bariatric surgery leads to remission of T2DM in the majority of patients and improvement in the rest[26]. Growing evidence from clinical and animal studies indicates that bariatric/metabolic surgery may be beneficial for T2DM in non-severely obese or even non-obese patients (BMI < 35 kg/m^2) [27, 28].

International Diabetes Federation (IDF) has released its position statement [29]: Surgery should be an accepted option in people who have T2DM and BMI of 35 more. Surgery should be considered as an alternative treatment option in persons with BMI 30 to 35 when diabetes cannot be adequately controlled by optimal medical regimen, especially in the presence of other major cardiovascular disease risk factors. The surgical approach is now being extended to overweight and mild to moderate obese (BMI <35 kg/m^2) patients with T2DM.

The concept of metabolic surgery was defined by Buchwald and Varco in 1978 in their book “Metabolic Surgery” as the operative manipulation of a normal organ or organ system to achieve a biological result for a potential health gain” [30]. Now, metabolic surgery is defined as any modification of the gastrointestinal (GI) tract, where rerouting the food passage seems to improve T2DM, based on mechanisms that are weight loss independent. This new frontier of bariatric/metabolic surgery includes the application of conventional bariatric procedures (RYGB, BPD, SG, MGB) and the introduction of new procedures (DJB, II-SG, II-DSG, BPD-SPP) designed with the specific aim of having metabolic effects irrespective of causing massive weight loss.

This study, to our knowledge the first multicenteric randomized clinical trial comparing surgically induced remission of diabetes with standard medical therapy for management of type 2 diabetes with BMI between 30-35 participants.

Even the procedures that typically produce the greatest reduction in BMI and excess weight in morbidly obese patients did not affect a similarly dramatic BMI reduction in the low-BMI patients [31].

Scopinaro et al. reported that BPD does not entail risk of excessive or undue weight loss because there is a maximum energy absorption capacity after the operation, which corresponds to a weight of stabilization of low BMI patients[32]. The similar homeostatic mechanism may explain weight stabilization without causing undesirable weight loss after surgical procedures including intestinal bypass. By the end of one year fellowship BMI stabilized at 26.0±2.5 kg/m^2 in BPD group, 24.65±2.1 kg/m^2 in GBP group and at 32.83±2.6 kg/m^2 in control group with P value of 0.004. no reported malnutrition in the surgical group with significant increase in the mean BMI of the control group.

Discontinuation of anti-diabetic medication and remission of T2DM after metabolic surgery were achieved in 86.8% and

64.7% of the patients with FPG and HbA1c approaching slightly above normal range. Moreover, metabolic surgery provided adequate glycemic control for 30.1% of the patients using insulin prior to surgery. It has been described that malabsorptive bariatric procedures have higher diabetes remission rates than restrictive ones[27,33]. Diabetes had been resolved in 45% patient out in BPD and 20% patients in GBP however worsening of diabetes has been observed in the control group, evidenced by increase diabetic medications and shift to insulin in 2 patients.

There is no strong evidence describing the durability of metabolic surgery in long term follow up. Two studies showed durable diabetes remission of T2DM during 5-18 years period after MGB and BPD[34,35]. By contrast, recent studies of bariatric surgery for T2DM patients with severe obesity showed that 24%-43% of the patients with initial remission or improvement of their T2DM subsequently developed T2DM recurrence or worsening during the mid- to long-term follow up period[36,37]. This clinical fellowship is only fourteen month duration, we are waiting long term results.

The aim of this study was to investigate if Surgery resolves or improves type 2 diabetes in patients with BMI 30 to 35, without provoking excessive weight loss. One year after operation, mean body weight and BMI being stable since the fourth month, none of the operated patients had any excessive weight loss, and, according to our definitions, full resolution was obtained in 45% of the cases in BPD group, control in 73%, and improvement in 27%.

When compared to the control group, all patients in the surgical group had a 1-year better outcome than all controls, the observed decrease of FSG and HbA1c being largely accounted for by an increase of the amount of therapy in the majority of patients.

As to the other components of the metabolic syndrome, waist circumference normalized in the vast majority of patients, and the percentage of hypertensive patients

was reduced to one-third of preoperative, with improvement in all the non-normalized cases. Postoperative serum cholesterol changes were those usually seen after BPD, as far as both total and HDL-cholesterol are concerned.

The conclusions that can be drawn from the present study are: Surgery beneficially affects type 2 diabetes also in patients with BMI 30 to 35, without causing any excessive weight loss; BPD is more effective than GBP in controlling diabetes in this category of patients. The main difference between the response to surgery of morbidly obese and low BMI type 2 diabetic patients seems to be mainly due to different diabetes biologic severity, and this difference is such that even suggests to consider these conditions as two different diseases.

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SURGICAL TREATMENT OF TYPE 2 DIABETES MELLITUS IN PATIENTS WITH BMI BETWEEN 30 TO 35 AGAINST STANDARD MEDICAL CARE; EXPERIENCE IN MULTICENTERIC RANDOMINZED CONTROL TRIAL

Hosam Elghadban MD, El-Laithy R. MD, Scopinaro N. MD, Dawoud I. MD and Ameen M. MD

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SOME METABOLIC EFFECTS OF GHRELIN

Mohamed A. Abo El-Enien M.Sc., Fayza R. El-Menabawy Ph.D, Soheir F. El-Bassiony Ph.D, Hanaa A. Abdel-Moneim Ph.D and Geehan A. El-Wakeel Ph.D

Department of Medical Physiology, Faculty of Medicine, Mansoura University, Egypt

Abstract

Background: The aim of the present work is to study the metabolic aspects of the ghrelin and to study some mechanisms that may be of help in the understanding and treatment of those pathological conditions characterized by insulin resistance and its evolution toward (type 2) diabetes. Materials and Methods: Twenty four male rats were divided into two main groups, twelve rats per each group. In vivo group was divided into control one and ghrelin- treated one, (0.2 μg per rat for 4 days), where fasting serum glucose and plasma free fatty acids (FFA) were measured. In vitro group included experiments on epididymal fat pad (EFP), hemidiaphragm and liver slices to study the effect of ghrelin (1 nM) on basal and insulin-induced glucose uptake and free fatty acids (FFA) release. Results: ghrelin caused a highly significant increase in both of fasting serum glucose level (157±13.4mg/dl) and also in fasting plasma FFA level (50.8 ± 15.5mg/dl). However, it caused a significant increase in insulin-stimulated glucose uptake by EFP in the presence of supraminimal and supramaximal insulin concentrations (2.543 ± 0.98 and 3.431 ± 0.25) mg/gm wet tissue / hour) and also by hemidiaphragm in the presence of supraminimal and supramaximal insulin concentrations (3.99 ± 0.42 and 5.112 ± 0.97) (mg/gm wet tissue/hour). Also, it increased net glucose output by liver slices in the presence of supraminimal and supramaximal insulin concentrations (-4.771 ± 1.9 and - 4.003 = 1.22) (mg/gm wet tissue / hour). It decreased FFA release from EFA release from EFP in the presence of supramiimal and supramaximal insulin concentrations (0.511 ± 0.21 and 0.435 ± 0.2) (mg/gm wet tissue/hour), hemidiaphragm in the presence of supraminimal and

supramaximal insulin concentrations (0.541±0.1 and 0.511±0.16) (mg/gm wet tissue/hour) and liver slices in the presence of supraminimal and supramaximal insulin concentrations (0.698 ± 0.14 and 0.691 ± 0.23) mg/gm wet tissue/ hour).

Conclusion : *Ghrelin increased serum glucose level and plasma FFA level. It potentiated the effect of insulin on glucose uptake in EFP and hemidiaphragm. It decreased hepatic insulin sensitivity and responsivity and enhanced the suppressive effect of insulin on FFA release from different peripheral tissues.*

Introduction

Ghrelin is a gut-brain peptide which has somatotropic and food intake increasing effects. But its impact on substrate metabolism is controversial: [1]found hyperglycemia in rats following chronic ghrelin administration nevertheless plasma insulin did not change. However,[2] supported the role of ghrelin in inhibiting insulin release. This was supported by[3] who found that ghrelin receptor antagonists, markedly lower fasting glucose concentration and attenuate plasma glucose elevation. On the contrary,[4] observed that ghrelin stimulated insulin release in the presence of high, but not basal, glucose concentration.

Ghrelin has been reported to increase body fat, also independently of changes in food intake[5]. A specific effect of ghrelin on lipid metabolism was suggested by the observation that rodents treated with ghrelin showed enhanced fat content independently of feeding behavior. However, under fasting it may enhance the growth hormone lipolytic action[6]. So, the aim of the present work is to clarify:

1) the effect of ghrelin on serum glucose level and plasma FFA level in rats 2) the effect of ghrelin on basal and insulin-stimulated glucose uptake by different tissues 3) the effect of ghrelin on FFA release in presence or absence of insulin in such tissues.

Materials and Methods

`Experimetal Animals :

Twenty four male Sprague-

Dawley rats, weighing 150 to 250 grams, were used in this work. The animals were housed in the laboratory of Medical Experimental Research Center (MERC) under controlled conditions, with constant temperature (24-26 and humidity (50-60 %). Standard rat chow and water were allowed. Rats were acclimated under these conditions for one week. The animals were studied in the fasting state, and these tissues were obtained:

- **Adipose Tissue:** was obtained from epididymal adipose tissue
- **Skeletal Muscle:** was obtained from hemidiaphragm
- **Liver Slices:** were obtained from right lobe of the liver.

Chemicals and Drugs Used

- Ghrelin: in the form of lyophilized powder of acylated purchased from Sigma Aldrish (100 μg). It was dissolved in 1% Acetic Acid and stored at - 22°C
- Insulin: actrapid insulin, in the form of ampoule containing 10 ml in a concentration of 100 IU per ml.

Experimental Groups :

I. In Vivo Experiments

1. Control group containing six rats
2. Ghrelin-treated group containing six rats treated with subcutaneous injection of ghrelin at a dose of 0.2 μg per day for four subsequent days (1)

- Blood samples were taken from the fasting rats through intracardiac blood sampling method for determination of:

- serum glucose level
- Plasma free fatty acids (FFA) level .

II. In Vitro Experiments :

A. Experiments on Epididymal Fat Pad (EFP)

1. Studying basal glucose uptake and free fatty acids (FFA) release
2. Studying glucose uptake and free fatty acids (FFA) release in the presence of ghrelin (1 nM)
3. Studying glucose uptake and free fatty acids (FFA) release in the presence of supraminimal concentration of insulin (100 μU per ml).

4. Studying glucose uptake and free fatty acids (FFA) release in the presence of ghrelin (1 nM) and supraminimal concentration of insulin (100 μU per ml)
5. Studying glucose uptake and free fatty acids (FFA) release in the presence of supramaximal concentration of insulin(10 mU per ml)
6. Studying glucose uptake and free fatty acids (FFA) release in the presence of ghrelin (1 nM) and supramaximal concentration of insulin(10 mU per ml)

B. Experiments on Hemidiaphragm :

1. Studying basal glucose uptake and free fatty acids (FFA) release (Control group)
2. Studying glucose uptake and free fatty acids (FFA) release in the presence of ghrelin (1 nM).
3. Studying glucose uptake and free fatty acids (FFA) release in the presence of supraminimal concentration of insulin (100 μU per ml)
4. Studying glucose uptake and free fatty acids (FFA) release in the presence of ghrelin (1 nM) and supraminimal concentration of insulin (100 μU per ml)
5. Studying glucose uptake and free fatty acids (FFA) release in the presence of supramaximal concentration of insulin (10 mU per ml)
6. Studying glucose uptake and free fatty acids (FFA) release in the presence of ghrelin (1 nM) and supramaximal concentration of insulin (10 mU per ml)

C. Experiments on liver slices

1. Studying net glucose uptake and free fatty acids (FFA) release (control group)
2. Studying net glucose uptake and free fatty acids (FFA) release in the presence of ghrelin (1 nM).
3. Studying net glucose uptake and free fatty acids (FFA) release in the presence of supraminimal concentration of insulin (100 μU per ml)
4. Studying net glucose uptake and free fatty acids (FFA) release in the presence of Ghrelin (1 nM) and su-

praminimal concentration of insulin (100 μU per ml)

5. Studying net glucose uptake and free fatty acids (FFA) release in the presence of supramaximal concentration of insulin (10 mU per ml).
6. Studying net glucose uptake and free fatty acids (FFA) release in the presence of Ghrelin (1 nM) and supramaximal concentration of insulin(10 mU per ml).

Incubation Medium

The incubation medium employed was Krebs Ringer Bicarbonate Buffered Solution (KRBBS)

Measured parameters :

Serum glucose level, plasma FFA level, tissue glucose uptake and FFA release.

Statistical Analysis :

Values were expressed in the form of mean (+/-) SD which are done by using excel program for figures and SPSS (SPSS, Sigma Plot Software, Inc, Chicago, IL) program statistical package for social science version 16.

Results as illustrated in tables 1, 2, 3 and 4 and figures 1, 2, 3 and 4

Table (1): Effect of ghrelin* on fasting serum glucose level (mg/dl):

	Fasting Serum Glucose Level (mg/dl)	
	Control group	Ghrelin-treated group
Mean	73	157
±SD	± 5.2	±13.4
P	-	< 0.001

P: as compared with control group

Table (2): Effect of ghrelin* on fasting plasma free fatty acids (FFA) (mg/dl):

	Fasting Plasma Free Fatty Acids (FFA) (mg/dl) level	
	Control	**Ghrelin-treated group**
Mean	20.7	50.8
±SD	± 2.05	± 15.5
P	-	< 0.001

P: as compared with control group.

Table (3): Effect of ghrelin (1 nM) on glucose uptake (mg/gm wet tissue/h.) by epididymal fat pad, hemidiaphragm and net glucose uptake by liver slices in basal conditions and in the presence of supraminimal (100 µU per ml) and supramaximal concentrations of insulin (10 mU per ml):

Tissue	Basal	Ghrelin	Supraminimal Insulin (100 µU per ml)	Ghrelin Supraminimal Insulin (100 µU per ml)	Supramaximal Insulin (10 mU per ml)	Ghrelin & Insuln(10 mU / ml)
EFP	1.111	1.39	1.990±0.52	2.543±0.98	2.733±0.45	3.431±0.25
Mean ±SD	±1.07	± 0.73				
P						
P1	-	NS				
P2			-	< 0.05	-	< 0.05
Hemidiaphr-agm	2.001	2.19	2.976±0.79	3.990±0.42	4.342±1.04	5.112±0.97
Mean ±SD	±0.66	±0.8				
P						
P1						
P2	-	NS				
			-	< 0.05	-	< 0.05
Liver	1.99	2.3±1.05	2.343±1.62	-4.771±1.9	2.730±0.86	-4.003±1.22
Mean ±SD	±					
P	0.99	NS				
P1	-		-	< 0.001		
P2					-	< 0.001

The results are Mean±SD, P: as compared with basal

P1: as compared with supraminimal insulin, P2: as compared with supramaximal insulin

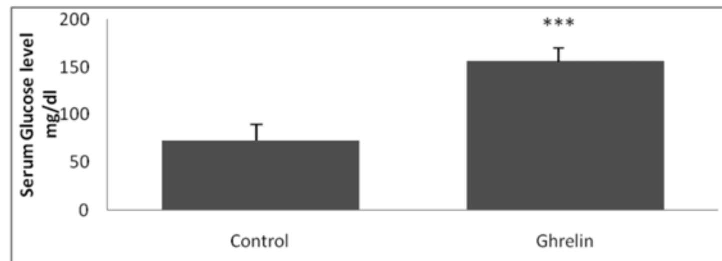
Table (4): Effect of ghrelin (1 nM) on free fatty acids (FFA) release (mg/gm wet tissue/h.) from epididymal fat pad, hemidiaphragm and liver slices in basal conditions and in the presence of supraminimal (100 µU per ml) and supramaximal concentrations of insulin (10 mU per ml):

Tissue	Basal	Ghrelin	Supraminimal Insulin (100 µU per ml)	Ghrelin Supraminimal Insulin (100 µU per ml)	Supramaximal Insulin (10 mU per ml)	Ghrelin Supramaximal Insulin (10 mU per ml)
EFP **Mean ±SD** **P** **P1** **P2**	1.115± 0.69 -	1.078± 0.432 NS	0.665±0.2 -	0.511±0.21 < 0.05	0.544±0.21 -	0.435±0.2 < 0.05
Hemidia -phragm **Mean ±SD** **P** **P1** **P2**	1.119 ±0.39 -	0.891 ±0.147 NS	0.773±0.23 -	0.541±0.1 < 0.05	0.652±0.24 -	0.511±0.16 < 0.05
Liver **Mean ±SD** **P** **P1** **P2**	0.99 ±0.26 -	0.828 ±0.243 NS	0.752±0.14 -	0.698±0.14 < 0.05	0.711±0.15 -	0.691±0.23 NS

P: as compared with basal P1: as compared with supraminimal insulin

P2: as compared with supramaximal insulin

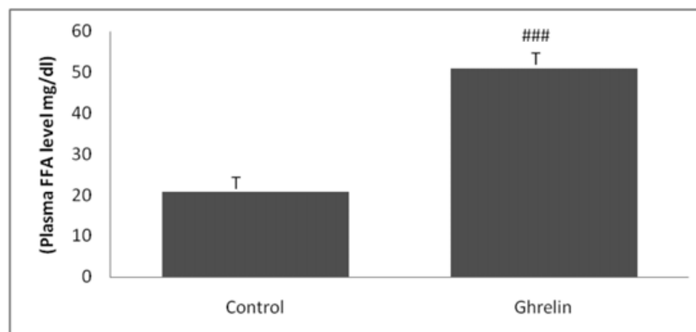
Figure (1): Effect of ghrelin* on fasting serum glucose level (mg/dl)



*Ghrelin was given by subcutaneous injection, (0.2 µg) per day for four subsequent days

***: Highly significant as compared with control group

Figure (2): Effect of ghrelin* on fasting plasma free fatty acids (FFA) (mg/dl)

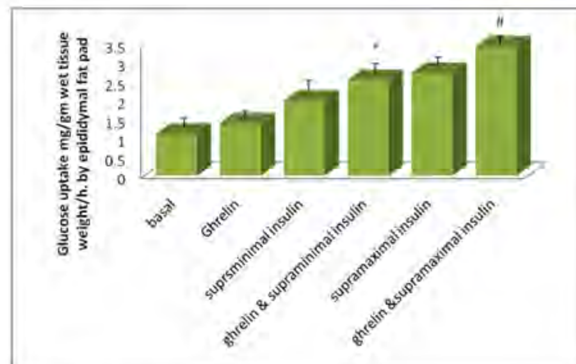


*Ghrelin was given by subcutaneous injection, (0.2 µg) per day for four subsequent days

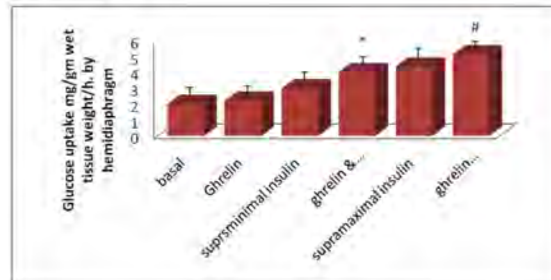
#: Significant as compared with control group ###: Highly significant

Figure (3): Effect of ghrelin (1 nM) on glucose uptake (mg/gm wet tissue/h.) by epididymal fat pad, hemidiaphragm and net glucose uptake by liver slices in basal conditions and in the presence of supraminimal (100 μU per ml) and supramaximal concentrations of insulin (10 mU per ml)

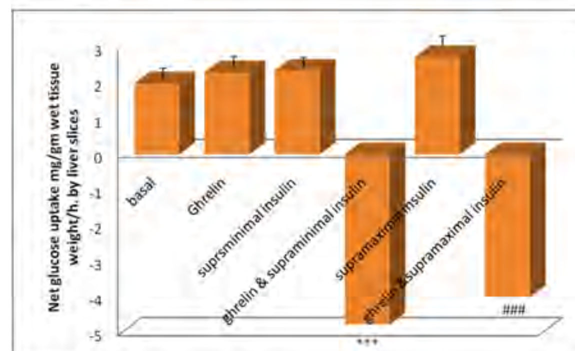
1. **Epididymal fat pad**



B) Hemidiaphragm

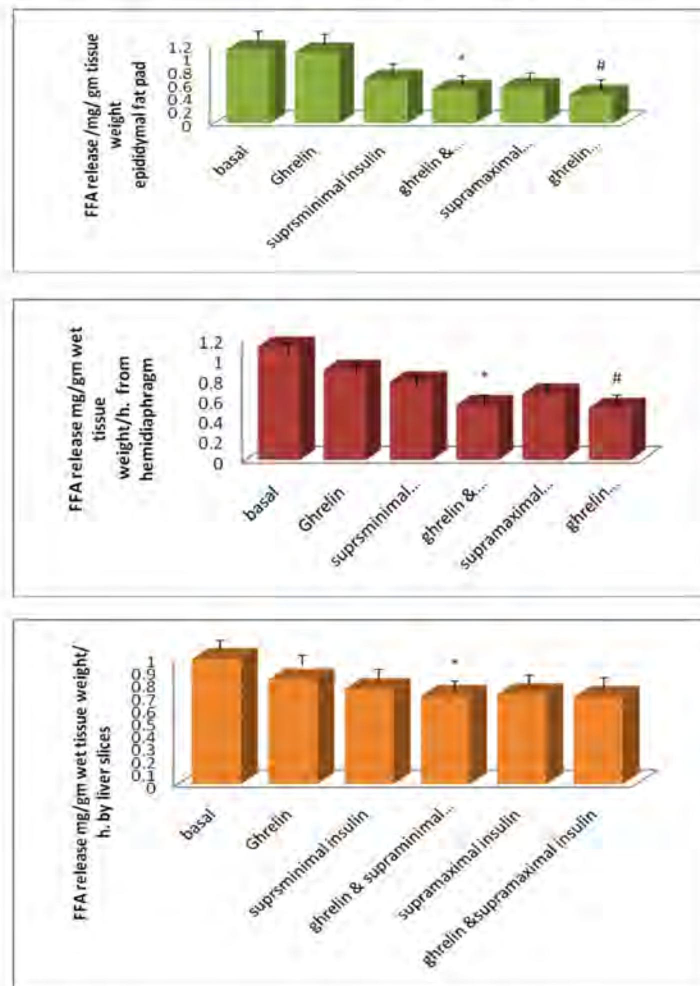


C) Liver slices



$: Significant as compared with basal
*: Significant as compared with supraminimal insulin
#: Significant as compared with supramaximal insulin

Figure (4): Effect of ghrelin (1 nM) on free fatty acids (FFA) release (mg/gm wet tissue/h.) from epididymal fat Pad, hemidiaphragm and liver slices in basal conditions and in the presence of supraminimal (100 μU per ml) and supramaximal concentrations of insulin (10 mU per ml)



*: **Significant** as compared with supraminimal insulin, #: **Significant** as compared with supramaximal insulin.

Discussion

Ghrelin is a gut peptide predominantly produced by the stomach, but also by other regions of the gastrointestinal tract[7,8]. Ghrelin circulates in the bloodstream in two forms: acylated and unacylated. Ghrelin has a fatty-acid modification that allows binding to and activation of the growth hormone secretagogue receptor type 1a (GHS-R1a[7].

In this study, ghrelin significantly increased serum glucose level. This is in agreement with the work of[9] who found an increase in serum glucose level following ghrelin administration both in rats and also in human studies. Also,[10] reported an increase in blood glucose level following chronic ghrelin demonstration in normal and obese subjects.

The increase in blood glucose level could be explained by decreasing insulin release from pancreas which may be either by central or peripheral mechanisms[11]: The central mechanism : the pancreas is innervated by parasympathetic nerve fibers originating from dorsal vagal complex that modulate pancreatic secretion via cholinergic synapses. Dorsal vagal complex contain ghrelin sensitive neurons that mediate an orexigenic effect and pancreatic enzyme secretion[12]. The peripheral mechanism: through inhibiting insulin secretion from the pancreas by both direct and indirect mechanisms : The direct effect: ghrelin receptors are expressed in both human and rat pancreatic islets[2]. The indirect effect : by acutely stimulating pancreatic polypeptides particularly somatostatin which could explain the suppression of insulin level[13].

The direct effect is explained by the insulinostatic action of ghrelin, through the following mechanisms: (1) ghrelin attenuates $[Ca2+]i$ in β-cells (2) ghrelin activates Kv currents in β-cells (3) ghrelin uses G-protein $G\alpha i2$ in β-cells[14].

Also, this increase in blood glucose level following ghrelin injection could be attributed to an increase in insulin resistance and decrease in insulin sensitivity independent of growth hormone

action. Studies in growth hormone deficient human subjects showed that injection of ghrelin caused an increase in blood glucose level followed by an eventual increase in insulin concentration. Both glycemia and insulin levels are raised compared to placebo control following a meal which is indicative of worsened insulin sensitivity [15].

Also, this condition of increased insulin resistance is observed in humans after coadminstration of ghrelin and growth hormone receptor antagonists which could suggest a direct role of ghrelin on insulin resistance [16].

The hyperglycemia could be attributed to increase in net hepatic glucose production. The underlying mechanism may be: blocking insulin's inhibitory effect on the gene expression of key gluconeogenic enzymes as found in hepatoma cell line by [17].

The effect of ghrelin on blood glucose level could be a direct one independent of growth hormone. This was also reported by a conclusive study by [18] performed in hypopituitary subjects in whom ghrelin infusion induced hyperglycemia under basal conditions. Further confirmations have been obtained in rodents where ghrelin was found to have similar rapid hyperglycemic effects in growth hormone deficient mice[2]. Controversial studies have proved an indirect effect mediated by growth hormone release by the work of [13].

In this study, ghrelin has no significant effect on basal glucose uptake by epididymal adipose tissue but significantly increased glucose uptake in the presence of both supraminimal and supramaximal insulin concentrations suggesting that ghrelin acts synergistically with insulin. This is in agreement with the work of [19] who demonstrated an increase in insulin-stimulated glucose uptake in isolated epididymal adipose tissue in a dose dependent manner. Rat epididymal fat pad expresses GHS-R 1(a) mRNA[19], suggesting that ghrelin may directly influence adipocyte function.

Studies using the adipocyte cell

line have demonstrated potentiation of insulin-stimulated glucose uptake[20]. Interestingly, these studies also found that ghrelin induced a significant increase in basal glucose uptake in these cells. In contrast,[21] found that ghrelin pre-treatment had no effect on insulin-stimulated glucose uptake in a brown adipocyte cell line. The differences between these results and ours may reflect differences in cell type. Also,[22] found that basal and insulin-stimulated glucose uptake by adipose tissue decreased with ghrelin.

In this study, ghrelin has no significant effect on basal glucose uptake by hemidiaphragm but significantly increased glucose uptake in the presence of both supraminimal and supramaximal insulin concentrations suggesting that ghrelin acts synergistically with insulin. This is in agreement with the work of[19] who demonstrated an increase in insulin-stimulated glucose uptake in isolated epididymal adipose tissue in a dose dependent manner.

The insulin stimulation of glucose uptake in adipose and muscle tissue occurs through complex signalling pathway acting through the insulin receptor tyrosine kinase. The primary effect is to promote the movement of the GLUT-4 protein from intracellular storage sites to the plasma membrane. Ghrelin induced increases IRS-1 and AKT phosphorylation, but when the adipocytes were treated with wortmannin, a PI3K inhibitor, completely blocked this ghrelin induced increase in glucose transport and phospho-AKT expression[20], suggesting that the direct effects of ghrelin on insulin-stimulated glucose uptake are mediated by the GHSR1a and PI3K/AKT activation.

The results of the present study demonstrated that ghrelin has no significant change in basal net glucose uptake by the liver slices. However, ghrelin increases net glucose production in the presence of insulin suggesting antagonism of insulin action.

Ghrelin could act via a non-GHS-R1a, since the GHS-R1a has not been clearly demonstrated in liver[17]. Therefore, it may act by

unidentified type (s) of ghrelin receptors.

The underlying mechanism may be: blocking insulin's inhibitory effect on the gene expression of key gluconeogenic enzymes as found in hepatoma cell line by [17].

Insulin inhibits gluconeogenesis, through the activation of the insulin receptor (IR). It acts predominantly by suppressing the expression of the genes for the key gluconeogenic enzymes phosphor-enol-pyruvate-carboxykinase (PEPCK) and glucose -6-phosphatase (G6Pase) [23]. In (rat hepatoma cell line) and (human hepatocellular carcinoma cell line) ghrelin was shown to affect insulin receptor substrate (IRS1) and its downstream molecules, including growth factor receptor-bound protein 2 (Grb2) and mitogen-activated protein kinase (MAPK).

In this study, ghrelin caused a highly significant increase in plasma FFAs level. This is in agreement with the work of [24]. This effect could be explained by indirect effect of ghrelin to stimulate lipolysis through the release of growth hormone and inhibition of insulin secretion [24].

We have investigated the direct effects of ghrelin on FFAs release from epididymal fat pad in vitro. It was observed that ghrelin caused a non-significant decrease in FFAs release. Addition of supraminimal and supramaximal concentrations of insulin caused a significant decrease in FFAs release. Addition of ghrelin (1 nM) to the incubation medium with insulin in both concentrations caused a more significant decrease in FFAs release.

[25] found that ghrelin suppressed glycerol release from cultured epididymal adipocytes. In addition, isoproterenol-stimulated lipolysis was significantly reduced by simultaneous ghrelin treatment in a dose-dependent manner in vitro [5]. Although the precise mechanisms governing the release of FFAs by ghrelin remain to be elucidated [26].

We have also investigated the effects of ghrelin on FFAs release from hemidiaphragm. It caused a non-significant decrease in free

fatty acids (FFAs) release. However, addition of ghrelin (1 nM) with insulin in both concentrations caused a more significant decrease in FFAs release suggesting potentiation of insulin action in inhibiting lipolysis.

[27] found that ghrelin induces lipid accumulation in specific abdominal depots; liver and skeletal muscle inducing muscle and liver steatosis. Also[28] proved that ghrelin induces GHS-R-dependent hepatic steatosis.

In the present study, we have investigated the effects of ghrelin on FFAs release from liver slices. It caused a non-significant decrease in free fatty acids (FFAs) release. Addition of supraminimal and supramaximal concentrations of insulin to the incubation medium caused a significant decrease in FFAs release. Addition of ghrelin (1 nM) with insulin in both concentrations to the incubation medium caused a more significant decrease in FFAs release. [28] reported that sterol-regulatory element-binding protein (SREBP1c), the master regulator of hepatic lipogenesis, was tripled by ghrelin exposure. However, SREBP1c does not regulate lipid synthesis in WAT [29].

The data in this work support a direct role for ghrelin in enhancing insulin-inhibited FFAs release in isolated epididymal adipose tissue, hemidiaphragm and liver slices. This is in agreement with the work of [17] who found that ghrelin induces adiposity in rodents and stimulates insulin-signaling cascade in various cell lines.

In the present study, ghrelin and insulin had the same effects on lipid metabolism in hemidiaphragm, adipose tissue, as well as, in liver slices, where both of them favor lipid storage as triacylglycerol (TAG). These effects may be located at the level of the receptor in the coupling between the receptor and the intracellular mediators resulting in the modulation of cellular lipid oxidation.

Conclusion

Ghrelin increases fasting blood glucose level and fasting plasma FFA level. It has no direct effect on basal glucose uptake or FFA release by peripheral tissues. It

potentiates insulin-stimulated glucose uptake by adipose tissue and muscle tissue. It enhanced the suppressive effect of insulin on FFA release from different peripheral tissues. The hyperglycemic effect of ghrelin is partially due to antagonism of insulin-stimulated net glucose uptake by liver. This antagonism is due to the decrease in both sensitivity and responsivity to insulin. So, ghrelin defends against low blood glucose level during fasting state. However, this effect is reversed after food intake while it enhances insulin-stimulated glucose uptake. Also, it defends fat loss by increasing substrate availability through glucose uptake and inhibiting FFA release. So, it preserves energy in the form of fat

Future Studies

- Comparing the effect of ICV administration of ghrelin and the effect of subcutaneous ghrelin administration.
- Prove that centrally adminstring ghrelin may induce fat accumulation which seems independent of increasing food intake (this work includes the measurement of body weight of rats).
- Test the role of sympathetic nervous system and adrenoreceptor expression and function in the fat accumulating effect of ghrelin administered centrally or peripherally or applied in vitro to adipocytes.

Recommendations

Any attempt to reduce stored fat by restricting caloric is less effective as ghrelin action in reserving energy in the form of fat increases by fasting. Thus, even if the one is able to overcome the orexigenic influence of ghrelin specially ghrelin induced desire to consume fat, the sustained circulating ghrelin converts ingested calories into stored fat. This unlucky sequence of events is exacerbated by the fact that postprandial suppression of circulating ghrelin is less pronounced in obese subjects and after the consumption of fatty food. In contrast, it is interesting to note that weight loss associated with maintenance on a low fat high carbohydrate diet is not accompanied by an increase in circulating ghrelin.

Therefore, it seems that ghrelin may defend stored lipid from utilization. Thus, interruption of ghrelin signaling at least intermittently is an essential element in order for such regimes to be effective.

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SOME METABOLIC EFFECTS OF GHRELIN

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OCCUPATIONAL DETERMINANTS OF PRETERM BIRTH AMONG WORKING WOMEN ATTENDING MANSOURA UNIVERSITY HOSPITAL

Ghada El-Khawaga MD and Ashraf Ghanem MD*

*Departments of Community Medicine and *Obstetrics and Gynecology,*

Faculty of Medicine, Mansoura University, Egypt

Abstract

Objective: *The purpose of this study was to detect the effect of various occupational factors, on the risk of preterm delivery among working women attending the delivery suite at the Obstetric and Gynecology Department at Mansoura University Hospital .*

Study design: *The study was a hospital-based non-matched case-control study conducted during the period from 1st of April 2011 to the 31st of March 2013.*

Setting: *Department of Obstetric and Gynecology, Mansoura University Hospital.*

Methods: *The study included 326 working pregnant women who delivered a preterm single baby (<37 weeks) (Cases) and 1014 working pregnant women who delivered a single baby at term (≥ 37 weeks) (Controls). Analysis of the working conditions were carried out for women who were working for at least the third month of pregnancy. Exclusion criteria were pregnant women with previous abortion, previous preterm delivery, women with multiple pregnancy, congenital malformation, placenta praevia, oligohydramnios, polyhydramnios, cerclage in situ, Preterm Rupture Of Membranes (PROM), stillbirth and complications during current pregnancy (bleeding and/or fetal problems). Occupational factors were measured using a pre-designed administered questionnaire, which included factors describing job and working conditions. The women were interviewed after delivery. Data including pregnancy outcome were obtained from clinical hospital records. Logistic regression analysis was used to adjust for well-known risk factors.*

Results: *Among working women, an excess risk for preterm delivery was observed for women working ≥ 40 hours per week $P < 0.05$ (OR 1.7, CI: 1.0-3.03), shift work $P \le 0.05$ (OR 1.4, CI: 1.02-1.8), or standing > 6*

hours per day P≤ 0.05 (OR 1.3, CI:1.0-1.8), demanding posture for ≥ 3 hours per day P< 0.05(OR 1.4, CI: 1.03-1.9), and carrying heavy weight P < 0.05 (OR 1.6, CI: 1.01-2.4). Excess risk was also observed for women having temporary contract P< 0.05 (OR 1.4, CI: 1.04-1.9). There was no association between preterm delivery risk and long commuting time to work, night work, work on assembly line, noise, cold or hot environment (P > 0.05), Also, no statistically significant difference was observed with the possibility to sit when standing, pushing or pulling objects, and absence of breaks.

Logistic regression analysis revealed strong predictive effect for both physical work demands (OR = 3.94, 95% CI: 1.03-18.19) and heavy weight carrying (OR =2.76, 95% CI: 1.98–8.74) on preterm delivery. Predictive effects were also observed between long working hours, prolonged standing (> 6 hours), and temporary contract on the risk of preterm delivery (P < 0.05).

Conclusion: *Certain occupational factors experienced by working pregnant women can increase the risk of having preterm delivery, this can provide relevant information on possible preventive measures to reduce the risk of preterm delivery among working pregnant women and its related serious sequel .*

Introduction

Preterm birth, defined as birth at < 37 completed weeks of gestation, is considered to be a major risk factor for subsequent morbidity and mortality of newborn[1]. It is the most important single determinant of adverse infant outcome in terms of both survival and quality of life[2]. In the UK, infant mortality among preterm births was 42/1000 live births in 2005, compared with 5/1000 live births overall. For very preterm births (at < 32 completed weeks of gestation), mortality in the first year was 144/1000 live births, compared with 1.8/1000 live births for babies born at term[3]. Very preterm birth accounts for 1.4% of UK births and 51% of infant deaths [4]. Risk of death or neurosensory disability increases with decreasing gestational age[2]. Moreover, preterm birth may have huge psychosocial and emotional effects on the family, as well as being costly for health services[5]. Consequently, prevention of preterm birth is important, not as an end in itself but as a means of improving outcome for the child.

Although preterm birth may be linked to different mechanisms, a certain number of risk factors of this pregnancy outcome have been identified, such as those related to maternal risk factors (e.g. age, body mass index, behavioural factors, marital status, education) and obstetric risk factors (e.g. parity, complications, previous events). Nevertheless, it is likely that risk factors remain undiscovered[6].

Studies on the relation between employment and preterm delivery have yielded conflicting results[7]. The decision to work during pregnancy reflects a large variety of factors such as education, social support, and health status that are independently linked to the risks of preterm birth[8,9]. In many studies, women who are employed have a lower risk of preterm birth than women who are not employed[10] and studies that have failed to find any association between working and adverse pregnancy outcome[11] have generally only compared working and non-working populations, rather than investigating the characteristics of the work itself[12]. Nonetheless, a broad range of studies have found that certain working conditions, in particular physically strenuous or fatiguing work, increase the risk of preterm birth[13]. Also, several published literature reviews and meta-analysis have pointed out that physical work demands, long working hours, shift work and/ or measures of job stress may be associated with these outcomes [8,9]. However, a large number of studies focused on the impact of occupation or job title on pregnancy outcomes[13,14], or on a reduced number (a maximum of 3 or 4) of occupational factors[15,16,17], or on occupational factors evaluated through a job-exposure matrix (i.e. derived from job titles) that may present several shortcomings in terms of exposure misclassification or underestimation of the actual risk[18,19]. Identifying employment related risk factors for preterm delivery is of particular importance because these risks are amenable to change through policies granting work leaves or modifying working conditions during pregnancy. In contrast, most other risk factors for preterm birth cannot easily be changed [20].

Studies in this area are lacking in Egypt, and as a growing percentage of women work outside home before, during and after pregnancy, work and its related occupational factors deserve to be studied in relation to preterm delivery among Egyptian women.

The aim of this study was to detect the effect of various occupational factors, on the risk of preterm delivery among working women attending the delivery suite at the Obstetric and Gynecology Department in Mansoura University Hospital (MUH).

Subjects and Methods

This study was conducted at the Department of Obstetric and Gynecology at Mansoura University Hospital (MUH) during the period from 1st of April 2011 to the 31st of March 2013. MUH is a pooling hospital which receives patients from Mansoura city and the adjacent districts. The study was a hospital-based non-matched case-control study. It included 1340 working pregnant mothers who attended the delivery suite during the study period.

The study included all working pregnant women fulfilling the criteria of eligibility which were defined as only women who worked (other than housework) for at least three months from the start of pregnancy, based on the assumption that there is a minimum duration of exposure before a working condition becomes a risk factor for preterm birth [20]. So, working conditions were ascertained for the first three months of the pregnancy.

Exclusion criteria were pregnant women with previous abortion, previous preterm delivery, women with multiple pregnancy, congenital malformation, placenta praevia, oligohydramnios, polyhydramnios, cerclage in situ, Preterm Rupture Of Membranes (PROM), stillbirth and complications during current pregnancy (bleeding and/or fetal problems).

Classification whether preterm or term, was based on accurate estimation of the gestational age. The latter was calculated from the mother's expected delivery date (that was derived from last menstrual period, and clinical

examination, as well as from ultrasound examination) compared with the actual date of the baby's birth.

Cases (preterm group): (N=326) were all singleton preterm deliveries (from 24 completed weeks up to < 37 weeks of gestation) over the study period. While, the controls (term group): (N=1014), defined as full term singleton birth (37 completed weeks of gestation or over) over the same period. For each case, 3 simple randomly selected unmatched controls were chosen.

A verbal consent was taken after explaining the study for the woman, requesting her participation, and verifying her eligibility.

For all mothers with preterm delivery and their controls, information on social and demographic characteristics, occupation, working conditions, lifestyle behaviors, and obstetric history was obtained by interviewing the woman at the maternity ward after the delivery using a self-completed questionnaire, while information on the pregnancy, delivery, the vital status of the newborn or the fetus, and gestational age at delivery was extracted directly from the hospital medical records.

The questionnaire documented the following working conditions: work contract (permanent, contract post); schedule (hours and consecutive days worked per week, evening (6.00 PM to 10.59 PM), or night work (11.00 PM to 5.59 AM); posture (sitting, standing, other demanding postures); physical effort (lifting [weight and frequency], pushing, and pulling objects); work organization (breaks, piecework or assembly line work); and environmental occupational conditions (e.g. temperature, exposure to environmental tobacco smoke). The questions were constructed after reviewing existing literature[21,22,23]. Occupation was coded using the International Standard Classification of Occupations, ISCO-8[24]. The final section of the questionnaire documented obstetric history, mother's medical profile (before and during pregnancy), newborn's characteristics (gender, weight, birth date, expected date of delivery according to the physician, congenital

anomalies), mother's lifestyle (drug consumption, physical activity, and consumption of caffeine in third trimester), and sociodemographic characteristics.

The dependent variable was preterm birth, while working conditions were the explanatory factors under investigation. The variables describing working conditions, presented by category in table 2, included the daily time commuting from home to work, weekly working hours, night work, shift work, standing position, strenuous postures (bending, twisting, kneeling, squatting or holding arms at shoulder level or above), carrying heavy loads (carrying up to 5 Kg, carrying 5-20 Kg, carrying >20 Kg), work with industrial machines, assembly line work, some specific exposures such as paints), noise (measured by the following question: "At work were you exposed to noise so loud that you had to raise your voice to talk to people?'; women who answered occasionally, often, or very often were considered as exposed), and some indicators of mental loads such as work at high speed, lack of pause (no rest room or other pauses at own convenience). Also, some well-known risk factors for preterm delivery, presented by category in table 1, derived either from the self-completed questionnaire: maternal age; Body Mass Index (BMI) calculated from the formula: weight in Kg/(height in meter)^2; smoking status: (non-smoker, smoker) and educational level (defined as illiterate / Read & write, <secondary, secondary, > secondary) as a marker of socio-economic status or extracted from the hospital records: planned pregnancy, parity, and region(urban/rural) were studied.

The analysis included the confounding factors presented in table 1 as well as the occupational factors presented in table 2.

Statistical analyses :

The preterm births group was compared with controls for all working conditions by simple bivariate analyses, using the Chi-square test; the same tests were used to examine the associations between well-known risk factors and preterm labour. Back-step multiple logistic regression model

using the Forward Wald statistical technique was used to study simultaneously occupational factors and well-known risk factors as potential predictive factors of preterm delivery. Odds-ratios (OR) and Wald 95% confidence intervals (CIs) were then calculated to characterize the significance, strength and precision of the associations. Statistical analysis was performed using SPSS 16 statistical software. $P \leq 0.05$ was considered statistically significant.

Results

The study included 1340 working pregnant women. The associations between well-known risk factors and preterm delivery are shown in Table 1. Both smoking and nulliparity were associated with preterm delivery [(P<0.01, 0.001 respectively)]. Significant associations were also observed for other variables: advanced maternal age, low BMI, and unplanned pregnancy. Among the potential confounders, the strongest associations (odds ratio > 1.5) with preterm delivery were observed for smoking and mother's age ≥ 35 years. Caffeine consumption during pregnancy were not associated with preterm delivery risk (data not shown).

The associations between occupational factors and preterm delivery are presented in Table 2. Of the occupational conditions present at the beginning of pregnancy, temporary contract tended to predict preterm delivery (P ≤0.05).Working >40 hours a week was found to be associated with preterm delivery (P≤0.05). Tendencies were also observed for shift work (P≤0.05). Also, demanding posture (bending, squatting, arms raised above shoulder level) for at least 3 hours per day (P≤0.05), standing >6 hours/ day (P≤0.05) and carrying heavy weight >20kg (P≤0.05) were significantly associated with preterm delivery.

There was no association between preterm delivery risk and the following conditions: work on assembly line, noise, long commuting time to work, night work, cold or hot environment, (P>0.05), Also, no statistically significant difference was observed with the possibility to sit when standing, pushing or pulling objects, ab-

sence of breaks and exposure to environmental tobacco smoke at work (data not shown).

The results of the logistic regression analysis including the six occupational factors selected as well as well-known risk factors are presented in Table 3. Significant and strong predictive effects regarding both physical work demands (OR = 3.94, 95% CI: 1.03-18.19) and heavy weight carrying (OR =2.76, 95% CI: 1.98-8.74) on preterm delivery were observed. Predictive effects were also observed between long working hours, prolonged standing (> 6 hours), and temporary contract on the risk of preterm delivery (P < 0.05). Shift work was not associated with preterm delivery.

The logistic regression analysis (Table 3) also showed that among the well-known risk factors, higher maternal age ≥ 35 and nulliparity remained significantly related to preterm delivery (P<0.05).

Table (1): The associations between well-known risk factors and preterm delivery

Variable	Cases (preterm group)*		Controls (term group)^		P	OR (95%CI)
	326	100	1014	100		
Age						
< 20	41	12.6	93	9.2		r
20-25	90	27.6	293	28.9	>0.05	0.7 (0.4-1.1)
25-30	97	29.7	308	30.4	>0.05	0.7 (0.5-1.3)
30-35	60	18.4	81	7.9	<0.05	0.4 (0.2-0.6)
≥ 35	38	11.7	239	23.6	<0.001	1.7 (1-2.9)
Parity			463	45.7		
0	185	56.7	551	54.3		r
1+	141	43.3			<0.001	0.6 (0.5-0.8)
BMI (kg/m^2)			200	19.7		
<20	84	25.8	597	58.9	≤0.05	1.5 (1- 2.2)
20-25	180	55.2	217	21.4	>0.05	1.1 (0.8-1.5)
25+	62	19.0				r
Smoking			967	95.4		
Non smoker	296	90.8	47	4.6		r
Smoker	30	9.2			<0.01	2.1 (1.3-3.1)
Education			134	13.2		
Illiterate/Read & write	61	18.7	269	26.5		r
<secondary	119	36.5	343	33.8	>0.05	0.97 (0.7-1.4)
secondary	93	28.5	268	26.5	<0.01	0.6 (0.4- 0.9)
>secondary	53	16.3			<0.001	0.4 (0.3-0.7)
Planned pregnancy			712	70.2		
Yes	208	63.8	302	29.8		1.3 (1.02 – 1.8))
No	118	36.2			≤ 0.05	r
Residence			575	56.7		
Urban	191	58.6	439	43.3	> 0.05	
Rural	135	41.4				r 0.9 (0.7- 1.2)

* Cases (preterm group) = preterm births 24-37 weeks ; ^Control (term group) = term births ≥ 37 weeks; OR = odds ratio; CI = confidence interval; r = reference group.

Table (2): The associations between occupational factors and preterm delivery.

Variable	Cases (preterm group)* No.	%	Controls (term group)^ No.	%	P	OR (95% CI)
Total	326	100	1014	100		
Occupation (ISCO-8 code)						
Professionals	44	13.5	160	15.8		r
Semi professionals	50	15.3	178	17.6	>0.05	1.02 (0.6-1.7)
Clerks	76	23.3	251	24.7	>0.05	1.1 (0.7-1.7)
Services & sale worker	66	20.3	181	17.9	>0.05	1.3 (0.8-2.1)
Craft & trade worker	27	8.3	97	9.6	>0.05	1.1 (0.6-1.9)
Manual worker	63	19.3	147	14.4	≤0.05	1.6 (1.0-2.5)
Daily commuting time (home to work)						
<60 min	153	47.0	486	47.9		r
60-120	151	46.3	463	45.7	>0.05	1.04 (0.8-1.4)
>120 min	22	6.7	65	6.4	>0.05	1.1 (0.6-1.9)
Weekly working hours						
< 30	26	8.0	94	9.3		r
30-40	236	72.4	786	77.5	>0.05	1.01(0.6-1.6)
>40	64	19.6	134	13.2	<0.05	1.7 (1.0-3.03)
Night work						
Yes	31	9.5	94	9.3	>0.05	1.03 (0.7-1.6)
Shift work						
Yes	90	27.6	221	21.8	≤0.05	1.4 (1.02-1.8)
Standing position						
<2h.	152	46.6	505	49.8		r
2-6h.	77	23.6	269	26.5	> 0.05	0.9 (0.7-1.3)
>6h.	97	29.8	240	23.7	≤ 0.05	1.3 (1.0-1.8)
Demanding posture						
Never	125	38.4	413	40.7		r
0-2.9 hours	66	20.2	278	27.4	>0.05	0.8 (0.6-1.1)
> 3 hours	135	41.4	323	31.9	<0.05	1.4 (1.03-1.9)
Heavy weight carrying						
0-5 kg	220	67.5	693	68.3		r
5-20 kg	66	20.2	240	23.7	>0.05	0.9 (0.6-1.2)
>20 kg	40	12.3	81	8.0	<0.05	1.6 (1.01-2.4)
Work with industrial machines						
Yes	64	19.6	188	18.5	>0.05	1.1 (0.8-1.5)
Work on assembly line						
Yes	25	7.7	73	7.2	>0.05	1.07 (0/7-1.8)
Contact with paints						
Yes	29	8.9	84	8.3	>0.05	1.1 (0.7-1.7)
Noise						
Yes	117	35.9	352	34.7	>0.05	1.05 (0.8-1.4)
Work at high speed						
Yes	162	49.7	487	48.0	>0.05	1.07 (0.8-1.4)
Work contract						
Permanent post	70	21.5	282	27.8		r
Contract post	256	78.5	732	72.2	<0.05	1.4 (1.04-1.9)
Cold work environment						
Yes	17	5.2	53	5.2	>0.05	1.02 (0.6-1.8)
Hot work environment						
Yes	26	8.0	84	8.3	>0.05	0.9 (0.6-1.6)

*Cases (preterm group) = preterm births 24-37weeks ; ^Control (term group)= term births ≥ 37 weeks;
OR = odds ratio; CI = confidence interval; r = reference group.

Table (2): The associations between occupational factors and preterm delivery.

Variable	Cases (preterm group)* No.	%	Controls (term group)^ No.	%	P	OR (95% CI)
Total	326	100	1014	100		
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Semi professionals	50	15.3	178	17.6	>0.05	1.02 (0.6-1.7)
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>40	64	19.6	134	13.2	<0.05	1.7 (1.0-3.03)
Night work						
Yes	31	9.5	94	9.3	>0.05	1.03 (0.7-1.6)
Shift work						
Yes	90	27.6	221	21.8	≤0.05	1.4 (1.02-1.8)
Standing position						
<2h.	152	46.6	505	49.8		r
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0-2.9 hours	66	20.2	278	27.4	>0.05	0.8 (0.6-1.1)
> 3 hours	135	41.4	323	31.9	<0.05	1.4 (1.03-1.9)
Heavy weight carrying						
0-5 kg	220	67.5	693	68.3		r
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>20 kg	40	12.3	81	8.0	<0.05	1.6 (1.01-2.4)
Work with industrial machines						
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Permanent post	70	21.5	282	27.8		r
Contract post	256	78.5	732	72.2	<0.05	1.4 (1.04-1.9)
Cold work environment						
Yes	17	5.2	53	5.2	>0.05	1.02 (0.6-1.8)
Hot work environment						
Yes	26	8.0	84	8.3	>0.05	0.9 (0.6-1.6)

*Cases (preterm group) = preterm births 24-37weeks ; ^Control (term group)= term births ≥ 37 weeks;
OR = odds ratio; CI = confidence interval; r = reference group.

Table (3): Logistic regression analysis of predictors of preterm delivery.

Variable	Adjusted Odds ratio	95 % confidence interval	Statistical significance
Age			
< 20	1		
20-25	0.89	0.18 – 4.47	
25-30	0.64	0.09 – 5.46	
30-35	0.28	0.38 – 7.65	
≥ 35	1.42	1.26 – 6.86	P < 0.05
Parity			
0	1.24	1.42 – 8.76	P < 0.05
1+	1		
BMI (kg/m²)			
<20	1.32	0.28 – 7.65	P > 0.05
20-24	1		
>25	1.86	0.62 – 7.16	P > 0.05
Active Smoking			
Non-smoker	1		
Smoker	2.06	0.52 – 8.92	P > 0.05
Education			
<secondary	1.56	0.36 – 8.79	P > 0.05
Secondary	0.38	0.16 – 1.84	P > 0.05
>secondary	1		
Planned pregnancy			
Yes	1		
No	0.56	0.18 – 4.32	P > 0.05
Weekly working hours			
< 40 h.	1		
≥ 40 h.	2.36	1.18 – 7.78	P < 0.05
Shift work			
Yes	1.72	0.45 – 6.86	P > 0.05
No	1		
Standing position			
<2h.	1		
2-6h.	0.66	0.12 – 3.48	P> 0.05
>6h.	0.88	1.52 – 7.36	P < 0.05
Heavy weight carrying			
0-5 kg	1		
5-20 Kg	0.98	0.18 – 4.32	P > 0.05
>20kg	2.76	1.98 – 8.74	P < 0.05
Work contract			
Permanent post	1		
Contract post	1.98	1.72 – 8.69	P < 0.05
Demanding posture			
Never	1		
0-2.9 hours	0.78	0.12 – 5.98	P > 0.05
> 3 hours	3.94	1.03 – 18.19	P < 0.05

Discussion

The relationship between employment and preterm birth remains enigmatic, despite a dramatic increase in the number of pregnant women in the Egyptian workforce. Manual workers, a category that includes industrial, agricultural, and unskilled workers had an excess of preterm births compared with professionals and semiprofessional (P<0.05) with an adjusted OR 1.6. This is in agreement with Saurel-Cubizolles et al.,[20] who reported similar finding in a multicenter study performed in 17 European countries. Women employed in low-skilled occupations were more likely to be exposed to shift work, high physical demands, and low job satisfaction, providing elements supporting the validity of the evaluation of working conditions.

This study showed that some occupational factors played a substantial role in predicting preterm delivery. These factors were temporary work contract, long working hours, shift work, long standing, heavy weight carrying and physical demands. Temporary contract was found to be significantly associated with preterm delivery. Employed women who had a temporary contract compared with those with permanent post were at a higher risk of having preterm delivery (P<0.05) with adjusted OR 1.4. This is in agreement with that found by Niedhammer et al.,[6] in Ireland who reported similar finding. Temporary contract may be considered as a marker of poor working conditions that may not have been evaluated per se in the present study, and may be associated with new challenges and adaptation to the job and may generate feelings of stress and anxiety to find another future job. This type of contract and perceived job insecurity have already been observed to be associated with other health outcomes [25].

Rregarding the number of hours worked/ week as a risk factors of preterm birth, in this study, the results are also consistent with those obtained with other populations in other countries. There is a significant relation between long working hours per week and preterm birth, with an adjusted OR of 1.7 with > 40

hours per week. Similarly, Niedhammer et al.,[6] in Ireland found that working long hours ≥40 hours per week was associated with increased rate of preterm delivery with adjusted OR 2.2. Also, Mamelle et al.,[26] in France reported a relative risk of 1.7 for preterm birth among women working >40 hours per week. Similar results were reported by McDonald et al.,[27] in Montreal, Canada whereas Klebanoff et a1., [28] in USA reported twice the risk of preterm birth among resident physicians working > 100 hours per week during the first trimester. Also, Peoples-Sheps et al.,[29] in USA found that women who worked 40 or more hours per week were more likely to have preterm delivery than those who worked fewer hours, and similarly a questionnaire-based case-control study of obstetric and neonatal nurses who were delivered prematurely and a group who were delivered at term demonstrated higher prematurity rates among nurses working >36 h/wk or >10 h/shift in USA[30].

In this study, standing was found to be significantly associated with preterm birth. As a categoric variable, standing > 6 hours per shift had significant odds ratio of 1.3. Similarly, other investigators have reported that standing for periods was significantly associated with preterm birth, and increased uterine contractions. Launer et al.,[31] in Guatemala reported that working in a standing position was a significant factor for preterm birth (adjusted OR 1.56). Teitelman et al.,[32] in USA reported a higher proportion of preterm births among women whose jobs required prolonged standing (7.7%) compared with those with sedentary (4.2%) or active (2.8%) jobs, with an adjusted OR for standing of 2.7. Using a comparable definition, Klebanoff et al.,[28] reported an adjusted OR of 1.3 for preterm birth for work that involved standing for ≥ 8 hours per day. Also, Henriksen et al.,[9] in Denmark have reported that prolonged standing to be a risk factor for preterm birth. Saurel-Cubizolles et al.,[20] found that standing more than 6 hours to be a risk factor for preterm birth. Also, Niedhammer et al.,[6] found that women with very physically active jobs had a higher risk for

preterm delivery compared with those with not very or not at all active job.

Physical exertion has been evaluated in several studies, most of which reported a significant correlation with preterm birth. In this study, both demanding posture > 3 hours and heavy weight carrying > 20 Kg were found to increase risk of preterm birth (P<0.05) with adjusted OR 1.4 and 1.6, respectively. Mamelle et al.,[26] reported a relative risk of 1.7 for preterm birth among women required to make strong physical efforts or carry heavy loads during pregnancy. McDonald et al.,[27] found that lifting heavy weights at least 15 times per day was associated with an increased risk of preterm birth (relative risk 1.25). Homer et al.,[15] in USA observed that women working in highly strenuous jobs were twice as likely to delivery prematurely. Ahlborg et a1.,[18] in Sweden showed that lifting weights exceeding 12 kg >50 times per week increased the risk of preterm birth (OR 1.7). Also, Escriba-Aguir et al.,[33] in Spain, found that exposure to medium or high level physical workload increases the risk of preterm birth, with an adjusted OR of 1.59 and 2.31, respectively. While, Saurel-Cubizolles et al.,[20] found loads carrying was not related to an excess risk of preterm birth.

Regarding the effect of shift work, this study found that shift work had a significant effect on preterm delivery (P < 0.05) with an adjusted OR 1.4. This is in agreement with Xu et al.,[34] in USA who found that rotating shift work had a significant effect on preterm birth: 20% for shift workers and 15% for regular schedule workers with an adjusted OR 2.0 and Zhu et al.,[35] in Denmark who found that shift work had a slight excess of significance on preterm delivery with an adjusted OR 1.09. While, Saurel-Cubizolles et al.,[20] found that shift work was not related to an excess risk of preterm birth.

As regards the effect of night work, the results in this study were contradictory to that reported by others. While Pompeii et al.,[36] in USA found that working at night during pregnancy may increase the risk of preterm delivery

by 50% with a relative risk 1.5, we found that night work does not affect risk of preterm delivery significantly. Conversely, Zhu et al.,(35) found that night work may prolong the duration of pregnancy with OR 1.09.

No other occupational factors were found to be predictive factors of preterm delivery in this study. This was especially the case for daily commuting time, work with industrial machines, work on assembly line, contact with paints, work at high speed, noise, and hot or cold work environment. This is in agreement to that found by Saurel-Cubizolles et al., (20). In this study, predictive effects were also observed for well-known non-occupational risk factors that were nulliparity, extremes of age, and smoking and low maternal BMI; these findings are in agreement with that reported by others[36,37,38] and reinforced the validity of our own results.

This study has several major strengths: the selection process yielded a sample of women at baseline, whose social circumstances may be considered as broadly representative of Egyptian women, the diversity of participating areas and the use of a common protocol, including an identical instrument for collecting information on the characteristics of the sample and working conditions. The same questions were asked of all women concerning their employment conditions. There is also no possibility of a reporting bias, as pregnancy outcomes were measured using hospital records, and thus independently of women's answers. The study included a large number of occupational factors and covered the main occupational factors that have previously been suspected to be risk factors. Evaluation of occupational factors was performed using a self-administrated questionnaire already used, and consistent results were found regarding social gradients in these factors in Lifeways working women. Furthermore, our study took into account education level (a marker of socio-economic status) in the multivariate analysis; consequently, the associations observed can be considered as independent of socio-

economic status. The study also included a large number of well-known risk factors of preterm delivery allowing us to control adequately for potential confounding in our analyses.

Meanwhile, this study also had some limitations. It is a retrospective study; consequently, there is the possibility of a recall bias: the evaluation of risk factors including occupational factors was not performed at the beginning of pregnancy and thus before the pregnancy outcome, which we are reporting, had actually occurred. Also, an information bias could exist if the mothers of preterm infants did not answer the questionnaire in the same way as controls. Joffe et al., (39) has drawn attention to the possible interviewer or recall bias that generally affects retrospective studies on the reproductive effects of women's work. In fact, only a few studies in this field have used a prospective design and these studies present another type of bias, that of study participants lost to follow-up[28,31,32]. Also, the fact that this study is a hospital-based, the sample may not be demographically typical of the general obstetric population and this is another limitation in this study. This study population is probably more representative of those patients seeking care at tertiary centers and university hospitals than of the general population. This issue obviously affects the generalizability of these findings to all obstetric patients.

In conclusion, the results in this study show that strenuous working conditions are an important risk factor for preterm birth among working women and suggest that some preventive measures might help reduce the incidence of preterm birth. Hence, we recommend reducing working hours per week or per shift, avoiding prolonged standing and heavy physical work, changing work areas, and/or <granting work leave during pregnancy, particularly late in pregnancy. The passage of Public Law to guarantee every pregnant woman work leave should become an urge need toward a national enlightened maternity policy. Such a policy, when appropriately instituted, should reduce the incidence of

those preterm births related to occupational factors and its related sequale. The impact of this policy and /or changing employment conditions during pregnancy on pregnant women exposed to high risk employment conditions should be considered in future analyses.

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ENDOGENOUS LIVER OVAL CELLS ACTIVATION IN RESPONSE TO CHRONIC LIVER INJURY: AN EXPERIMENTAL IMMUNOHISTOCHEMICAL STUDY IN ALBINO RATS

Hussein Abd Ellatif M.Sc., Huda Eltahry Ph.D., Kamal Botros Ph.D., Dalia Saleh, Ph.D. and Gamal Shiha MD*

Departments of Anatomy Embryology and Internal Medicine,*

Mansoura University, Egypt

Abstract

Background: *The liver has a tremendous regenerative capacity through proliferation of all existing cell lines within the liver, including hepatocytes, epithelial cells that line the canaliculi, endothelial cells, Kupffer and hematopoietic stem cells. In addition, the liver contains "stem" cells or liver progenitor cells (oval cells in rodents) that can be activated by liver damage.*

Aim of this work: *Is to study the endogenous oval cells population in a model of induced chronic liver injury.*

Materials and Methods: *Eighteen adult albino rats were divided equally into three groups (control, liver injury, liver injury and hepatocyte proliferation block groups). The liver injury was induced by ip injection of 2ml/kg BW carbon tetrachloride (CCL4) dissolved in corn oil (1:1) twice/week for 4 weeks. The hepatocytes block group was treated with acetylaminofluorene (2-AAF) in a dose of (10 mg/kg BW) as daily oral gavage for two weeks starting 2 weeks following CCL4 administration and extended for another 2 weeks. There days from the end of the experiment all rats were sacrificed by neck dislocation, blood and liver samples were collected and were biochemical and histopathological analysis, respectively. Plasma aspartate transaminase (AST), alanine transaminase (ALT) activities, serum albumin and total bilirubin levels in the plasma were determined in the blood samples. Sections from the livers were stained by H&E for histopathological evaluation, OV-6 immunoperoxidase stain for activated oval cells. Data were subjected to*

statistical analysis.

Results: *There was a significant increase in the plasma levels of ALT and AST in the liver injury group (155 ± 9.4 and 408 ± 302.9) and in the liver injury and hepatocyte block group (114 ± 37.5 and 265 ± 187.3) compared to the control (39 ± 3.68 and 40.5 ± 3.7), respectively. On the other hand there was no change in the albumin (3.9 ± 0.2) or bilirubin (3.9 ± 0.1) levels in the liver injury group or in the liver injury and hepatocytes block group (3.9 ± 0.2 and 0.31 ± 0.16) compared to the control (0.38 ± 0.2 and 0.35 ± 0.08), respectively. No oval cells were seen in the control or the liver injury group by the stains used. On the other hand, oval cells were seen as small single or forming duct like structures in the periportal region of the liver injury and hepatocyte block group, these cells were positively stained with OV-6.*

Conclusion: *It is concluded that induction of oval cell proliferation was achieved in chronic liver injury model using CCL4/2AAF protocol. The activated oval cells were seen mainly in the periportal region either as single cells or forming duct like structures and were positively stained with OV-6 immune peroxidase stain.*

Key words: *Liver; Chronic injury; Oval cells, OV-6.*

Introduction

The liver is the largest organ in the body and is specialized to perform wide range of functions including; detoxification, synthesis of plasma proteins and bile acid formation[1]. The predominant cells of the mature liver are the parenchymal hepatocytes which constitute approximately 80% of the total hepatic cell volume[2].

The regenerative capacity of the adult mammalian liver is immense. Liver regeneration is a misnomer as the liver actually heals by deoxyribonucleic acid (DNA) synthesis and mitosis rather than regeneration in the true sense.

The healing process in the liver is characterized by the proliferation of all existing cell lines within the liver, including hepatocytes, epithelial cells that line the canaliculi, endothelial cells, Kupffer and hematopoietic stem cells [3].

It is generally accepted that in addition to these cells, the liver contains "stem" cells or liver progenitor cells (oval cells in rodents) that can be activated by liver damage. Unique to the liver is that pre-existing mature cells constitute the primary option of response to injury while progenitor cells function as a reserve compartment that is activated when the regenerative capacity of mature cells is compromised [4].

Oval cells (also known as transit amplifying cells) are the progeny of stem cells. They can divide rapidly but in contrast to stem cells do not posses the ability to self-renew. They have the potential to generate more than one differentiated cell type but cannot be serially transplanted. During liver regeneration, oval cells are essential at forming a second line of defense[5]. They are capable of differentiating into hepatic lineages (i.e. hepatocytes and cholangiocytes) and also some non-hepatic lineages such as intestinal and pancreatic cell types. It may take years for significant recovery to be achieved; the time varies depending on the underlying cause of the liver injury and its severity [6].

The aim of this work is to study the endogenous oval cells population in a model of induced chronic liver injury.

Materials and Methods

Animal preparation:

Eighteen adult female albino rats (Cux1: HEL1) 12 weeks of age, weighing (200-250 g). Rats were bred and maintained in an air-conditioned animal house (Medical Experimental Research Center, MERC, Mansoura University) (under controlled temperature 25 ± 2°C) with specific pathogen-free environment and were subjected to a 12:12-h daylight / darkness cycle and allowed free access to rat chow and water. The principles of laboratory animal care were followed in all experimental protocols and were approved by ethics committee of animal research in MERC.

Animal groups:

Rats were randomly divided into the following groups:

- Control Group (n=6). The

rats were injected intraperitoneally with corn oil (2 ml/kg B.W.) twice a week throughout the whole experiment.

- Liver injury group (n=6). The rats were injected intraperitoneally with CCL4 (Sigma cat: 48604) in a dose of (2ml/kg B.W.) dissolved in corn oil (1:1) twice/week for 4 weeks.

- Liver injury and hepatocytes block group (n=6). The rats received CCL4 to induce chronic liver injury as before for two weeks in addition, rats received acetylaminofluorene (2-AAF) (A7015, Sigma Aldrich) to inhibit hepatocytes proliferation. The drug was administered in a dose of (10 mg/kg B.W.) as daily oral gavage for two weeks starting. 2-AAF was dissolved in a small volume of dimethyl sulfoxide (DMSO; Sigma cat: D8418) and suspended in corn oil to a final concentration of 2 mg/ml.

Biochemical analysis:

Heparinized blood samples were obtained from all animals at the time of scarification. Plasma aspartate transaminase (AST), alanine transaminase (ALT) activities, serum albumin and total bilirubin levels in the plasma were determined using commercial available kits (Sigma chemical co. kits Nos 58 and 59). Sham control animals were also subjected to the same tests.

Specimens' collection:

Animals from all groups were sacrificed after three days from the end of the experiment by cervical dislocation. Blood samples were collected by heart puncture. After coagulation, sera were collected for further biochemical analysis. Livers were harvested, cut into pieces, preserved in 10% formalin and processed for histopathological studies.

Staining:

The fixed liver tissue was dehydrated in ascending grades of alcohol and then cleaned by xylene then embedded in paraffin. 5-μm thick sections were mounted on clean glass slides. Sections were stained with hematoxylin and eosin (H&E) for histopathological changes, anti huma/rat OV-6 immunohistochemical stain for detection of oval cells.

OV-6 immunohistochemistry:

Tissue sections were rehydrated in descending concentrations of ethanol and endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol. Tissue was microwaved to boiling for 20 minutes in 0.1 mol/L of Tris EDTA pH 9.0, for antigen retrieval. The antibody was diluted in phosphate-buffered saline plus 0.1% non fat dried milk and applied at 4°C for 12 hours. Primary antibody dilution was 1:10

Tissue was incubated with biotinylated anti-mouse secondary antibody in a species specific manner (R and D syetem. MAB002). The label was peroxidase conjugated streptavidin. Color development was performed with diaminobenzidine peroxidise substrate (D-4293, Sigma Chemical Co.).

Fnally, sections were counter stained for 2 minutes with hematoxylin, dehydrated through graded alcohols and mounted under glass coverslips.

Statistical analysis:

Data were expressed as mean ± SD. Multiple comparisons were done using SPSS 15.0 computer Software. Results were considered significant at p?0.05.

Results

Biochemical analysis:

There was a significant increase in the plasma levels of ALT and AST in the liver injury group (155 ± 9.4 and 408 ± 302.9) and in the liver injury and hepatocyte block group (114 ± 37.5 and 265 ± 187.3) compared to the control (39 ± 3.68 and 40.5 ± 3.7), respectively. On the other hand there was no change in the albumin (3.9 ± 0.2) or bilirubin (3.9 ± 0.1) levels in the liver injury group or in the liver injury and hepatocytes block group (3.9 ± 0.2 and 0.31± 0.16) compared to the control (0.38 ± 0.2 and 0.35 ± 0.08), respectively (Table 1).

Histopathological assessment:

Examination of the liver of the control group showed normal liver architecture. The hepatocytes were arranged in cords or plates radiating from the central veins throughout the whole tissue. No signs of dilatation or congestion of portal

veins. Examination of the hepatocytes showed normal vesicular nuclei (Fig. 1).

Examination of sections of the liver from animals subjected to chronic liver injury (CCL4 model) showed areas of variable degrees of ballooning, hydrobic degeneration and necrosis of the hepatocytes. Mild dilatation and congestion of portal vein with inflammatory cells infiltration in the peri-portal region were seen (Fig. 2).

In the animals subjected to liver injury and hepatocytes block the heaptocytes showed condensed chromatin with signs of DNA adducts (marginal heterochromatin attached to the nuclear membrane) (Fig. 3).

Changes in oval cell population in HE stained liver sections:

No evidence of oval cells in the control or in the CCL4 treated group. In the liver injury and hepatocytes block group, the oval cells appeared as small oval cells proliferating in files between hepatocytes in the periportal regions or extending beyond the periportal region. The cells had scanty basophilic cytoplasm, dark staining nucleus with large nucleo-cytoplasmic ratio. The cells radiate from the periportal region in the form of individual cells or ductular like structures (Fig. 3). Some cells also appeared as individual oval shaped cells with hepatocyte phenotype (Fig. 4).

OV-6 immunoreactivity:

Only the bile ducts within the portal triad in the control liver were positive for OV6 and the reaction is limited to the interface with the portal space at the level of canal of Hering and ductular canalicular junction (Fig. 5). In the experimental groups, the proliferating cells react to OV6 with formation of reactive ductules and small hepatocyte like cells (Figs. 6 and 7).

Measured parameters in the experimental groups, results are expressed as mean ± SD

Parameter	Control	Liver injury (CCL4)	Liver injury and hepatocytes block (CCL4/2AAF)
ALT (U/L)	39 ±3.68	155 ± 9.4	114 ± 37.5
AST (U/L)	40.5 ±3.7	408 ± 302.9	265 ± 187.3
Albumin (g/dl)	3.9 ± 0.1	3.9± 0.2	3.9± 0.2
Bilirubin (mg/dl)	0.35 ± 0.08	0.38± 0.2	0.31± 0.16

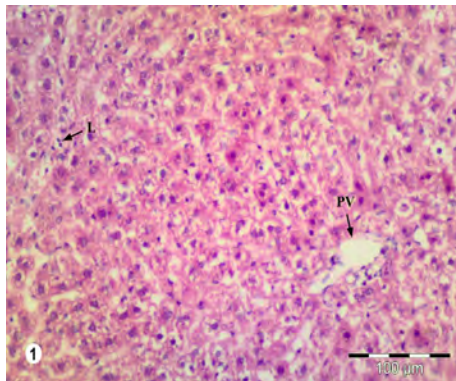

Fig. 1 : A photomicrograph of liver tissue from control rat showing normal liver architecture, minimal lymphocytic infiltration (L) in the liver parenchyma with no signs of ductular or individual oval cell response around the portal vein (PV) (Hx & E stain; X100).

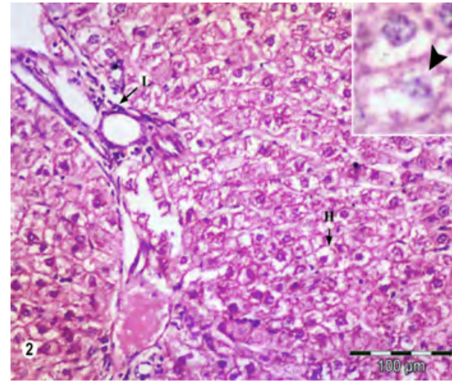

Fig. 2 : A photomicrograph of liver tissue from a rat with liver injury (CCL4 treated) showing hydrobic degeneration (H) of hepatocytes with increased lymphocytic infiltration (L). No signs of ductular or individual oval cell response. Inset: showing hepatocytes with hydropic degeneration and disintegrated nucleus (arrow head). (Hx & E stain; X100; Inset, X400).

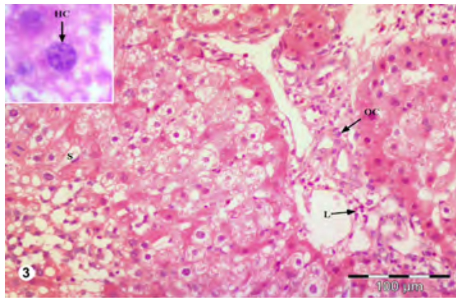

Fig. (3) : A photomicrograph of liver tissue of rat subjected to liver injury and hepatocytes block (CCL4/2AAF treated) showing oval cells (OC) surrounding the portal tract, lymphocytic infiltration is also evident (L) with widening of liver sinusoids (S). Inset: showing evidence of hepatocytes block in the form of condensed chromatin and heterochromatin (HC) (Hx & E stain; X100; Inset, X400).

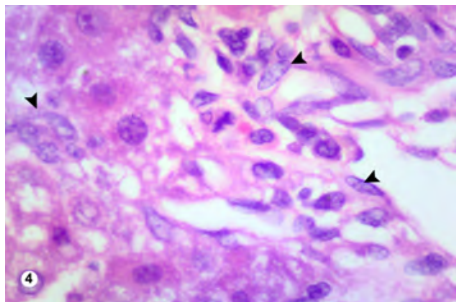

Fig. (4) : A photomicrograph of liver tissue of rat subjected to liver injury and hepatocytes block (CCL4/2AAF treated) showing oval cells (arrowheads) with scanty basophilic cytoplasm and high nucleo/cytoplasmic ratio. Hx & E stain; X100; Inset, X400).

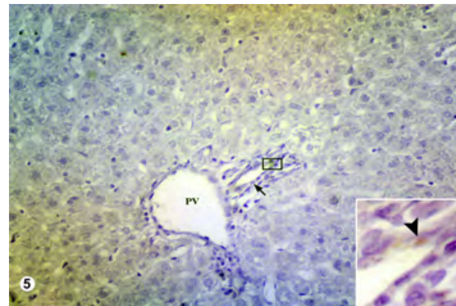

Fig. (5) : A photomicrograph of liver tissue from control rat showing faint immunoreactivity to OV-6 antibody which is limited to cells lining the intraportal bile ducts (arrow), note the portal vein (PV). Inset: High magnification of the rectangle showing the positively stained cells (arrowhead).
(OV-6 immuneperoxidase stain; X100; Inset, X400).

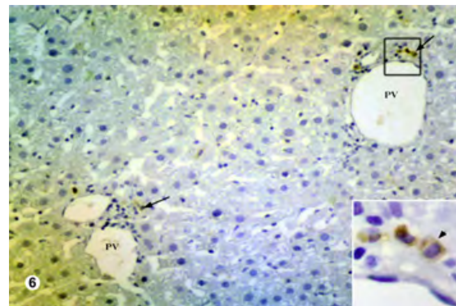

Fig. (6) : A photomicrograph of liver tissue from a rat with liver injury (CCL4 treated) showing mild immunoreactivity to OV-6 antibody which is limited to the cells lining the intraportal bile duct (arrows) surrounding the portal vein (PV). Inset: High magnification of the rectangle showing the positively stained cells (arrowhead).
(OV-6 immuneperoxidase stain; X100; Inset, X400).

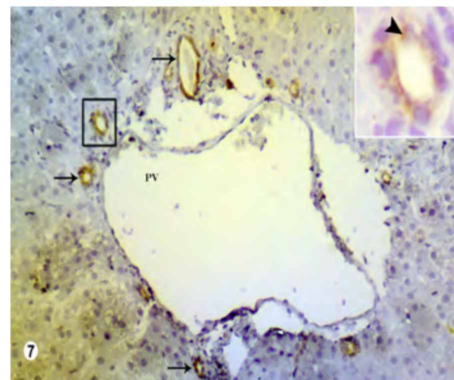

Fig. (7): A photomicrograph of liver tissue of rat subjected to liver injury and hepatocytes block (CCL4/2AAF treated) showing increase in OV-6 immunoreativity with oval cells forming more reactive ductules (ductular like oval cells) (arrows). The newly formed ductules are seen surrounding the portal vein (PV). Inset: High magnification of the rectangle showing the positively stained cells (arrowhead).
(OV-6 immuneperoxidase stain; X100; Inset, X400).

Discussion

The chronic liver injury induced by CCL4 without block to the hepatocytes was also used in this study and oval cell response to this model of injury was not evident by the methods of detection used. On the other hand, using CCL4/AAF protocol induced an increase in the oval cell number and activity in response to this model of chronic liver injury. These results are in consistent with former studies which stated specific protocols for oval cells induction.

Biochemical analysis (ALT, AST, albumin and bilirubin) and observation of inflammatory cell infiltration was conducted and the current results show that liver damage and inflammation are rapidly induced following liver injury. While oval cell numbers remained low during the first 3 days following the liver injury, both serum ALT, AST and inflammatory cell numbers increased during this period. This suggests that expansion of the oval cell compartment begins shortly following liver damage and inflammatory cell infiltration, supporting the view that injury-initiated inflammatory signalling events are responsible for activating the oval cell response.

Methods for activation of oval

cells involve the same principle, inhibition of the proliferative potential of mature hepatocytes followed by liver injury. Chemical inhibition of hepatocyte proliferation is made possible by the unique expression of mixed function oxidases (P450s) within the parenchymal cells of the liver. Popular oval cell induction models in the rat include choline-deficient diet followed by ethionine exposure, galactosamine, 2-acetylaminofluorene (2AAF)/CCl4, 2AAF/partial hepatectomy (PH), and allyl alcohol [7].

The liver has a remarkably high capacity to regenerate upon various injuries, such as partial hepatectomy or toxic insults. In rodent models, after 70% partial hepatectomy, the liver can completely recover its initial volume and function within a week or so. During this recovery process, hepatocytes, as well as cholangiocytes, in the remaining liver undergo a few cycles of cell division to sufficiently restore the lost tissue. Thus, the liver regeneration can usually be achieve by proliferation of the differentiated, postmitotic hepatocytes that remain intact, without necessitating an involvement of stem/progenitor cell populations. On the other hand, when the liver suffers from severe and/or chronic damages, hepatocytes proliferation is suppressed. It is under this condition when the facultative stem/progenitor cells are known to emerge and contribute to the liver regeneration process[8].

Libbrecht (2000) stated that oval cells (Progenitor cells) activation or ductular reaction is seen in the majority of chronic liver diseases. The degree of oval cell activation increases with the severity of the disease. In moderate and severe degrees of inflammation, intermediate hepatocytes occur, having a phenotype intermediate between oval cell / ductular cells and mature hepatocytes [9].

2AAF is metabolized by hepatocytes to an N-hydroxyl derivative, which interferes with the cyclin D1 pathway. Biliary epithelial cells and oval cells lack the ability to convert the 2AAF to its toxic metabolite. Therefore, the administration of 2AAF prior

to CCL4 inhibits hepatocyte proliferation and force oval cell recruitment to mediate liver regeneration.This procedure results in a robust oval cell response following CCL4 (peaking between day 9 and 11 post injury) and within 14 days, oval cell start to differentiate into hepatocytes [10].

Another explanation to the selective oval cells activation was noticed by Fausto (2005) who stated the involvement of TWEAK (TNF weak inducer of apoptosis), a member of the TNF family, in the proliferation of oval cells [11]. It appears that TWEAK selectively promotes proliferation of oval cells without having an effect on hepatocytes. The effect is mediated by the TWEAK receptor Fn14. Both TWEAK and its receptor increase in the standard liver regeneration but it appears to persist longer in the oval cell response[12].

In the present study, oval cells appeared in the H&E stained liver injury sections as small cells around the ducts and vessels in the portal areas with scanty lightly basophilic cytoplasm and pale blue staining nuclei. Many studies have characterized these cells and have established them as facultative liver stem/progenitor cells that are likely to play a relevant role in liver regeneration from various types of injuries [13].

Oval cells are considered to be capable of differentiating into two hepatic epithelial lineages, i.e., hepatocyte and cholangiocyte. In possible relation to this notion, oval cells express both hepatocyte (albumin) and cholangocyte (CK19) markers. The immature hepatocyte marker alpha-fetoprotein (Afp) is known to be expressed in oval cells in rats, but not in mice. Similarly, expression of the hepatoblast marker (Dlk1) has been shown in a subpopulation of rat oval cells but is not found in mouse oval cells [14].

There are several monoclonal antibodies that have long been used as “golden standards” to recognize oval cell markers, such as OV-1 and OV-6 in rats and A6 in mice. OV-1 antibody reacts

with an unknown antigen expressed on the surface of oval cells and thus can be used to isolate these cells, while OV-6 antibody recognizes a common epitope in the cytoskeleton components CK14 and CK19. In this study the small recognized oval cells were positively stained with OV-6 [15].

In the present study, the oval cells were seen radiating from the periportal region in the form of individual cells or ductular like structures especially in the CCL4/ AAF chronic liver injury group. The ductular reaction is the term given to a duct-like proliferation of a population of liver progenitor cells in the setting of fibrosis. Many investigators have shown a direct association between the extent of the ductular reaction and the severity of fibrosis in cases with chronic liver diseases suggesting that progenitor cell activation plays an important if undefined role in fibrosis. The extent of the ductular reaction appears to correlate directly with impaired hepatocyte replication consistent with the notion that progenitor cells are only recruited when replication of mature liver cells is inhibited [16].

Other groups of investigators have not observed mutually exclusive expression of hepatocytic and biliary markers during progenitor cell proliferation but instead describe “intermediate hepatocytes” that express both sets of markers. These intermediate hepatocytes appear in contiguity with ductular progenitor cells when liver inflammation and necrosis is moderate or severe suggesting that differentiation of progenitor cells (oval cells) towards the hepatocytic lineage is directly related to the extent of parenchymal injury [17].

Regardless of the details of progenitor cell activation and marker expression, these observations strongly support the concept that hepatic progenitor cells (oval cells) of the ductular reaction contribute directly to the fibrogenic process, most likely through cell-cell cross talk between the progenitor cells and nearby mesenchymal populations and via recruitment of inflammatory cells [18].

The signals that mediate progenitor cell quiescence and activation, however are still unclear. Nguyen et al. (2007) made an important observation that rat liver progenitor cells (oval cells), unlike hepatocytes are resistant to the growth inhibitory effects of the growth factor TGF-B providing at least a partial mechanistic basis for their differential activation in the TGF-B rich environment of fibrosis and cirrhosis [19].

During oval cell activation in the 2AAF/CCL4 rat model, oval cells proliferate and radiate from the periportal region (possibly from the canal of Hering) toward the pericentral region of the liver. Because oval cells express CXCR4 on their surface, it is possible that oval cells arising from the periportal region respond to the SDF-1 gradient across the liver lobule and migrate into the parenchyma, where the microenvironment is favourable for their proliferation or differentiation and that goes in parallel with our work. The effect of SDF-1 on cell survival/ anti-apoptosis remains controversial. Recent studies found that activation of the SDF-1/ CXCR4 axis prevents certain hematopoietic stem/progenitor cells or cell lines from apoptosis in vitro whereas others did not [20]. Moreover, there is evidence showing that SDF-1 acts synergistically with other cytokines such as granulocyte-macrophage colony-stimulating factor, SCF, and thrombopoietin, enhancing the survival of CD34 progenitor cells [21]. From all of previous data, it could be explained why oval cells started their proliferation in the periportal region and then they migrated toward the central vein and invade the liver parenchyma in a concentration dependant manner on SDF-1/CXCR4 pathway.

In conclusion, induction of oval cell proliferation was achieved in chronic liver injury model using CCL4/2AAF protocol. The activated oval cells were seen mainly in the periportal region either as single cells or forming duct like structures and were positively stained with OV-6 immune peroxidase stain.

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ENDOGENOUS LIVER OVAL CELLS ACTIVATION IN RESPONSE TO CHRONIC LIVER INJURY: AN EXPERIMENTAL IMMUNOHISTOCHEMICAL STUDY IN ALBINO RATS

Hussein Abd Ellatif M.Sc., Huda Eltahry Ph.D., Kamal Botros Ph.D., Dalia Saleh Ph.D. and Gamal Shiha MD

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CISPLATIN-INDUCED ACUTE KIDNEY INJURY MODEL IN RATS: A BIOCHEMICAL AND HISTOPATHOLOGICAL STUDY

Mohamed Abouelkheir, Dina A. ElTantawy MD*, Karawan M. Abdelrahman MD, Mohamed Sobh MD and Mohamed-Ahdy Saad MD**

Departments of Clinical Pharmacology, Pathology,*

*Medicine, Section of Nephrology, UNC**,*

Faculty of Medicine, Mansoura University, Egypt

Abstract

Background. *Cisplatin has been used to induce an animal model of toxin-induced acute kidney injury (AKI). Cisplatin-induced AKI is characterized by lack of correlation between functional parameters and the degree of kidney injury. The aim of the present study is to explain such lack of correlation by evaluating the extent of apoptosis in cisplatin-induced AKI in comparison to the pathological finding by light microscope.*

Methods. *A single intraperitoneal injection of cisplatin was used to induce AKI in rats. Animals received either saline, cisplatin (5 mg/kg), or cisplatin (7.5 mg/kg). Surviving animals were sacrificed 5 and 10 days after drug injection. Survival rate, serum creatinine, blood urea nitrogen (BUN), body weight, apoptosis, and renal tissue damage were determined.*

Results. *In rats sacrificed at the 5th day, serum creatinine and BUN were elevated but did not show any significant differences between the two cisplatin-injected groups. Mortality was significantly higher in the 7.5-mg group. Renal tissue damage in the 5 mg-group was limited to the layer of the outer strip of the outer medulla while in the 7.5-mg group, damage involved all the layers of the kidney. Apoptosis in the 5 mg-group involved all the kidney layers.*

Conclusion. *In a rat model of cisplatin-induced AKI, tubular cell*

apoptosis might extend beyond the pathological finding by light microscope. This unrecognized renal tissue affection might partially explain the lack of correlation between kidney function parameters and the degree of renal tissue damage in this model.

Keywords: *acute kidney injury; animal model; apoptosis; blood urea nitrogen; cisplatin; creatinine ; rat*

Introduction

Acute kidney injury (AKI) is a common devastating disorder in clinical medicine. It was estimated that about 5% of all hospitalized patients and between 30 to 50% of patients in the intensive care units (ICU) were diagnosed to have AKI[1]. Regardless the cause and despite major technical improvements in dialysis and intensive care, the mortality and morbidity among patients with severe AKI remain high[2,3]. Mortality rates among patients with AKI in the ICU approximated 50-70%[4]. Among patients who survived, thirteen percent remained dialysis-dependent[5]. The pathophysiological mechanisms that participate in AKI include alterations in renal perfusion, tubular dysfunction and cell death, intratubular obstruction, and inflammation[2].

Animal models are mandatory to improve our understanding of human AKI despite their limitations[6,7]. Three basic types of animal models of AKI are now well established: ischemia; toxins and sepsis models. Each model has advantages and disadvantages but none has been proven to be the ideal model of AKI. When an animal model is used to study AKI, one feature of the model that should be considered is whether the degree of functional impairment is correlated to pathological degree of injury or not[8]. In human, the degree of functional impairment in AKI is poorly correlated to pathological degree of injury. Several explanations have been provided to explain the lack of correlation between pathology and the degree of functional impairment in AKI. Explanations were almost always limited to the disadvantages of ordinary biochemical assessment of the kidney functions (9, 10).

Cisplatin is a widely used chemotherapeutic agent. Its chief dose-limiting adverse effect is nephrotoxicity. Cisplatin induces tubular apoptosis and necrosis leading to AKI [11]. It was estimated that between 19 and 33 % of AKI cases are attributed to drug nephrotoxicity, including cisplatin [12]. The mechanisms of cisplatin-induced AKI are complex and involve multiple pathways [13, 14]. Cisplatin has been used to induce an animal model of toxin-induced AKI[15,16,17]. As a model of AKI, cisplatin model is simple and reproducible. The model simulates the sequence of events, pathological changes, biochemical abnormalities, and predisposing factors that occur in human[18]. As in human, the degree of functional impairment in cisplatin-induced AKI is poorly correlated to the degree of renal injury[8]. In this study, two different doses of cisplatin were compared. In addition to serum creatinine, blood urea nitrogen (BUN), histologic changes in the kidney, and mortality, apoptosis was additionally evaluated to find out if other factors could additionally explain the lack of correlation between the ordinary kidney function tests and histological changes in the kidney in this model.

Materials and Methods

The experimental protocol was approved by the Local Ethical Committee, Faculty of Medicine, Mansoura University.

Animals:

The study was performed on 30 adult female Sprague-Dawley (SD) rats weighing approximately 160 to 200 g. Rats received a standard rat chow and had free access to water.

Agents:

Cisplatin was obtained from Hospira (Hospira UK Limited, Warwickshire, UK) in 1 mg/ml concentration. The drug was diluted in 0.9% sodium chloride (1:2) just before use. The injected volume ranged from 2.4 to 3 ml.

Cisplatin-induced acute tubular injury:

For induction of acute renal failure, SD rats received a single intraperitoneal (I.P.) injection of cisplatin diluted in saline. Renal affection was confirmed in day 5

of the experiment by measurement of serum creatinine.

In vivo study design:

Animals were divided into 3 groups as the following:

1. Group I (control) (*n* = 6), these animals were not subjected to any intervention except that an equal volume of saline was injected I.P.
2. Group II (5-mg group; (*n*=12): rats received a single I.P. injection of cisplatin (5 mg/kg) diluted in saline [19].
3. Group III (7.5-mg group; *n*=12): rats received a single I.P. injection of cisplatin (7.5 mg/kg) diluted in saline [20].

In each group, sacrifice was planned to take place at days 5 and 10 after injection of cisplatin.

For each group, body weight, serum creatinine, and BUN were measured. Blood samples (0.2 ml) were collected for biochemical measurement by retro-orbital venous plexus puncture through the medial canthus in halothane-anesthetized rat. At the day of sacrifice, rats were weighed and sacrificed using an overdose of thiopental (12 mg/100 g of the body weight). Blood samples were taken by heart puncture, centrifuged at 3000x g for 15 minutes, and frozen till the time of biochemical analysis. Both kidneys were harvested for preparation of pathological samples.

Biochemical parameters:

Blood samples were used for determination of serum creatinine and BUN. These parameters were measured using an automated spectrophotometer (Slim Plus, Italy). Serum creatinine levels were determined according to the method described by Murray [21] using original kits (Diamond Diagnostics, Egypt). BUN levels were determined according to the method described by Tabacco [22] using specific kits (Stanbio Lab., Texas, USA).

Renal Morphology:

For all groups, kidneys were perfused through the abdominal aorta using saline 0.9% till complete clearance of the perfusion fluid, and then 10% neutral buffered formalin for in situ fixation. The kidney was harvested, cut longitudinally, and sent for patho-

logical evaluation in 10% neutral buffered formalin. Samples were processed and embedded in paraffin wax and sections (4 μm thick) were stained with hematoxylin and eosin for light microscopic observation.

Apoptosis:

Tubular cells apoptosis in the control group and in the renal tissue samples from the 5th day of the experiment of the group that received 5 mg/kg of cisplatin was assessed using a specific apoptosis detection kit (Takara Bio. Inc., Japan). Detection of apoptosis is based on the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method.

Statistical analysis

Statistical analysis was carried out using the SPSS software (version 16.0, SPSS, IL, USA). Data were tested for parametric distribution by Kolmogorov-Smirnov test. Biochemical parameters and body weight were evaluated by independent-samples t test. Values were expressed as mean ± SD. For the survival rate, the significance was computed with a log-rank test. P-values < 0.05 were considered statistically significant.

Results

Survival:

Kaplan-Meier survival curves of rats that received cisplatin (5 and 7.5 mg/kg) are shown in Figure 1. Rats that received the 5-mg/kg dose of cisplatin showed a greater survival than rats that received the 7.5-mg/kg dose ($P < 0.001$). All rats in the 5mg-group away from the five rats which were sacrificed at the 5th day were still alive. In the 7.5-mg group, four rats were already dead. The remaining three rats of the 7.5-mg group died in the day 6 (n=2) and 7 (n=1). In the 5-mg/kg group, two rats died at the 6th day (n=1) and the 7th day (n=1) and five rats were sacrificed at day 10 of the experiment.

Body weight:

In the first five days, changes in weight were parallel in both groups. In the 5-mg group, weight was reduced from 169.6±9.6 g before injection of cisplatin to 132.9±8.9 g by the 5th day. In the 10th day, surviving rats were able to regain some weight 153±7.6 g.

In the 7.5-mg group, weight was reduced from 172.5±10.1 g before injection of cisplatin to 137.5±10.6 g by the 5th day. No statistically significant differences in the body weight were evident between the two groups at days 0 (*P* = 0.477) or day 5 (*P* = 0.293) of the experiment.

Serum creatinine and BUN:

At day 5 of the experiment, serum creatinine and BUN were markedly elevated (between 8 to 9 folds) in both groups. The levels of serum creatinine and BUN at day 5 in the 5-mg group showed no statistically significant difference when compared to the 7.5-mg group (Table 1). By day 10, serum creatinine, but not BUN, levels of the surviving rats in the 5-mg group were completely normalized.

Pathological changes:

Normal histology of the different layers of the kidney was demonstrated in Figure 2. In rats sacrificed at day 5, the cisplatin-induced lesion in the 5-mg group was restricted to the outer strip of the outer medulla (OSOM). It was in the form of prominent vacuolation, necrosis, degeneration, and loss of architecture of tubules. Almost all the proximal tubules were affected. In addition, inflammatory cells infiltration and tubular casts were also evident. The cortex and the inner medulla were free at the level of light microscopy at x400 magnification (Fig. 3). Regarding the 7.5-mg group, the lesion in the 5th day was extensive. In addition to affecting all the tubules in the OSOM in a way similar to the 5-mg group, other kidney layers were also severely affected. Necrosis, inflammatory cells infiltration, and tubular casts were also evident in the cortex and inner medulla. Most of the vascular structures were congested and hemorrhage was also evident (Fig. 4). In rats sacrificed at day 10, the lesion in the 5-mg group was still restricted to OSOM. Necrosis and degeneration were less evident while inflammatory cells infiltration appeared to be more. Other kidney layers remained unaffected.

Apoptosis:

Using TUNEL method, apoptotic cells were evident at the 5th day in the kidney of the group that received 5 mg/kg of

cisplatin in comparison to the control group. The apoptotic cells were not restricted to the OSOM. On the contrary to microscopic examination where the cortex and the inner medulla were free, evidence of cellular apoptosis in the cortex and the inner medulla indicated that these two layers were affected by cisplatin (Fig. 5).

Table 1. Changes in serum creatinine (mg/dl) and BUN (mg/dl) at days 0 and 5 after cisplatin (5 and 7.5 mg mg/kg) administration.

	Day	5-mg cisplatin group	7.5-mg cisplatin group	F	P value
Serum creatinine (mg/dl)	0	0.48±0.1 (*n* = 12)	0.51±0.1 (*n* = 12)	0.092	0.576
	5	4.21±0.99 (*n* = 12)	4.5±1.1 (*n* =10)	0.163	0.525
BUN (mg/dl)	0	18.5±2.2 (*n* = 12)	18.8±2.3 (*n* = 12)	0.050	0.719
	5	268.3±52.0 (*n* = 12)	295.2±59.8 (*n* = 10)	0.205	0.280

** Significant difference (P<0.05) between the two groups.*
Data are represented as Mean ± SD
n = number of animals

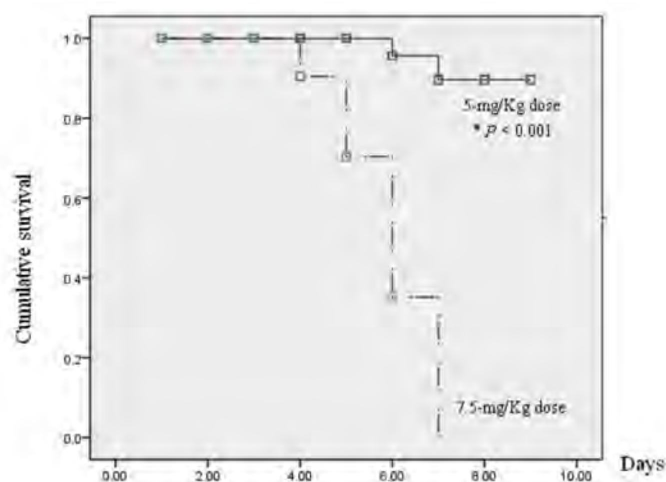

Fig. 1. Survival was greater in rats which received cisplatin in the 5 mg/kg dose in comparison to rats which received the 7.5 mg/kg dose.
** significant difference (P<0.001) from the 7.5-mg group.*

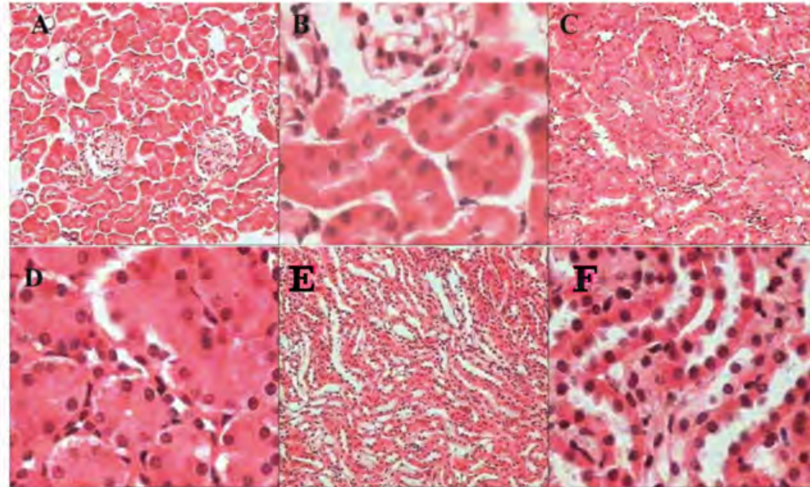

Fig. 2. Photomicrograph of the appearance of the normal layers in the rat kidney stained with hematoxylin and eosin. The photomicrograph shows the cortex (A, B), the OSOM (C, D) and the inner medulla (E, F). Magnification: x100 (A, C, E), x400 (B, D, F).

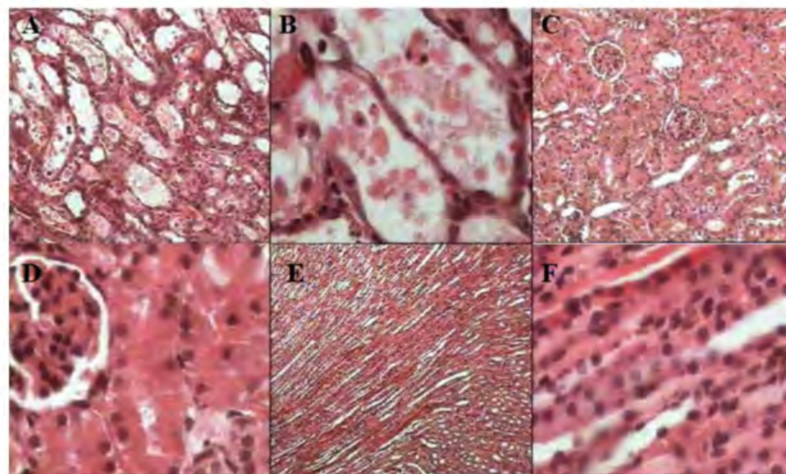

Fig. 3 : Histopathological changes of the different layers in the rat kidney after 5 days of administration of 5 mg/kg of cisplatin. The OSOM showed tubular necrosis, degeneration, and tubular casts (A, B). The cortex (C, D) and the inner medulla (E, F) were free (H & E). Magnification: x100 (A, C), x400 (B, D, F), x40 (E).

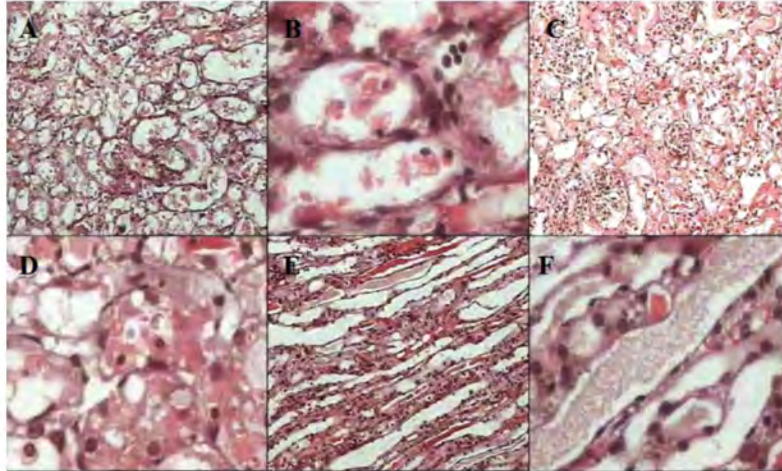

Fig. 4 : Histopathological changes of the different layers in the rat kidney after 5 days of administration of 7.5 mg/kg of cisplatin. The OSOM layer was the most affected layer showing tubular necrosis, degeneration, tubular casts, and hemorrhage (A, B). The necrotic changes involved also the cortex (C, D) and the inner medulla (E, F). H & E stain. Magnification: x100 (A, C, E), x400 (B, D, F).

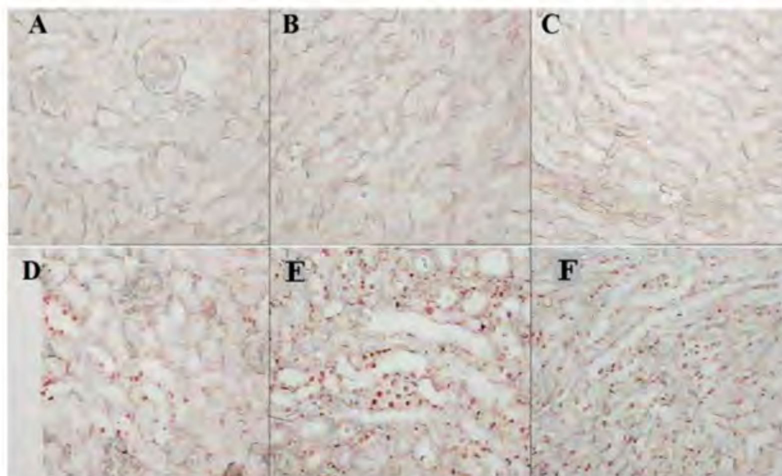

Fig. 5 : TUNEL assay for detection of apoptotic cells in the cortex (A, D), OSOM (B, E), and the inner medulla (C, F) of the kidney section from rats after 5 days of injecting cisplatin in the 5 mg/kg dose (D, E, F) in comparison to control (A, B, C). Magnification x400.

Discussion

Animal models are mandatory to improve our understanding of human AKI [2,6]. There are three well established types of animal models of AKI: ischemia; toxins and sepsis models[8]. In this study, AKI was induced using two different doses of cisplatin (5 and 7.5 mg/kg)[19,20]. Serum creatinine, BUN, pathological changes, apoptosis and mortality were all evaluated.

In the present study, the levels of serum creatinine and BUN at the 5th day after injecting cisplatin showed non-significant differences between the 5- and the 7.5 -mg groups. However, both survival rate and histopathological changes differ in each group. Survival rate showed a highly significant difference between the two groups. When sacrificed animals were excluded, more than 58% (7 out of 12) of animals died during the 10 days of the experiment in the 7.5-mg group. In the 5-mg group, only 16% (2 out of 12) of animals died during the same period. At the pathological level, renal tissue damage was extensive and involved all the layers of the kidney after 5 days of injecting cisplatin in the 7.5-mg group while renal damage was limited to the OSOM in the 5-mg group. However, TUNEL assay revealed that apoptosis extended to involve all the kidney layers in the 5-mg group.

The degree of functional impairment in cisplatin-induced AKI is poorly correlated to pathological degree of injury. Only two models of AKI, namely warm ischemia and radiocontrast media-induced AKI, were reported to be characterized by correlation between functional injury and pathology[8]. Explanations of such lack of correlation between the pathology and functional impairment have been extensively discussed in several studies[9,10,23] but almost always focused on limitations related to the biochemical parameters alone.

Regarding serum creatinine, it was reported that creatinine is not the ideal indicator of renal function during AKI[9]. Moran and Myers[24] reported a slow rise of serum creatinine even after a sudden fall in the glomerular filtration rate (GFR). In addition, serum

creatinine was found to be affected by many non-renal factors. For example, edema that usually develops in patients with AKI leads to dilution of serum creatinine and slows recognition of AKI[9]. Serum creatinine is cleared from plasma by both glomerular filtration and tubular secretion[25]. It was found that when GFR decreases, a compensatory increase in the tubular secretion of creatinine would also slow the rise of serum creatinine levels[23]. Moreover, Liver dysfunction and low muscle mass lead to reduction of creatinine production. Finally, several drugs have been found to interfere with tubular handling of creatinine [8, 26, 27].

In a similar way, BUN does not appear to match the degree of renal injury[10]. The levels of BUN were found to be affected by several factors. Exogenous urea load or endogenous production were found to increase with a high protein diet or with enhanced tissue breakdown secondary to hemorrhage, trauma, or even glucocorticoid therapy [28,29]. In addition, tubular reabsorption of BUN was found to increase if GFR was reduced [10].

Considering the pathological changes, renal tissue damage in cisplatin nephrotoxicity is characterized by both necrosis and apoptosis of the tubular cells. Cisplatin is capable of inducing the three pathways of apoptosis [14]. It was reported that the dose of cisplatin might determine whether the cells die by necrosis or apoptosis [30]. Using oil-immersion technique, light microscopic examination could reveal the morphological changes in the apoptotic cells with several limitations [31]. However, the most accepted method for detection and quantification of apoptosis is TUNEL method [32, 33].

Away from oil-immersion, light microscopic examination of the different kidney layers revealed that the 5-mg/kg dose of cisplatin affected only the OSOM layer. However, Using TUNEL assay for detection of apoptosis revealed that the same dose of cisplatin affected all the kidney layers. To our knowledge, all studies on cisplatin-induced AKI adopted scoring systems of renal tissue injury which did not consider apoptosis.

It might be possible that the ordinary scoring systems that grade the degree of renal injury underestimate the actually affected cells secondary to ignoring the apoptotic cells. It is logic to expect that cells that undergo apoptosis are non-functioning. In the present study, we suggest that the inability of considering the apoptotic cells in scoring of renal injury might additionally explain the lack of correlation between renal function parameters and the pathological changes in cisplatin-induced AKI.

In the present study, we found that although serum creatinine and BUN did not show any significant differences regardless the dose of cisplatin, mortality was significantly higher in the group that received 7.5 mg/kg of cisplatin. The ability of ordinary kidney function parameters to predict the morbidity or mortality in AKI has been evaluated in human. Risk factors that might increase mortality were found to be mainly other comorbid illness, oliguria, malignancy, sepsis, mechanical ventilation, and multiorgan failure [34,35]. Eighty percent mortality of patients with acute renal failure was found to be associated with mechanical ventilation[36]. In a validated index to predict mortality from renal disease, renal dysfunction accounts for 21% of the index, and comorbid illnesses account for the remainder[37]. In this study we suggest that, even in previously healthy rats, ordinary kidney function parameters might be poor predictors of mortality in cisplatin-induced AKI.

In clinical studies, the cause of death after AKI was identified in 93.4% of cases. Death occurs mainly secondary to sepsis (41.1%), cardiovascular disease (19.2%) and malignancy (12.9%). As a primary cause of death, AKI accounted for only 3% of cases [38]. Few animal studies have focused on the cause of death after isolated AKI. These studies suggested that a distant cardiopulmonary dysfunction was incriminated[39,40]. No studies evaluated the brain as the cause of death following AKI. We suggest that brain autopsy may reveal if the brain is involved in death after AKI or not.

Our data demonstrated that, in a rat model of cisplatin-induced AKI, the extent of apoptosis did not match the degree of renal tissue damage. This model is characterized by lack of correlation between functional parameters and the degree of kidney injury. Such character was attributed to limitations related to the biochemical assessment of renal function. The present study suggested that limitations related to pathological assessment of injury, as considering apoptosis, might be also incriminated in this character. Considering molecular events, namely apoptosis, may allow more precise correlation between the degree of renal dysfunction and the extent of renal tissue damage.

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CISPLATIN-INDUCED ACUTE KIDNEY INJURY MODEL IN RATS: A BIOCHEMICAL AND HISTOPATHOLOGICAL STUDY

Mohamed Abouelkheir, Dina A. ElTantawy MD, Karawan M. Abdelrahman MD, Mohamed Sobh MD and Mohamed-Ahdy Saad MD

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TREATMENT OF ACUTE DISTAL FEMUR FRACTURE IN ELDERLY PATIENTS: A COMPARATIVE STUDY BETWEEN LOCKED COMPRESSION PLAT AND RETROGRADE NAIL

Ibrahim Awad MD, Elshenawy Mostafa MD, Akram Ramadan MD and Alaa Abou Ouf MSc.

Department of Orthopedic and Trauma Surgery,

Mansoura university, Egypt.

Abstract

Background: *The treatment of fractures of the distal femur is more challenging when dealing with elderly patients having co morbidities both local (e.g. osteoporosis, osteoarthritis, and ipsilateral implants) and systemic illness. Locked compression plat and retrograde nail using indirect reduction of the metaphyseal fracture component, offering relative stability and a less invasive approach. Randomized comparison of these common methods of fixation has not been reported.*

Methods: *fifty one elderly patients with acute post traumatic distal femoral fractures were randomized to receive either a retrograde intramedullary nail fixation (RIN group, 26 fractures) or a distal femoral locked compression plat (LCP group, 25 fractures). The groups were followed for 8-40 months.*

Results: *there was no significant difference in epidemiologic parameters. The operative parameters were favorable with nail. No difference in rate of reoperation in both groups. Final outcome was marginally significant with nail than plate.*

Conclusion: *Based on our study; accepted outcome had been achieved with both methods compared with results of previous studies. However in our series nail showed more favorable outcome, less surgical morbidities, better rehabilitation.*

Introduction

Supracondylar fractures of the femur are very serious injuries. For a long time they were considered difficult to heal and often led to a degree of disability. These difficulties become greater when they are associated with elderly patients who present with a high degree of osteopenia.[1]

Surgical methods involving open reduction and internal fixation have been advocated using various implants including angle blade plats[2], the Zickel device[3], Rush rods[4] and AO dynamic condylar screw[5]. The diversity of approaches advocated for the surgical fixation of supracondylar femoral fractures indicates the lack of a gold standard for this challenging fracture type. In recent years, efforts have focused on minimally invasive methods to increase the rate of fracture union and reduce the incidence of infection.[6,7] The advent of locking implant constructs has revolutionized the area of peri-articular fracture fixation. By creating a fixed angle construct, the mechanical stability of the plat screw implant is increased.[8] LCP allows for percutaneous insertion of implants thus become less destructive to soft tissues. Its locked screw heads makes multiple fixed angel screw-plat constructs functioning as one unit.[9]

The popularity of closed intramedul- lary nailing of long bones has extended to femoral nails inserted in a retrograde manner as an operative solution for distal femoral fractures. Although much has been written on the relative value of nail and plate fixation of the distal femur, randomized comparison of these treatment methods in elderly has not been reported.[10]

Materials and Methods

A prospective study for comparison between fixation of distal femoral fractures in elderly patients using femoral retrograde intramedullary nails (RIN) and distal femoral locked compression plats (LCP). The study was done from September 2009 to may 2012 and Conducted on 51 elderly patients with distal femoral fractures randomized case after another (alternately) in a consecutive series to have either femoral retrograde In-

tramedullary nails (RIN) or distal femoral locked compression plats (LCP). The AO/OTA classification was used to grade the fractures. Periprosthetic fractures in both groups were classified according to Su et al., (2004)[11]. Patients were operated on in a supine position with the knee flexed 45°. A small bag was placed under the ipsilateral hip to prevent external rotation of the proximal fragment. A total of 26 patients with 26 fractures were treated with RIN (fig. 1). The surgical approach depends on the fracture type; Percutaneous transpatellar approach was used in 22 fractures (81.48%). Medial parapatellar approach was used in 5 intraarticular fractures (18.51%); two fracture type C1 & three fractures type C2 because closed reduction of articular surface was not anatomical. An intramedullary nail of adequate length and diameter was used; the longest and thickest nail was preferred as it gain better purchase in the femur, aid alignment and achieve optimal stabilization. The nails ranged from 200 mm to 380 mm in length. Distal femur LCP was used in 25 patients with 25 fractures (fig. 2). Minimal Invasive Percutaneous Plat Osteosynthesis (MIPPO) approach was used for plat insertion and screws fixation. Distal incision was done according the requirements of fracture reduction. Intera-articular fractures (AO/OTA types C1and C2) that required open reduction; the anterolateral parapatellar approach was used in 7 patients (28%) and Lateral MIPO approach was used in extra-articular fractures AO/OTA type A fractures, and type C fractures which are non displaced or successfully reduced with reduction clamp. It was used in 18 patients (72%). The proximal screws inserted through multiple 1cm stab incisions. Interaoperative plate length is determind under fluoroscopic control; we used Locking Compression Distal femur plates (LCP) 5 holes in 3 cases, 7 holes in 8 cases, 9 holes in 9 cases, and 13 holes in 5 cases Tourniquet and primary autologous bone grafting was not used. wound drainage was used in all patients. Operating time (skin incision to closure), blood loss (weighting of surgical gauges, suction blood volume) and hospital stay were recorded. Antibiotic (Cefuroxime 750mg 8 hourly) was

given for 5days. Anticoagulant (Enoxaparin 40mg subcutaneously once a day) was continued until they are fully mobilized. Suction drain was removed 48–72 hours and sutures after two weeks. Unless there are other injuries, or complications, joint mobilization may be started immediately postoperatively. Both active and passive motion of the knee and hip can be initiated immediately postoperatively. Emphasis placed on quadriceps strengthening and straight leg rises. Weight bearing was not suggested until early callus formation was seen on radiographs. The patients were then started partial-weight bearing, and gradually progressed to full-weight bearing for another 6 to 8 weeks.

Results

Epidemiologic parameters were similar in both groups. The average age of the patients in the nail group was 76.9 years (range, 63-97 years) 5 males and 21 females and in the plat group 75 years (range, 62-96 years); 10 males and 15 females.

The etiology of the fracture in nail group was a fall at home in 24 patients (88%) and RTA in 2cases (12%). In plat group the etiology of the fracture in nail group was a fall at home in 22 patients (92.4%) and RTA in 3 cases. The distribution of the simple and complex fracture types was similar in both treatment groups with no significant difference. The interval between injury and surgery was on average 3.4 days (range, 0-9 days). There were only 1case in each group without any medical co morbidity (i.e., 3.70% in RIN group and 4% in LCP group). All other cases in both groups had medical co morbidities. (i.e., 96.30% in RIN group and 96% in LCP group). The P value was insignificant. Operative time, blood loss, blood transfusion, and image intensifier exposure time was highly significant (table 1). In RIN group; Out of 26 patients with 26 fractures, 1 patient (3.8%) lost follow up because of death in 8th weeks postoperative due to multimorbidities not related to original injury. The mean follow up period was 18.84 months (range, 1 0- 36 months). In LCP group; Out of 25 patients with 25 fractures, 2 patients(8%) lost follow up; one be-

cause of death in 9th postoperative day due to multimorbidities not related to original injury. the mean follow up period was 17.44 months (range, 8-40 months). The final results were rated according to the functional score of Sanders et al., (1991)[12] in RIN group; 1 case (4%) was Excellent, 15 cases were Good (60%), 8 cases were Faire (32%) and 1 cases were Poor (4%). In LCP group; 11 cases were Good (47.83%), 9 cases were Faire (39.13%) and 3 cases were Poor (13.04%). P value was marginally significant 0.050. Knee motion is one of the most important objective finding in assessment of our results. At the last follow-up visit, the active range of motion was measured with a goniometer. In RIN group; the range of motion in our series ranged from 90°-130° knee flexion with an average of 110°, while extension lag ranged from 0°-20° with an average of 2.9°. In LCP group; the range of motion in our series ranged from 70°-130° knee flexion with an average of 103°, while extension lag ranged from 0°-20° with an average of 2.9°. P value was insignificant in both flexion (0.119) and extension (0.229).

Estimation of frontal plane angulation was done by measuring the anatomical Lateral Distal Femoral angle aLDF, then applying computer assisted goniometer soft ware to measure the aLDF angle, In RIN group; Mean aLDF angle was 79.3°, range 60-93°. 15 cases (60%) had frontal plane deformity at union. Mean frontal plane deformity was 3.1°, with varus angulation in 7 cases (28%) ranging from 0 to 13°; mean varus angle 7.17o. Valgus angulation in 8 cases (32%) ranging from 0 to 20°; mean valgus angle 3.7°. In LCP group; mean aLDF angle was 83°, range 71-91°. 14 cases (60.87%) had frontal plane deformity at union. Mean frontal plane deformity was 3.9o, with varus angulation in 12 cases (52.17%) ranging from 0 to 8°; mean varus angle 3.32°. Valgus angulation in 2 cases (8.70%) ranging from 0 to 9°; mean valgus angle 0.8°. There was no statistical significance regarding total angulation, varus, and valgus angulation. Average time of appearance of callus In RIN group was 5.5 weeks with a range of 4 to 10 weeks. In LCP group, average time was 7.95 weeks with a range of 4 to 16 weeks. P value was sig-

nificant (0.002). In RIN group, average time of fracture union was 19.5 weeks with a range of 12 to 28 weeks. Out of 25 cases; 24 cases (96%) progressed to union, one case (4%) had metal failure and loss of reduction, and no cases of delayed union. In LCP group, average time was 27.65 weeks with a range of 12 to 32 weeks. Out of 23 cases; 19 cases (82.6%) progressed to union, 3 cases (13%) had non union, and one case (4.4%) of delayed union. P value was significant (0.002) as regard healing time.

No cases of infection in RIN group. In LCP group; 1 case of deep infection in the early postoperative period, debridement, irrigation, and change of loose screws was done in the 3rd week postoperative. In RIN group; 6 patients required 2nd surgical procedures. Loosening and failure of distal locking screws in 6 cases; only 2 patients required removal. Two cases required removal of one the distal locking screws due to pain on the medial aspect of the knee. One case (case no. 9) had revision into TKR. One case required nail removal due to anterior knee pain. In LCP group; 6 patients required 2nd surgical procedures. One patient had a stress riser fracture at proximal end of the plat, required open reduction and fixation by another plate anterior to span the fracture and the plat. One case required lateral patellar release due to tethering of the lateral retiaculum by the plat. Two cases required bone grafting. One case required revision into buttress plat and external fixator. Then TKR after nonunion and metal failure. One case required debridement of deep infection.

Table 1: Blood loss, blood transfusion, OT duration and image time of RIN group and LCP group, number, mean, standard deviation and significance.

Group Statistics						
Group		*N*	*Mean*	*Std. Deviation*	*Std. Error Mean*	*sig(2-tailed)*
Blood Loss	RIN	26	234.8148	178.54907	34.36179	**0.000**
	LCP	25	459.6000	122.01366	24.40273	
Blood Transfusion	RIN	26	.1481	.36201	.06967	**0.020**
	LCP	25	.4400	.50662	.10132	
Operative Duration	RIN	26	100.1481	26.58470	5.11623	**0.000**
	LCP	25	157.6000	27.27636	5.45527	
Image Time	RIN	26	120.2593	39.21400	7.54674	**0.050**

Table (2): Average knee motion with supracondylar nail in different series.* contained elderly patients only, ** contained young and elderly patients,# studies on LCP. - Not reported.

Series	Patient/fractures	Mean flexion	Mean extension lag
*Danziger et al., (1995)** [25]*	24/25	108°	4°
Dunlop and Brenkel, (1999) [15]*	29/30	104°	4°
Kumar et al,(2000) [16]*	16/16	106°	6.9°
*Henry, (2000)** [10]*	104/111	100°	-
*Seifert et al., (2003)** [26]*	47/48	113°	-
Armstrong et al., (2003) [27]*	27/27	97°	4°
*Markmiller et al.,RIN (2004)** [9]*	16/16	103°	10°
Christodoulou et al., (2005) [1]*	35/35	80°	-
Nathan et al.,(2006) [13]*	12/12	108°	5°
*Our series RIN group**	26/26	113°	2.5°
Our series LCP group#*	25/25	103°	2.9°
*Yeap et al., (2007) *#[28]*	11/11	107°	-
*Markmiller et al., LCP(2004)** #[9]*	16/16	110°	10°
*Kanabar et al., (2007) *#[29]*	17/17	93°	-

Table (3): union and non union rates in reported in different series using RGN.*studies designed for elderly. ** Studies designed for elderly and young patients.

Series	Patient/ fractures	Mean union time in weeks	% of union	% of non union
Ginning and Hansen, (1999) [14]	25/26	12	87%	-
Dunlop and Brenkel, (1999) [15]*	29/30	-	92%	6.66%
Kumar et al,(2000) [16]*	16/16	15	93.7%	6.3%
Lauri et al., (2004) [33]	44/46	17.5	84.78%	4.35%
*Markmiller et al., (2004)** [9]*	16/16	-	93.75	6.25%
Christodoulou et al., (2005) [1]*	35/35	17	85.7%	5.7%
*Singh et al.,(2006) * [17]*	16/16	11	100%	0%
Nathan et al.,(2006) [13]*	12/12	-	100%	0%
*Our series RIN group**	25/25	19.5	96%	4%

Table (4): union and non union rates in reported in different series using LCP.*studies designed for elderly. ** Studies designed for elderly and young patients.

Series	Patient/ fractures	Mean union time in weeks	% of delayed Union	% of union	% of nonunion
Sayed et al.,(2003)** [34]	25/25	13	-	-	28%
Markmiller et al., (2004)** [9]	16/16	13.8	12.5%	87.5%	0%
Wong et al, (2005)* [20]	16/16	30	0%	100%	0%
Yeap et al (2007)** [28]	11/11	18	0%	100%	0%
Kanabar et al, (2007)** [29]	17/17	17	5.88%	82.35%	11.76%
Elganiany and elgeidi (2010)* [21]	13/13	20-32	0%	91.7%	8.3%
*Our study**	23/23	26.6	4.35%	86.90%	8.7%

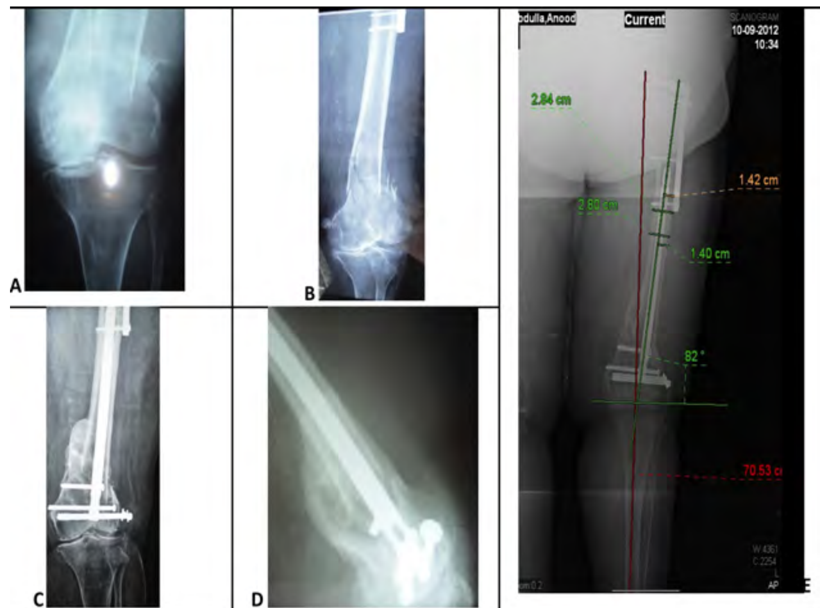

Figure (2): Case no. 3; A, B) preoperative X ray. C,D) Full union. E) Standing scanogram. LDFA82°.

Figure (3): Case no. 3 (LCP group); A, B) preoperative X ray. C, D) Full union. E) Standing scanogram. LDFA87°.

Discussion

In elderly patients, fractures of the femoral supracondylar region are predominantly caused by low-energy trauma predisposed to by osteoporosis. These fractures are difficult to treat due to bad bone stock and impaired vascularity and are often comminuted and unstable(13). The objective of this study is to present the results of surgical management of supracondylar fractures of the femur (types A and C1 and C2 according to the AO/OTA classification) in elderly patients we compared those two minimally invasive treatment techniques for distal femoral fractures (distal femoral nail and plate fixator). Several studies of biomechanical investigations and clinical studies of experience with these new implants have been published, but to our knowledge there are no published clinical studies comparing these two fixation methods done generally and in elderly patients in particular.

Several studies(14,15,16,17, 18,19) had been conducted on treatment of distal femoral fracture in elderly patients using RIN; the average age of patients included in these studies was about 75 to 80 years. Although large num-

ber of studies reported in literature about the use of locked plats in fixation of distal femoral fractures; we could hardly found two studies [20,21] reporting these results in elderly population. No significant difference in age between the RIN and LCP groups in our study. P value (0.7). In our RIN group we had 15 fractures type A and 10 fractures type C and 2 periprosthetic fractures. We had 12 fractures type A and 10 fractures type C2 and 3 periprosthetic fractures in our LCP group. The P value was 0.931. Average operative time in RIN group was 100.41 minutes (range, 60-145 minutes) which is nearly similar to that of Gynning and Hansen[14]. (1999) and Walcher et al., (2000)[22], while our time is shorter than that of Gellman et al., (1996)[23] and Markmiller et al., (2004)[9] their average operative time was around 142 minutes; However our time was longer than that of Sameh et al., (2007)[18] was 70 minutes; this could because all their fractures was type A and all nails used was long nails with proximal end at the lesser trochanter with no proximal locking. In LCP group average operative time was 157.6 minutes (range, 110-230 minutes) which is comparable to that of Kregor et al, (2004)[24] had average time 153 minutes (range 52-546 minutes), The P value was highly significant when comparing; operative time, fluoroscopy exposure time, blood loss, and blood transfusion; all results was in favor of RIN over the LCP. This results however explained by the more simple technique of locked nail fixation in comparison to the technically demanding procedure of locked plat fixation especially when using minimal invasive approaches. The average hospital stay in our study was 21.5 days (range, 11-38 days) in RIN group and 31 days (range 14-90 days) in LCP group; P value was significant 0.043. Elderly people usually associated medical problems which require a lot of investigations and preparation, and also they need prolonged post operative rehabilitation. we have longer duration because we conducted early postoperative rehabilitation inpatient, longest stay (90 days) in a case complicated by fracture at the proximal end of the plat 2 weeks post operative. There are few accounts in the literature that

detail the hospital stay, only Dunlop and Brenkel, (1999)[15] reported average hospital stay in their elderly group of 19 days in the acute ward and 72 days in the rehabilitation ward. In our series, the range of knee motion was found in the elderly to be affected by the associated osteoarthritis, osteoporosis, dementia, and rehabilitation difficulties in elderly age group. No significant difference in range of motion between the two groups (Table 2).

In our RIN series we did not encounter any cases of superficial or deep infection in RIN group. This was similar to results many authers[14,16,30]. While Nathan et al., (2006)[13] reported one case of deep infection and Sameh et al., (2007)[18] reported one case of superficial wound infection in their series. In our LCP group we had one case of deep infection. Schutz et al., (2005)[31] reported 3 cases of superficial infections treated with antibiotics, Henderson et al., (2011)[32] reported 7 cases of infection; in a retrospective study of 86 fractures including 18 open fractures fixed with locked plat. Initial callus started earlier in the nail group average 5.5 weeks versus 7.95 weeks in the plat group P value was significant (0.002). This intern resulted in earlier weight bearing and better rehabilitation. Kumar et al. (2000)[16] reported appearance of callus 3 to 6 weeks after surgery. In the retrograde nail group; time of full union was 19.5 weeks with a range of 12 to 28 weeks versus 26.6 weeks with a range of 12 to 32 weeks in LCP group. P value was significant (0.002) as regard healing time. In RIN group out of 25 cases; 24 cases (96%) progressed to union, one case (4%) did not progress to union because of metal failure, and no cases of delayed union. These results were similar to results of other authors[14, 16, 30]. The only reported delayed union was by Lauri et al., (2004)[33]; reported 5 cases (10.9%) of delayed union. In LCP group out of 23 cases; 19 cases (86.96%) progressed to union, 3 cases (13%) had non union. and one case (4.35%) of delayed union. Highest incidence of nonunion reported by Sayed et al., (2003)[34] was 7 cases (28%) (Table 3, 4). higher values of coronal plane angulations in the nail group than the plat group; mean varus angu-

lation was 7.17° in RIN group and in LCP group 3.32° while mean valgus angulation was in RGN group was 3.7° and in LCP group was 0.8°. These values are also supporting the biomechanical studies of various retrograde nailing systems compared with plat systems predominantly have shown a lower torsional and axial stiffness of nails but a better bending stiffness, particularly for physiologic and critical modes of varus loading. The overall reoperation rat was almost comparable in both groups; however all procedures in the nail group were simple day case surgeries except one case, while all those in plat group were major surgeries.

This could give some upper hand to the nail over the plat especially when treating elderly patients with multiple medical problems. Final outcome was rated according to Sanders et al., (1991)[12]. P value was marginally significant 0.050. The higher incidence of fair and poor results could be related to many factors; most of reported studies are not designed for elderly patients, meanwhile about 54% of our patients were house hold or totally dependent before injury. Decreased excellent result in our groups could be related to our more accurate evaluation of angulation will hardly give any result without angulation .

In conclusion; Both femoral retrograde interlocking nail and locked compression plat of distal femur appear to have statistically insignificant differences regarding knee motion, pain, function and rate of need of 2nd surgery. However retrograde nail is preferable to locked plat in terms of operative time, blood loss, and image intensifier exposure time, early appearance of callus, weight bearing, and shorter time required for full union. Also no incidence of delayed union, or nonunion. Locked plat is preferable to nail in terms of angulation and shortening. However still there is incidence of delayed union and nonunion. The incidence of reoperation was similar to nail group, however the procedures were not minor like the nail group. Based on our study; accepted outcome had been achieved with both methods compared with results of previous

studies. However in our series nail showed more favorable outcome, less surgical morbidities, better rehabilitation. Both methods have been used successfully in peri-prosthetic fractures; however the choice of the implant depends on the type of the prosthesis, and type of fracture aiming to achieve healing of the fracture without affecting the validity of the prosthesis. Clinicians should resist the 'cookbook' method of addressing these injuries and analyze patient factors, fracture geometry, and implant limitations when forming treatment plans. A surgeon should realize his comfort level with the injury and familiarization of implant choices. More similar studies are needed for results confirmation.

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TREATMENT OF ACUTE DISTAL FEMUR FRACTURE IN ELDERLY PATIENTS: A COMPARATIVE STUDY BETWEEN LOCKED COMPRESSION PLAT AND RETROGRADE NAIL

Ibrahim Awad MD, Elshenawy Mostafa MD,
Akram Ramadan MD and Alaa Abou Ouf MSc.

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PREDICTORS OF IMPROVED SEMINAL PARAMETERS AND FERTILITY POTENTIAL AFTER DIFFERENT VARICOCELECTOMY TECHNIQUES

Amro Elhadidi MD, Adel Zalata MD, Moheb Mansour MD, Mohammad Elgendy MD and Ramadan Ellithy MD

Departments of General Surgery; Andrology & Dermatology and Medical Biochemistry. Faculty of Medicine, Mansoura University, Egypt

Abstract

Introduction : *Varicocele is one of the most common causes of infertility. In this study, we evaluated and compared the operative time, semen analysis results and complications of two different methods of varicocelectomy; Loupe assisted subinguinal and Palomo high retroperitoneal varicocelectomies. Also we investigated predictors of improved fertility potential in relation to preoperative variables.*

Materials and Methods: *from March 2011 to April 2013, Patients were randomly assigned to undergo either subinguinal or Palomo varicocelectomy. We follow up patients up to one year with postoperative assessment of seminogram and fertility potential at 3 and 6 months. Surgical findings, complications, recurrence rates and pregnancy rate were compared.*

Results: *two hundred patients assigned for this study and randomly distributed in two group showed comparable results as regard improvement of postoperative seminogram, hormonal assay and fertility potential. The operative time was longer in subinguinal group 67 ± 17 (50-130) and 62±15 (45±115) for bilateral cases with $p \le 0.01$. As regard postoperative complications, no disease recurrence occurred in group 1 while there is a one case in group 2. In logistic regression analysis there was a positive correlation between pregnancy rate and preoperative sperm concentration, serum inhibin B level, serum testosterone level and acrosin activity. A negative correlation with serum FSH, seminal*

No, olive tail moment (OTM), sperm tail length and DNA percentage in tail was observed.

Conclusion: *loupe assisted subinguinal and Palomo high retroperitoneal ligation gives comparable results as regard improvement of seminogram, hormonal assay, acrosin activity and DNA fragmentation. As regard fertility potential subinguinal varicocelectomy had superior results. Prediction of improvement of fertility potential can be made and significantly direct patients toward proper management.*

Keywords: *Semen-sperm-varicocele -DNA fragmentation - Nitric oxide - serum inhibin B*

Introduction

The prevalence of varicocele is approximately 15% in the general population, 19%-41% in men with primary infertility, and 45%-81% in men with secondary infertility. A correlation between varicocle and primary infertility is well documented[1]. Altered seminogram in particular, total sperm count, motility, and morphology suggest that varicocele may be the cause of infertility[2]. Traditionally, varicocele is diagnosed by clinical examination of the patient and graded according to the Dubin and Amelar classification[3]. Beside the improvement in seminogram in patients treated with varicocelctomy, certain other parameters like DNA fragmentation and oxidative stress (OS) showed also a significant improvement after varicocelectomy[4]. Pregnancy rate after varicocele repair has been shown to be a major factor for estimation the treatment success; by these a normal conceiving way and reduction in the assisted reproductive techniques (ART) with its high cost can be obtained[5]. The techniques currently used to treat varicocele are the inguinal (Ivanissevich) approach, high retroperitoneal (Palomo) approach, subinguinal ligation using microsurgical or loupe assisted techniques[6], laparoscopic ligation of the spermatic veins[7] and procedures involving retrograde and antegrade sclerotization, such as the Tauber technique[8]. Te best treatment modality for varicocele in infertile men should include improvement of seminogram and fertility potential with lower

rates of complications [9]. In this study we evaluate two methods of varicocelectomy techniques for improvement of seminal parameters and pregnancy rate. We also try to evaluate the correlation between preoperative variables and postoperative improvement of fertility potential to reach how can predict which patients with primary infertility and varicocle will get benefit from varicocele repair.

Materials and Methods

Patients :

A total of 200 unilateral and bilateral varicoceles were operated on, from 2011 march till 2013 April at Mansoura University Hospital. Cases were randomly selected for one of either approach; loupe assisted subinguinal (group 1) or Palomo high retroperitoneal (group 2) approach. Indications for varicocelectomy were the same in both groups and included primary infertility and varicocele associated with documented abnormalities in sperm parameters. Diagnostic protocol included physical examination, color Doppler ultrasonography (CDUS). Preoperative assessment of hormonal profile (serum FSH, serum testosterone, serum LH, serum prolactin, and serum inhibin B) associated with estimation of seminal plasma level of Nitric oxide; acrosin activity and assessment of DNA fragmentation level were included. Prediction of postoperative fertility potential was assessed using a logistic regression analysis. Serum follicle-stimulating hormone (FSH) and luteinizing hormones were measured using chemiluminescence assays. Testosterone was analyzed by radioimmunoassay. Assessment of acrosin activity is done by gelatinolysis. Sperm nuclear DNA fragmentation test using COMET Assay (single cell gel electrophoresis assay).

Operation :

Loupe assisted subinguinal approach :

In group 1, the patient was placed in the supine position under spinal anesthesia. The incision was made transversely with a length of approximately equal to 2.5 cm at the level of external inguinal ring, just outside the pubic tubercle. By retracting the edges of the wound, the spermatic cord was dissected subinguinally

without breaching the inguinal canal. Looping of spermatic cord then occurred. The external spermatic vein is assessed and if dilated a double ligatures was applied. Then the internal spermatic fascia is approached through meticulous dissection. With the aid of a x 3.0 loupe all dilated veins was dealt with. The arteries were identified by their clearly visible pulsations. The dilated internal spermatic veins were identified and dissected carefully with mosquito clamps. With careful manipulation, each dilated vessel was isolated and a double ligature of 3-0 Vicryl was used to control the vessel and then cutted with a sharp scissor. The compartment of the vas deferens was protected and left untouched except when abnormally engorged veins were evident. After the procedures performed inside the spermatic cord were completed, the wound was closed with 3-0 Vicryl sutures.

Palomo high retroperitoneal approach :

In group 2, Under spinal anesthesia the patient was placed in a supine position, a short transverse incision about 3.5 cm was performed just medial to the anterior superior iliac spine approximately at the level of the internal inguinal ring. The external oblique aponeurosis was incised in the direction of its fibers and the internal oblique muscle with the beneath transversus abdominis were splitted and retracted to expose the transversalis fascia and peritoneum. From the most lateral point the fascia transversalis was detached using a gauze soaked with saline then pealing of the peritoneum was continued to expose the testicular vessels. The testicular vessels were visualized along the peritoneal reflection, and dissected off the surrounding fat by sharp and blunt dissection. The veins were ligated and a segment was removed. Care had to be taken not to injure the inferior epigastric vessels which were near to the testicular vessels specially as we go more and more close to the internal ring where they pass below and medial to the internal ring. Preservation of testicular artery was done in all patients. After all visible veins and venous

collateral were ligated, the internal oblique muscle with the underneath transversus one were approximated with interrupted 2/0 Vicryl sutures. The external oblique aponeurosis was closed using continuous Vicryl 2/0. The subcutaneous tissue and the skin were then closed.

Statistical Analyses

The significance of the difference between variable in each group was assessed using the Wilcoxon test (paired samples). The significance of the difference between the groups was assessed using the Mann-Whitney U-test. Receiver operating characteristics (ROC) analysis was carried out using the MedCalc program (MedCalc Software, Belgium). The 95% Confidence interval, sensitivity, specificity, Significance level P (Area=0.5), and the area under the ROC curve were obtained for each parameter. A multivariate logistic regression analysis was employed to determine the significant predictor of the postoperative pregnancy occurrence. In all analyses, the level of statistical significance was set at $P<0.05$.

Results

Patients :

Preoperative characteristics were well balanced in the two randomized groups, as shown in (Table 1). The mean ages of the patients were 28.6 ±4.8 years, 28 ±4.9 years in groups 1 and 2, respectively. The mean time of period of infertility was (3.15±1.72) with range (1-10) years and (3.1±2.3) with range (1-10) years in both groups respectively. The follow–up duration was 1 year. Follow-up visits were at the first and second postoperative weeks and every 3 months, afterwards. Follow-up visits were at the first and second postoperative weeks and every 3 months, afterwards.

Operative Time :

The operation time was calculated from incision to skin closure in both groups. The mean operative times were 37 ± 10 (20–53) and 32±13 (18–65) in unilateral cases in both group respectively. While the operative time was 67± 17 (50-130) and 62±15 (45±115) for bilateral cases with $p \le 0.01$. There was no significance in the mean time of hospital stay ($p=0.8$) in both groups. Patients return to

normal activities occurred after a mean of 5.5 and 7.2 days after subinguinal and Palomo surgery, respectively

Postoperative Complications :

Four patients in group 1 developed scrotal edema, which were treated by rest, non-steriod anti-inflammatory drugs, and scrotal supports. Also, no patient in this group had hydrocele. No varicocele recurrences developed in group 1. In group 2, there were 2 (2%) patients with mild hydrocele which treated conservatively and 1 patient developed recurrence in this group which treated by subinguinal approach. No patient had wound infection or orchitis in both groups.

Semen Parameters

The two study groups were comparable regarding the preoperative semen parameters, including sperm count, motility, and morphology. A comparison among the two study groups between the mean preoperative and postoperative semen parameters showed significante improvement in sperm concentration , motility and morphology at preoperative (0) ,3, 6 months follow up period. Also, the two groups had comparable improvement in sperm motility, concentration and/or morphology at preoperative (0), 3 and 6 moths postoperatively (table 2).

Seminal Nitric oxide:

The level of seminal plasma nitric oxide (NO, μmol-1) was decrease significantly in both groups (107.3 ± 17.5 to 91.9 ± 16.4) and (110.5 ± 20.1 to 90.6 ± 18.3) with p=0.0001.

Hormonal parameters:

There is a significant decrease in serum FSH and a significant increase in the level of serum testosterone before and after treatment at both groups. The two groups had comparable results in FSH, LH, and prolactin at preoperative (0) and at 3, 6 months postoperatively. As regard serum testosterone there was a significant difference between the group 1 and group 2 at 0, 3 and 6 month (P = 0.0012, P = 0.0014 and P = 0.0002 respectively). This may explain the superiority of subinguinal approach as regard pregnancy rate (table 3).

Acrosin activity and serum inhibin B level :

A significant increase in the acrosin activity from 4.5± 0.9 to 8.5 ± 2.4 and 4.6±0.9 to 8.61 ± 2.12 in both group respectively. As regard serum inhibin B level (pg/ml) there was a significant improvement (136.2 ±7.8 to 153.9 ±18.9 and from 135.5± 8.4 to 150.9 ±17.99 with p< 0.0001). Also, a significant decrease in DNA fragmentation in both groups was noticed (table 4).

Pregnancy Rate:

The pregnancy rate at 1 year was not significantly different (0.225) among the two groups and was 35% and 27 % in the subinguinal and Palomo groups, respectively.

The predictive value of each parameter in relation to pregnancy outcome was detected by calculating the area under the ROC curve (Table 5 and Figure 1 A, B).

Table 1: Patients characteristics .

Variables	Subinguinal ligation	Palomo ligation	p-value
Patients numbers	100	100	
Varicocele side 1- Left 2- Bilateral	 9(9%) 91(91%)	 9(9%) 91(91%)	
Age (year) • Mean ±SD • Range	 28.6±4.8 20-40	 28±4.9 20-40	0.428
Period of infertility(year) • Mean ±SD • Range	 3.15±1.7 1-10	 3.1±2.3 1-10	0.891
CDUS • GRADE 1 • GRADE 2 • GRADE 3	 2 (2%) 16(16%) 82(82%)	 0(0%) 15(15%) 85(85%)	

Table 2: Sperm characteristics comparison at 0, 3, 6 months between the two groups .

Variable	Subinguinal ligation	Palomo ligation	p-value
Sperm concentration (10^6 /ml)(0 month)	**12.95±5.19**	**12.1±5.1**	0.2788
Sperm concentration (10^6 /ml)(3 month)	34.3±7.9	**33.5±6.6**	0.5791
Sperm concentration (10^6 /ml)(6 month)	**36.5±8.9**	**35.8 ±7.3**	0.6268
Sperm motility (%)(0 month)	33.5±3.95	34.1±3.8	0.2567
Sperm motility (%)(3 month)	38.96±6.8	37.7±5.3	0.1589
Sperm motility (%)(6 month)	41.3±9.22	40.3 ±7.6	0.4680
Sperm morphology (%) (0 month)	33.3±2.22	33.3±3.3	0.7635
Sperm morphology (%) (3 month)	35.6±2.1	36.2±2.9	0.958
Sperm morphology (%(6 month)	38.8 ± 5.21	39.2±5.4	0.6104

Table 3: hormonal assay comparison at 0, 3, 6 months

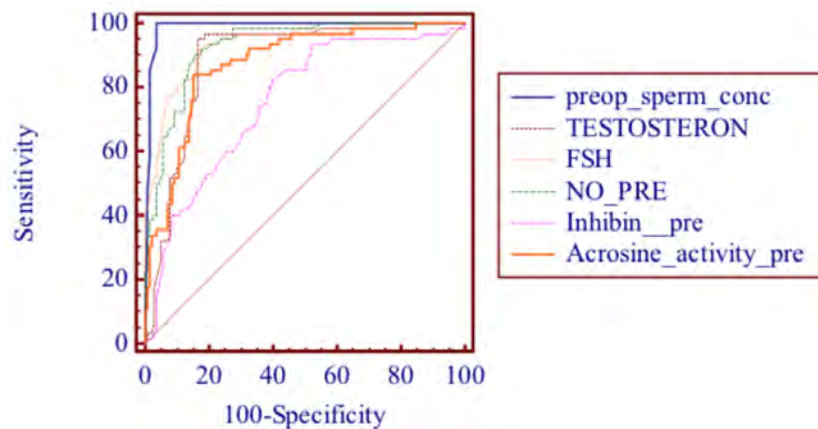
Variable	Subinguinal ligation	Palomo ligation	p-value
FSH (µIU/ML) (0 month)	**5.65 ±3.1**	**5.8±2.85**	0.5658
FSH (µIU/ML) (3 month)	**4.4 ±2.7**	**4.9±2.6**	0.0520
FSH (µIU/ML) (6 month)	**4.2 ±2.6**	**4.7±2.6**	0.0952
LH(µIU/ML) (0 month)	4.75±2.25	4.8±2.2	0.8536
LH(µIU/ML) (3 month)	4.5±2.1	4.6±2.1	0.6958
LH(µIU/ML) (6 month)	4.4±2	4.55±2.	0.6441
Prolactine (ng/ml) (0 month)	10.7±3.9	10.6±3.9	0.7535
Prolactine (ng/ml) (3 month)	10.4±3.9	9.9±3.7	0.3406
Prolactine (ng/ml)(6 month)	10.1±3.7	9.7±3.5	0.5461
Testosterone(ng/ml) (0 month)	**22.5±7.3**	**19.3±6.6**	0.0012
Testosterone (ng/ml) (3 month)	**26.1±8.4**	**22.4±7.9**	**0.0014**
Testosterone (ng/ml) (6 month)	**28.2±8.5**	**23.6±8.7**	0.0002

Table 4: DNA fragmentation in pre and post-operative period

Variable	Before treatment	After treatment	P value
Subinguinal ligation			
OTM	2.9 ± 1.1	1.13 ± 0.16	< 0.0001
Tail length	1.75 ± 0.64	0.5± 0.17	< 0.0001
DNA percentage in tail	18.3 ± 3.7	5.1± 1.3	< 0.0001
Palomo ligation			
OTM	2.9± 0.98	1.15 ± 0.13	< 0.0001
Tail length	1.8± 0.67	0.53 ± 0.2	< 0.0001
DNA percentage in tail	19.1± 4.07	4.99 ± 1.3	< 0.0001

Table 5: Diagnostic accuracy of independent factors

Independent variables	Area under the ROC curve (AUC)	Standard Error	95% Confidence interval	Significance level P (Area=0.5)
Preoperative sperm concentration	0.988663	0.00672	0.962282 to 0.998342	<0.0001
FSH	0.934783	0.0178	0.891148 to 0.964778	<0.0001
Serum testosterone	0.885870	0.0252	0.833451 to 0.926398	<0.0001
NO level	0.928530	0.0175	0.883564 to 0.960096	<0.0001
Serum inhibin	0.755493	0.0364	0.689886 to 0.813361	<0.0001
Period of infertility	0.891129	0.0244	0.839506 to 0.930684	<0.0001
Acrosine activity	0.873831	0.0261	0.819697 to 0.916478	<0.0001
OTM	0.874299	0.0244	0.820228 to 0.916866	<0.0001
Tail length	0.872020	0.0265	0.817639 to 0.914973	<0.0001
DNA percentage in tail	0.850865	0.0308	0.793808 to 0.897185	<0.0001



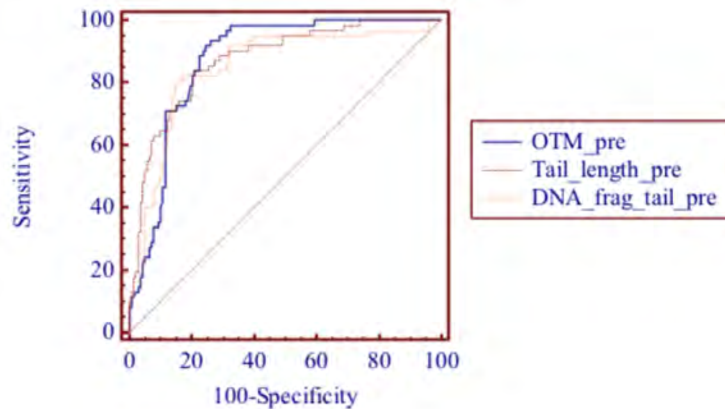


Figure (1 A, B): ROC curves of some preoperative predictive variables

Discussion

Varicocele is the most frequent physical finding in infertile men, being present in approximately 15% of men in the general population and 35% in infertile men(10). The pathophysiology of varicocele-associated infertility is not completely understood, but an increase in testicular temperature secondary to a venous reflux at the pampiniform plexus is the most accepted explanation(11). Varicocele repair by occlusion of the affected spermatic vein(s) is the treatment of choice for most physicians, and can result in improvements in scrotal discomfort, improvement in spermatogenesis, Leydig cell function, testosterone levels in most cases and spontaneous pregnancy(12,13,14,15). Moreover, several studies suggest that pregnancy rates following varicocelectomy range from 40% to 60% within 1 year following surgery and up to 70% within 2 years of surgical repair (16,17) .

Several attempts have been made to predict which varicocele patients are likely to benefit from varicocele repair in terms of an improved seminogram and fertility potential; such prediction has proved to be very difficult. Fujisawa et al.(18) reported an exaggerated gonadotrophin response to gonadotrophin-releasing hormone, which could be normalised by varicocele treatment, implying the impairment of gonadotrophin control mechanisms in infertile

men with varicocele . Giannakis et al.[19] showed that a main parameter predicting the effect of varicocelectomy on spermatogenesis is testicular telomerase activity.

Ishikawa and Fujisawa, [20] previously showed that age was not a significant factor to predict improvement in semen characteristics. In this study, age was also not a predictor for postoperative pregnancy rate, but the most important was the period of infertility which correlated negatively with postoperative fertility potential.

Marks et al. [16] also reported that testicular atrophy in an infertile patient indicated a decreased potential for seminal improvement and pregnancy after varicocele repair . Uygur et al.[21] in his study has been shown that a larger varicocele caused irreversible testicular damage and showed less improvement in postoperative seminal parameters. However in this study no correlation was found between testicular volume and fertility potential.

Sertoli cell dysfunction is sometimes detected in animal models with varicoceles. Intratesticular androgen-binding protein concentrations in subjects with varicoceles are significantly lower than in controls [22] . In Leydig cell secretory function, intratesticular testosterone concentrations in subjects with varicocele were significantly lower than in controls. In addition, secretory deficiency of Leydig cells in animals with varicoceles has been demonstrated[23]. Considering that varicoceles may be a factor in the progressive deterioration of testicular function (both spermatogenesis and steroidogenesis) over time (World Health Organization, 1992)[24], the testicular function of high serum FSH and low testosterone patients may be strongly affected by varicoceles. Kondo et al. [25] in his study concluded that a pre-operative low serum FSH and high testosterone concentration were predictors of postoperative improvement of fertility potential, and suggested that patients with normal Sertoli and Leydig cell functions always benefit from varicocele repair. In this study; high preoperative serum FSH had a negative correlation with

postoperative fertility rate while high preoperative testosterone concentration was a good positive predictive variable.

After the specific immunoassay for inhibin-B became available in 1995, a number of studies demonstrated that the serum inhibin-B level is a more sensitive predictor of the spermatogenic state than FSH [26]. In this study there was a significant increase in serum inhibin post-varicocelectomy and also S.inhibin-B show a good predictive value for postoperative pregnancy occurrence. Dubin & Amelar, [27] suggested that pregnancy rates were higher when sperm counts exceeded 10 million / ml, being lower in severely oligozoospermic cases in whom sperm counts were less than 10 million / ml; however, Segenreich et al. [28] reported a good outcome despite counts below this value. In our study high preoperative sperm concentration was a strong predictor for postoperative pregnancy rate with a sensitivity of 100% and specificity of 96%. Smit et al.[29] showed that the DNA fragmentation index decreased significantly after surgery from 35.2% to 30.2% (p = 0.019). After varicocelectomy 37% of the couples conceived spontaneously and 24% achieved pregnancy with assisted reproductive technique. The mean postoperative DNA fragmentation index was significantly higher in couples who did not conceive spontaneously or with assisted reproductive technique (p = 0.033). In the present study the Comet assay showed that the mean values of sperm DNA fragmentation were significantly predictors for postoperative pregnancy and a preoperative low value of sperm DNA fragmentation correlated well with the conceiving rate.

Acrosin is a trypsin- like serine proteinase found within the acrosome of human spermatozoa[30]. It is an important proteolytic enzyme that can hydrolyze the zona pellucida in the oocytes. It also plays a vital role in the process of fertilization and involved in acrosome reaction [31]. Its absence or reduced activity may be a clinical cause of male infertility. Thus measuring acrosine activity is suitable approach for

evaluating the fertilizing capacity of human spermatozoa [32].

Zalata et al. [33] measured the acrosine activity by using the gelatinolysis technique. They reported that the presence of oxidative stress in an individual with abnormal semen parameter is associated with impaired sperm function as measured by its acrosin activity. In the present study varicocele is likely to be the source of reactive oxygen species (ROS). It is important to recognize that abnormal spermatozoa may induce oxidative stress by either removing the scavenging capabilities of the seminal plasma or by increasing the ROS generation .

Shiraishi & Naito[34] observed that increased production of nitric oxide (NO) in the testis is involved in the enlargement of varicocele and indirectly deteriorates spermatogenesis. Moreover, based on an experimental model of varicocele in rats, Kisa et al. [35] concluded that NO is an important mediator in the pathogenesis of varicocele and that ROS are effective via NO pathways. In this study elevating level of seminal seminal NO was associated with negative correlation and decrease in the fertility potential for men with varicocele and primary infertility

Finally, important predictive variables like preoperative sperm concentration, acrosine activity, hormonal status of the patient and DNA fragmentation must be assessed thoroughly in all varicocele patients associated with primary infertility for better direction of surgical management and also for better postoperative outcome .

Conclusions

Varicocele repair improved sperm concentration and motility. The results of our study indicated that other parameters must be investigated well beside routine pre-operative senimogram and duplex scan .

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PREDICTORS OF IMPROVED SEMINAL PARAMETERS AND FERTILITY POTENTIAL AFTER DIFFERENT VARICOCELECTOMY TECHNIQUES

Amro Elhadidi MD, Adel Zalata MD, Moheb Mansour MD, Mohammad Elgendy MD and Ramadan Ellithy MD

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IMPLICATION OF NITRIC OXIDE IN WOUND HEALING IN DIABETIC RATS

Hasnaa A. Attia M.Sc., Abd El Hakim Z. Gabr MD, Fathy A. Ebrahim MD, Maha. M. Amin MD and Amany M. Shams MD

Department of Anatomy and Embryology, Faculty of Medicine,

Mansoura University, Egypt

Abstract

Background: *Effective wound healing leads to restoration of tissue integrity and occurs through a highly organized multistage process involving various cell types. Nitric oxide (NO) is essential to all phases of wound healing. Diabetes characterized by impaired wound healing that thought to be due to nitric oxide deficiency at the wound site.*

Aim of the work: *This study investigated whether exogenous nitric oxide supplementation with the nitric oxide donor molsidomine (N-ethoxycarbomyl-3-morpholinyl-sidnonimine) could reserve the impaired healing in diabetes.*

Material and Methods: *The wound healing was studied by creating a dorsal skin incision in diabetic and non diabetic rats. Three groups were used Group I (Control non diabetic animals),Group II (Unmanipulated diabetic animals) and Group III (Diabetic animals treated with molsidomine). Specimens were collected after 2nd, 5th and 10th day. Histological sections were stained with hematoxylin and eosin and anti inducible nitric oxide synthase (iNOS) immunohistochemistry.*

Results: *the regeneration of injured epidermis was faster in diabetic molsidomine treated group than in unmanipulated diabetic. Molsidomine promotes keratinocyte proliferation and migration at wound edge with rapid sealing of the wound gap in treated diabetic group when compared with unmanipulated diabetic group. Also, it enhanced neoangiogenesis.*

Conclusion: *The nitric oxide donor molsidomine can partially reverse impaired healing associated with diabetes.*

Introduction

Healing of skin wounds is a complex programmed sequence of cellular and molecular processes that include inflammation, cell migration, angiogenesis, provisional matrix synthesis, collagen deposition, and re-epithelialization[1]. Nitric oxide (NO) is a highly diffusible intercellular signaling molecule implicated in a wide range of biological effects. It is generated by the enzyme nitric oxide synthase (NOS), which catalyzes the conversion of L-arginine to L-citrulline. Three NOS isoforms have been characterized. Two enzyme isoforms are constitutively expressed (endothelial and neuronal NOS), whereas one isoform is an inducible enzyme (iNOS), initially found in macrophages (Griffith and Stuehr, 1995)[2]. Frank et al. (2002)[3] noticed the important role of nitric oxide (NO) in wound healing. Nitric oxide derived from iNOS during repair has been shown to mediate vasodilation[5], angiogenesis[6], inflammation[4], re-epithelialization[3], collagen deposition[7], and various immune responses[8]. Many impaired wound healing states have been associated with depleted NO production. There are strong correlations between reduced cutaneous NO availability and well-documented impairments in diabetic wound healing. In diabetes, the complex overall process of wound healing, and its constituent phases are all impaired. Decreased chemotaxis, phagocytosis, bacterial killing and levels of local antioxidant[9,10] during early phase of repair have all been related to impaired healing in diabetes. Growth factor depletion [11,12], increased glucocorticoid levels[13], decreased cellular proliferation[14,15] and up-regulation of apoptosis[16] characterize the later phases of healing in diabetes. The excessive adiposity of diabetes may also play an inhibitory role in diabetic wound healing[17]. Normalization of blood glucose concentrations may help to reverse some of the impaired mechanisms of wound healing, e.g., the processes of collagen metabolism and subsequent cross-linking are benefited by treatment of diabetes with insulin[18,19]. Schaffer et al. (1997)[7] and Stallmeyer et al.(2002)[20] discovered that the diabetic state is characterized by greatly reduced expression of NOS and a resultant re-

duction in availability of NO in the cutaneous wound environment. Also, Witte et al. (2002)[8] confirmed that diabetic wound healing is characterized by a NO-deficient state that associated with reductions in wound breaking strength and collagen deposition that has been improved with molsidomine treatment which significantly increased fresh wound breaking strength with elevated wound hydroxyproline content and wound collagen content in diabetic animals. In addition, molsidomine improved collagen remodeling capability in diabetic animals that reflected by an increase in activity of matrix metalloproteinase-2[8].

Materials and Methods

*** Animals used:-**

Forty adult female albino rats, weighting 150-200 gm aging 6-10 weeks were used. The experimental protocol and animal care were in compliance with the requirements of regulations of the Committee on animals experimentation of Mansoura Univeristy.

• Experimental design:-

A- Induction of diabetes mellitus in the rats:

The animal model of diabetes was done by using Streptozotocin (STZ). STZ was dissolved in sodium citrate buffer, pH 4.5 and injected within 15 min of preparation. Twenty-six rats were fasting for 12 hours. Then, streptozotocin (STZ) was injected intraperitoneally at a dose 40 mg/kg daily under light ether anesthesia. Diabetic status was confirmed by measuring the blood glucose (Accu-Chek blood glucose meter, Roche Diagnostic, Germany) from day 3 after the first injection of STZ. Rats were confirmed diabetic when fasting blood glucose (FBG) >13.9 mM (250 mg/dl) for 2 consecutive days[21].

B. Wounding of rats:

Thirty-six rats were used. The animal's back was shaved. Using a pen, a mark was made on midline of the back of each rat. The mark was 4cm caudal to the animal’s base of the skull, 1cm long. The wounds were performed on these marks. They were 1 cm full thickness dorsal wounds (cutting through the epidermis, dermis and panniculus carnosus muscle).

*** Experimental groups:**

Group I (Control non diabetic animals): Twelve rats were used as control.

Group II (Unmanipulated diabetic animals): Twelve diabetic rats did not receive any treatment.

Group III (Diabetic animals treated with molsidomine):

Twelve diabetic rats were treated with molsidomine (1mg per kg body weight per day) orally. The treatment was started on the day of wounding till the 10,th day post wounding.

It was decided to examine epidermal wounds at two, five and ten days after wounding, using four animals for each time points.

*** Microscopic examination:**

Paraffin sections were cut at 6 microns and stained with Hematoxylin and Eosin stains and 4 microns for immunohistochemical stain.

Histopathological analysis:

1- **Epidermal Closure:** complete basal-layer formation, relative closure parameters were obtained by assessing the appearance of epidermal wound edges (epidermal migration).
2- **Granulation tissue (capillaries and fibroblast):** Hematoxylin and Eosin staining was used to assess granulation-tissue formation.
3- **Inflammation:** Hematoxylin and Eosin staining was used to assess the inflammatory response (polymorph nuclear leukocytes) at the wound area.

*** Morphometric study:**

Examination of sections from each specimen were done on an Olympus CX31 light microscope. Pictures were obtained by a PC-driven digital camera (Olympus E-620).The computer software (Cell*, Olympus Soft Imaging Solution GmbH) allowed morphometric analysis to be performed. Image J was used to analyze images and the results of all were averaged (mean). The surface area of neovascularization (newly formed loops of capillaries) and staining pattern of epithelial layer of skin were analysized in each group for comparison and the data obtained

were subjected to statistical analysis.

• Quantitative Evaluation of iNOS:

Sections were digitized using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 40 X objective. The result images were analyzed on Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for iNOS immunohistochemical staining analysis and stain quantification.

• Statistical Analysis:

Statistical analysis was done for the morphometric analysis of neovascularization and iNOS expression intensity using the arithmetic mean, standard deviation (SD), analysis of variance (ANOVA) and t-test according to Munor et al. (2002)[22]. All the statistical analysis was done using SPSS (statistical package for social science) program version 20. The data were parametric by using Kolmogrov-Smirnov test and expressed as Mean±SD. Significance was considered when p value was less than or equal 0.05. Insignificance was considered when p value more than 0.05.

Results

1. Haematoxylin & Eosin stained sections:-

• Second day after wounding:

There is increase in the thickness of epidermis at the wound margins of unmanipulated normal and diabetic molsidomine treated groups than unmanipulated diabetic group. The epidermal thickness of diabetic molsidomine treated group higher than that of unmanipulated normal and unmanipulated diabetic groups. The epidermis at the wound margin is formed of 3-5 layers in unmanipulated normal and unmanipulated diabetic groups increasing to 6-8 layers in diabetic molsidomine treated group. (Figs.1,2,3).

There is no evidence of complete re-epithelialisation in all groups. In unmanipulated normal and diabetic molsidomine treated groups, the leading edge sealed most of the wound gap while, in unmanipulated diabetic group, the leading edge was short and covered only a small part of the

wound gap with lag in keratinocyte migration (Figs.1,2,3).

There is a demarcation line of polymorph nuclear leucocytes between the vital tissue and the necrotic debris in the superficial part of dermis. This demarcation line was more prominent in unmanipulated normal and diabetic molsidomine treated groups than unmanipulated diabetic group (Figs. 1,2,3).

There is a fibrin network filling out the whole depth of the wound gap in unmanipulated normal and diabetic molsidomine treated groups. While, in unmanipulated diabetic group, the fibrin network is present at the bottom of the gap and is not filling its whole depth (Figs.1,2,3).

*** Fifth day after wounding:**

There is increase in the thickness of epidermis at the wound margins of unmanipulated diabetic and diabetic molsidomine treated groups than unmanipulated normal group. The epidermal thickness of diabetic molsidomine treated group higher than that of unmanipulated normal and unmanipulated diabetic groups. The epidermis at the wound margin is formed of 8-10 layers in unmanipulated normal group and it is formed of 10-12 layers in unmanipulated diabetic group increasing to 12-14 layers in diabetic molsidomine treated group. There was also complete bridging of the incisions with newly synthesized epithelial cells in unmanipulated normal and diabetic molsidomine treated groups. However, the newly synthesized epithelial cells failed to bridge the wound gap in unmanipulated diabetic group (Figs.4,5,6).

Attenuation of the initial inflammatory response was observed in both unmanipulated normal and diabetic molsidomine treated groups. While in unmanipulated diabetic group, the initial inflammatory response was still prominent in the upper part of the wound (Figs.4,5,6).

In unmanipulated normal and diabetic molsidomine treated groups, the whole depth of the incision was completely filled with newly synthesized immature granulation tissue containind prolife-

rating fibroblasts, endothelial cells and loops of newly synthesized capillaries with blood cells in some of these loops. While, in unmanipulated diabetic group, the granulation tissue located mainly in the bottom of the wound gap with less number of newly synthesized capillaries than unmanipulated normal and diabetic molsidomine treated groups (13.3±2.4, 16.13±2.1 respectively) (Table 1). The number of loops of newly synthesized capillaries in the granulation tissue of molsidomine treated group was significantly higher than unmanipulated diabetic and unmanipulated normal groups ($p < 0.05$).

*** Tenth day after wounding:**

here was reduction in thickness of epidermis at the wound edge in all groups. There was also complete bridging of the incisions with newly synthesized epithelial cells in all skin sections. The new epidermis, in unmanipulated diabetic and diabetic molsidomine treated groups was thicker than unmanipulated normal. The new epidermis in diabetic molsidomine treated group was thinner than that of unmanipulated diabetic and nearly of the same thickness of epidermis at the wound edge (Figs. 7,8,9).

The wound gap was completely filled with mature granulation tissue in all groups but, the amount of newly deposited extracellular matrix was less in unmanipulated diabetic group than unmanipulated normal and diabetic molsidomine treated group. The number of newly synthesized capillaries showed significant increase in the unmanipulated diabetic group ($p < 0.05$) as compared to unmanipulated normal and diabetic molsidomine treated (45.3±13.1) (Table 1).

2- Immunohistochemistry of inducible nitric oxide synthase (iNOS):-

*** Second day after wounding:**

Skin sections of unmanipulated normal, unmanipulated diabetic, and diabetic molsidomine treated groups showed marked positive cytoplasmic (iNOS) immunoreactivity in inflammatory cells (polymorph nuclear leukocytes and macrophages) and endothelial cells at the site of incision. Also, epidermal basal and suprabasal

cells at the wound edge showed moderate positive cytoplasmic immunoreactivity, however, the leading edge showed very weak immunoreactivity in skin sections of all groups (Fig 10, 11 and 12). The immnunorectivity in epidermis at the wound edge in unmanipulated normal, unmanipulated diabetic, and diabetic molsidomine treated groups was (3.4±0.68, 7.2±1.1 and 14.6±2.1 respectively) (Table 2) showing significant increase ($p<0.05$) in diabetic molsidomine treated group as compared to unmanipulated normal and unmanipulated diabetic groups.

• Fifth day after wounding:

In unmanipulated normal group, most of newly formed epidermal cells sealing the wound showed negative (iNOS) immunoreactivity, but, some cells showed very weak positive cytoplasmic (iNOS) immunoreactivity in the form of light brown cytoplasmic granules (Fig 13). In unmanipulated diabetic group, the epidermal basal and suprabasal cells at the wound edge showed very strong positive cytoplasmic (iNOS) immunoreactivity in the form of dark brown cytoplasmic granules. In addition, epidermal cells close to the wound edge showed strong positive immunoreactivity but weaker than the wound edge (Figs 14). In diabetic molsidomine treated group, some of newly formed epidermal cells sealing the wound showed moderate positive cytoplasmic (iNOS) immunoreactivity in the form of brown cytoplasmic granules, however, others showed negative immunoreactivity (Fig 15). The immnunorectivity in neoepidermis and epidermis at the wound edge in unmanipulated normal, unmanipulated diabetic, and diabetic molsidomine treated groups was (1.7±0.27, 38.5±8.5 and 6.1±1.8 respectively) (Table 2) showing significant increase ($p<0.05$) in unmanipulated diabetic group as compared to unmanipulated normal and diabetic molsidomine treated groups.

*** Tenth day after wounding:**

In unmanipulated normal group, newly formed epidermal cells at the site of wound showed negative (iNOS) cytoplasmic immunoreactivity, however, few sparsely distributed suprabasal cells close to the wound edge

showed very weak positive (iNOS) cytoplasmic immunoreactivity in the form of cytoplasmic light brown granules (Fig 16). In unmanipulated diabetic group, most of newly formed epidermal cells at the site of wound showed negative (iNOS) cytoplasmic immunoreactivity, however, some cells mainly suprabasal neoepidermal cells showed weak positive (iNOS) cytoplasmic immunoreactivity in the form of cytoplasmic light brown granules that stronger than unmanipulated normal (-ve control) group (Fig 17). In diabetic molsidomine treated group, newly formed epidermal cells at the site of wound showed negative (iNOS) cytoplasmic immunoreactivity, however, few sparsely distributed suprabasal cells close to the wound edge showed very weak positive (iNOS) cytoplasmic immunoreactivity in the form of cytoplasmic light brown granules (Fig 18). The immnunorectivity in neoepidermis and epidermis at the wound edge in unmanipulated normal, unmanipulated diabetic, and diabetic molsidomine treated groups was (0.7±0.27, 2.67±0.57 and 0.83±0.25 respectively) (Table 2) showing significant increase ($p<0.05$) in unmanipulated diabetic group as compared to unmanipulated normal and diabetic molsidomine treated groups.

Table (1): Newly formed loops of capillaries (neovascularization).

Time Point Animal Group	5th, day unmanipulated normal (N)	5th, day unmanipulated diabetic (D)	5th, day diabetic molsidomine treated (D+M)	10th, day (N)	10th, day (D)	10th, day (D+M)
Mean	37.6	13.3	80.5	53.02 [a b]	145.07 [a b]	45.3 [a b]
± SD	4.1	2.4	8.5	6.9	20.3	13.1
P1		0.000	0.000		0.000	0.41
P2			0.000			0.000

Table (2): Inducible nitric oxide synthase (iNOS) expression..

Time Point Animal Group	2nd, day (N)	2nd, day (D)	2nd, day (D+M)	5th, day (N)	5th, day (D)	5th, day (D+M)	10th, day (N)	10th, day (D)	10th, day (D+M)
Mean	**3.4**	**7.2**	**14.6**	**1.7 [a]**	**38.5 [a]**	**6.1 [a]**	**.7 [a b]**	**2.67 [a b]**	**.83 [a b]**
± SD	**.68**	**1.1**	**2.1**	**.27**	**8.5**	**1.8**	**.27**	**.57**	**.35**
P1		**0.000**	**0.000**		**0.000**	**0.02**		**0.000**	**0.8**
P2			**0.000**			**0.000**			**0.000**

* All result are expressed as mean ± standard deviation (SD).
* Non significant : at P > 0.05.
* Significant (*) : at P < 005.
* P1 significance between unmanipulated normal (N) and unmanipulated diabetic (D) or diabetic molsidomine treated (D+M) groups.
* P2 significance between unmanipulated diabetic (D) and diabetic molsidomine treated (D+M) groups.
* a = significance between 2nd day group and 5th day or 10th groups for unmanipulated normal (N) and unmanipulated diabetic (D) or diabetic molsidomine treated (D+M) groups.
* b = significance between 5th day group and 10th groups for unmanipulated normal (N) and unmanipulated diabetic (D) or diabetic molsidomine treated (D+M) groups.

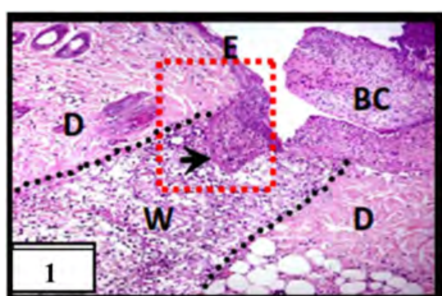

Fig. 1: Photomicrograph of a section in normal skin wound **(2nd day)** after wounding showing epidermis **(E)** and dermis **(D)** at the wound edge, fibrin network **(W)** filling the wound gap and blood clot **(BC)** and short spur of migrating keratinocytes **(short arrow)** at site of the wound (Hx&E x 100).

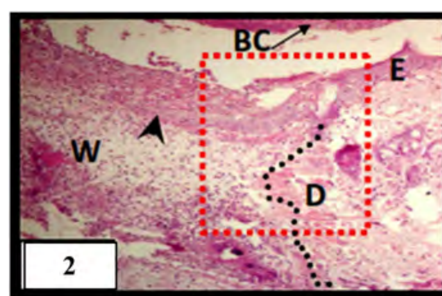

Fig. 1: Photomicrograph of a section in unmanipulated diabetic group **(2nd day)** after wounding showing epidermis **(E)** and dermis **(D)** at the wound edge, fibrin network **(W)** filling the wound gap, blood clot **(BC)** and short spur of migrating keratinocytes **(arrow head)** at site of the wound (Hx&E x 100).

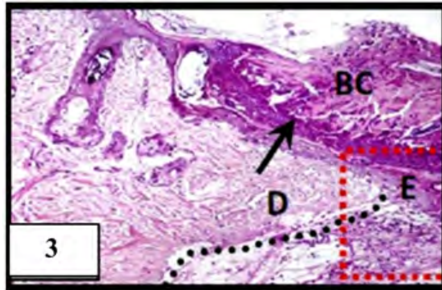

Fig. 3: Photomicrograph of a section in diabetic molsidomine treated group **(2nd day)** after wounding showing epidermis **(E)** and dermis **(D)** at the wound edge, fibrin network **(W)** filling the wound gap, dashed line between the dermis and fibrin network, blood clot **(BC)** and demarcation line formed of polymorph nuclear leukocytes **(arrow)** (Hx&E x 100).

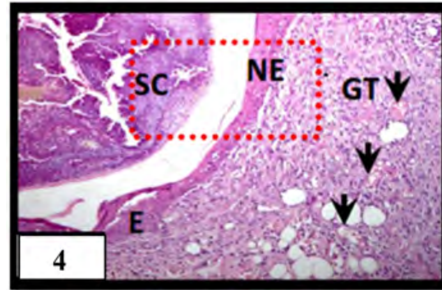

Fig. 4: Photomicrograph of a section in normal skin wound **(5th day)** after wounding showing scab **(SC)**, epidermis **(E),** dermis **(D)** at the wound edge, granulation tissue **(GT)** at the wound gap (Hx&E x 100).

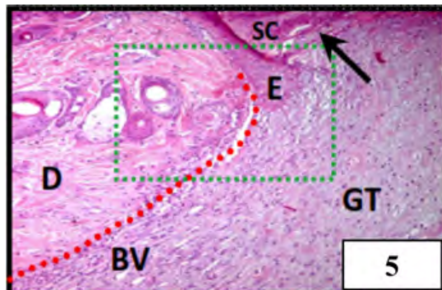

Fig. 5: Photomicrograph of a section in unmanipulated diabetic group **(5th day)** after wounding showing scab **(SC),** epidermis **(E),** dermis **(D)** at the wound edge, short spur of migrating keratinocytes **(arrow)**, granulation tissue **(GT)** filling the wound gap completely and newly formed blood vessels (BV) (Hx&E x 100).

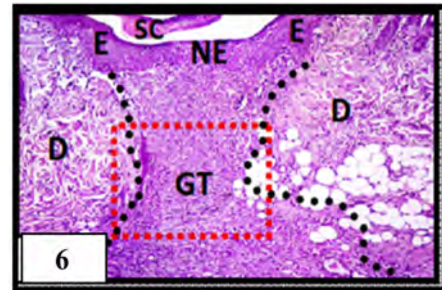

Fig. 6: Photomicrograph of a section in diabetic molsidomine treated group **(5th day)** after wounding showing epidermis **(E),** dermis **(D)** at the wound edges, scab **(SC),** newly formed keratinocytes **(NE)** and granulation tissue **(GT)** filling the wound gap (Hx&E x 100)

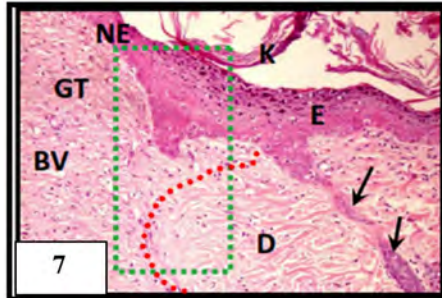

Fig. 7: Photomicrograph of a section in normal skin wound **(10th day)** after wounding showing keratin **(K)**, epidermis **(E),** epidermal extensions **(arrows)** into dermis **(D)** at the wound edge, neoepidermis **(NE)**, granulation tissue **(GT)** filling the wound gap completely and new loops of capillaries (**BV**) (Hx&E x 100).

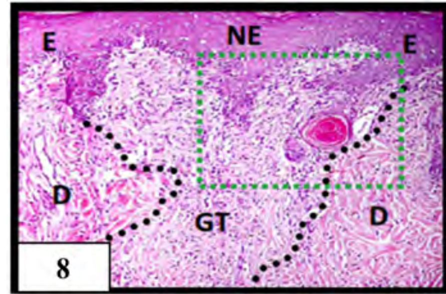

Fig. 8: Photomicrograph of a section in unmanipulated diabetic group **(10th day)** after wounding showing epidermis **(E),** dermis **(D)** at both wound edges, neoepidermis **(NE)**, granulation tissue **(GT)** filling the wound gap completely (Hx&E x 100).

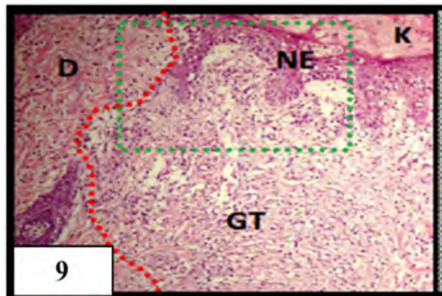

Fig. 9: Photomicrograph of a section in diabetic molsidomine treated group **(10th day)** after wounding showing keratin **(K),** dermis **(D)** at wound edge, neoepidermis (**NE),** granulation tissue **(GT)** filling the wound gap completely (Hx&E x 100).

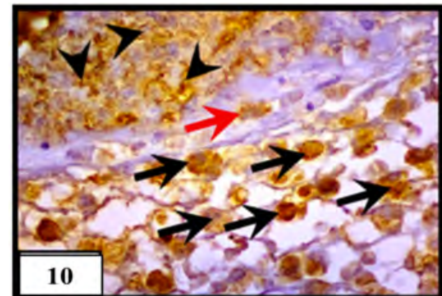

Fig. 10: photomicrograph of a section in normal skin wound **(2nd day)** after wounding showing strong positive (iNOS) immunoreactivity in polymorph nuclear leukocytes present in the demarcation line **(arrow heads)** in the form of cytoplasmic brown granules, strong positive immunoreactivity in macrophages, endotheial cells present at the site of the wound **(black arrows)** and very weak positive immunoreactivity in cell leading edge **(red arrow)** (immunohistochemical technique for iNOS with haematoxylin counterstain, x 400).

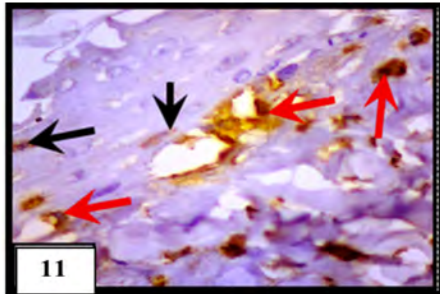

Fig. 11: photomicrograph of a section in unmanipulated diabetic group **(2nd day)** after wounding showing strong positive immunoreactivity in macrophages , endotheial cells present at the site of the wound **(red arrows) a**nd very weak positive immunoreactivity in cells of leading edge **(black arrow)** (immunohistochemical technique for iNOS with haematoxylin counterstain, x 400).

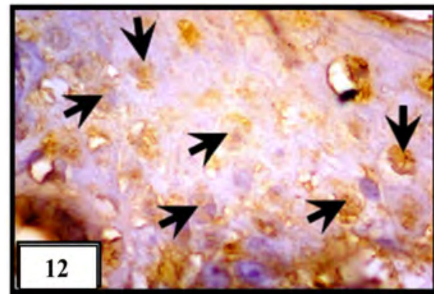

Fig. 12: photomicrograph of a section in diabetic molsidomine treated group **(2nd day)** after wounding showing strong positive (iNOS) immunoreactivity in epidermal cells in the form of cytoplasmic light brown granules **(arrows)** (immunohistochemical technique for iNOS with haematoxylin counterstain, x 400).

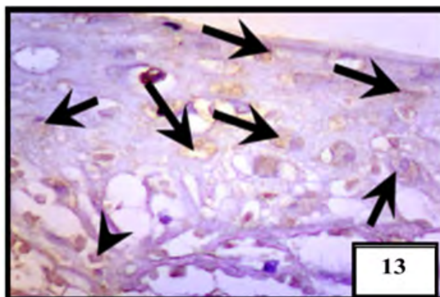

Fig. 13: photomicrograph of a section in normal skin wound **(5th day)** after wounding showing weak positive (iNOS) immunoreactivity in neoepidermal cells in the form of cytoplasmic light brown granules **(arrows)** and positive immunoreactivity in endothelial cells **(arrow head)** of newly formed loops of capillaries (immunohistochemical technique for iNOS with haematoxylin counterstain, x 400).

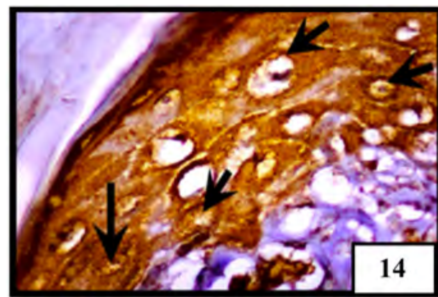

Fig. 14: photomicrograph of a section in unmanipulated diabetic group **(5th day)** after wounding showing very strong positive (iNOS) immunoreactivity in epidermal cells at the wound edge in the form of cytoplasmic dark brown granules **(arrows)** (immunohistochemical technique for iNOS with haematoxylin counterstain, x 400).

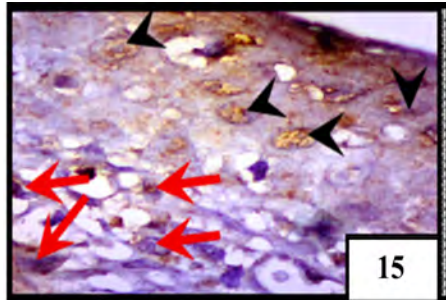

Fig. 15: photomicrograph of a section in diabetic molsidomine treated group **(5th day)** after wounding showing moderate positive (iNOS) immunoreactivity in neoepidermal cells in the form of cytoplasmic brown granules **(arrow heads)**, positive immunoreactivity in endothelial cells **(arrows)** of newly formed loops of capillaries (immunohistochemical technique for iNOS with haematoxylin counterstain, x 400).

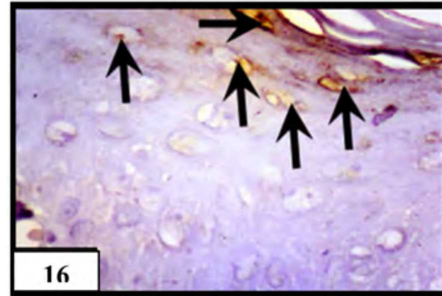

Fig. 16: photomicrograph of a section in normal skin wound **(10th day)** after wounding showing very weak positive (iNOS) immunoreactivity in neoepidermal cells in the form of cytoplasmic light brown granules **(arrows)** (immunohistochemical technique for iNOS with haematoxylin counterstain, x 400).

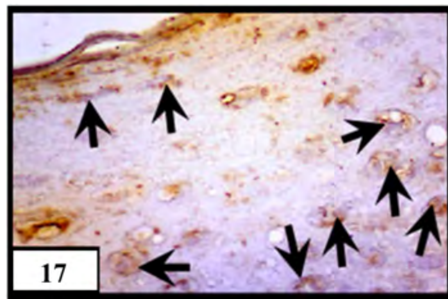

Fig. 17: photomicrograph of a section in unmanipulated diabetic group **(10th day)** after wounding showing weak positive (iNOS) immunoreactivity in neoepidermal cells in the form of cytoplasmic light brown granules **(arrows)** (immunohistochemical technique for iNOS with haematoxylin counterstain, x 400).

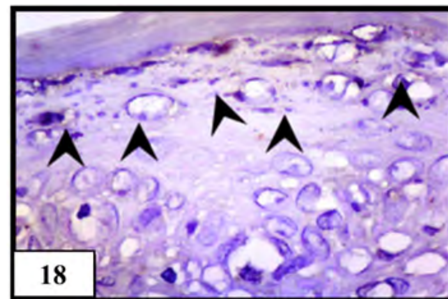

Fig. 18: photomicrograph of a section in diabetic molsidomine treated group **(10th day)** after wounding showing very weak positive (iNOS) immunoreactivity in neoepidermal cells in the form of cytoplasmic light brown granules **(arrow heads)** (immunohistochemical technique for iNOS with haematoxylin counterstain,

Discussion

Strong correlations are present between reduced cutaneous NO availability and well-documented impairments in diabetic wound healing [9,10]. The diabetic wound healing is characterized by a nitric oxide-deficient state characterized by reductions in wound breaking strength and collagen deposition and a severely impaired inflammatory process[8]. In addition, abnormalities in endothelial and inducible NOS expression have been demonstrated in diabetic animal wound tissue when compared to non-diabetic counterparts [8,20].

The present work studied the sequence of wound healing by histological and immunohistochemical methods in normal rats, unmanibulated diabetic rats and diabetic rats treated with a nitric oxide donor molsidomine to explore whether the impaired wound healing in diabetes is due to reduced endogenous (NO) production secondary to deficient (iNOS) and (eNOS) or substrate availability that can be restored by exogenous (NO) or impaired cellular responses to (NO).

In the tenth day after wounding, diabetic molsidomine treated wound was similar to unmanipulated normal (-ve control) as they showed, regeneration of the epidermis finished and the differentiation process of keratinocytes was confirmed by the normal process of the keratinization with regression in the number of fibroblasts and endothelial cells in the granulation tissue, while the amount of collagen increased. Moreover, the organization of collagen into newly formed fibrils was observed. In agreement with the finding of Witte et al. (2002)[8] who showed wound breaking strength and wound collagen content in molsidomine-treated diabetic animals, reflected by an increase in activity of matrix metalloproteinase-2, indicating that NO improved collagen remodeling capability and wound breaking strength.

In unmanipulated diabetic group, the epidermis completely regenerated by the tenth day after wounding, but still thick. It can be explained by delayed apoptosis and differentiation that need certain level of NO upon which the keratinocytes changes its behavior

from proliferation to cytostasis and begin to differentiate. Also, this hyperproliferative behavior may be a compensatory mechanism of the impaired granulation tissue deposition. In addition, increased collagen deposition at the site of the wound, is not sufficient to establish a mature matrix that will provide support to the wound but rather, occurs as compensatory attempt by the granulation tissue to overcome the healing impairment[23].

In the present work, iNOS immunohistochemical study of skin sections during the 10th day showed increased expression in unmanipulated diabetic group more than diabetic-molsidomine treated and unmanipulated normal groups as in the 5th day, but with significant increase in expression of iNOS in diabetic-molsidomine treated rat than unmanipulated normal group. It can be explained by delayed differentiation and apoptosis phase that was started in the unmanipulated normal group by the 7th day. In support with this concept, the thickness of epidermis at the site of the wound was still thicker than epidermis near to the wound which need apoptosis with inhibition of proliferation of keratinocytes that achieved by a local feedback mechanism stimulating iNOS with increased production of NO[23]. Also it can be explained in unmanipulated diabetic and diabetic ethanol treated groups by retarded process of healing. Frank et al. (1998)[24] revealed that iNOS was significantly expressed during inflammation, reepithelialization, and granulation of tissue. So with this retarded healing the expression of iNOS will remain high as in chronic ulcers[25].

It can be concolloded that the nitric oxide donor molsidomine can partially reverse impaired healing associated with diabetes.

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IMPLICATION OF NITRIC OXIDE IN WOUND HEALING IN DIABETIC RATS

Hasnaa A. Attia M.Sc., Abd El Hakim Z. Gabr MD, Fathy A. Ebrahim MD, Maha. M. Amin MD and Amany M. Shams MD

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COMBINED EFFECT OF VITAMIN E AND C (ANTIOXIDANT) ON KIDNEY OF DIABETIC RATS: HISTOLOGICAL AND ULTRA STRUCTURAL STUDY

Fathy A. Ibrahim Ph.D, Samira L. Abd Elhady Ph.D, Amany M. Shams El Din Ph.D and Medhat F.A. Taha M.Sc

Department of Anatomy & Embryology, Faculty of Medicine,

Mansoura University, Egypt

Abstract

Background: *Diabetes mellitus is a heterogeneous group of disorders characterized by high blood glucose levels. Diabetic nephropathy is one of its important complication, that consider one of the predisposing factor for chronic renal failure. Vitamin E and C are known to have antioxidant properties.*

Aim of the work: *This work is aimed to clarify the effect of combination of Vitamin C, a hydrophilic antioxidant and Vitamin E, a lypophilic antioxidant, on histological and ultrastructural changes in renal tissue of streptozotocin -induced diabetic rats.*

Material and Methods: *sixty adult male albino rats were used in these work and divided into five equal groups 12 rats were used as control group 1. 48 rats were received STZ (40 mg/kg) to establish diabetic model. The STZ-induced diabetic rat were divided into 4 groups, Group II. Streptozotocin (STZ) induced diabetic rats Group III:STZ diabetic rats received subcutaneous insulin injection of 2U/Kg/day for 2month. Group IV: STZ induced diabetic rats received vitamin E in a dose of 250 mg/kg/day and vitamin C in a dose of 1mg/gm/day for 2month.Group V: diabetic animals received SC injection of 2U/Kg and vitamin E in a dose of 250 mg/kg/day and vitamin C in a dose of 1mg/gm/day for 2month.After 2month of treatment kidney of each rat subjected for light and electron microscopic investigation.*

Results: *In control rat, renal cortex showed renal corpuscle and proximal convoluted tubule. In streptozotocin induced diabetic rats in*

Group II the lesion was mainly in renal cortex. Renal corpuscle revealed shrinkage of glomeruli with wide capsular space, while proximal convoluted tubules revealed pyknotic nuclei, degenerated desquamated cells with large number of cast were detected in the lumen and loss of apical brush border. Electron microscopic examination revealed thickening of glomerular basement membrane, widening of subpodocytic space and disruption of minor process. The nuclear membrane of podocyte was irregular and its cytoplasm showed many vacuoles. Proximal convoluted tubule showed detachment of apical part of lining cells and marked cytoplasmic vacuolation with loss of microvilli on the apical surface. The lumen contained also necrotic tissue and cast. Group III: show degenerative changes in renal corpuscle and proximal convoluted tubule less deteriorated than in diabetic group. Group IV: showed more degenerative changes in renal corpuscle and proximal convoluted tubule than in insulin treated group but less than diabetic non treated group group II Group V: showed renal corpuscle and proximal convoluted tubule more or less normal appearance.

Conclusion: *combination of vitamin E and C with glycemic control through insulin decrease degenerative changes of the diabetes on renal cortex of rats.*

Introduction

Diabetic nephropathy is one of the predisposing factor for chronic renal failure. Renal injury is observed in 35% of patients with Type I and Type II diabetes. Long-term glycemia, genetic factors, race, sex and hypertension have been implicated in the development of diabetic nephropathy[1].

Diabetic nephropathy presents itself with ischemic nephropathy, nodular glomerulosclerosis and renal failure. Clinically, 30-300 mg/day or 20-200 μg/min microalbuminuria indicates diabetic nephropathy[2]. Diabetes mellitus is an important etiopathological factor in oxidative stress[3]. As a result of lipid and protein oxidation, the levels of superoxide dismutase (SOD), glutathione (GSH-) and catalase (CAT) increase in kidneys [4].

Streptozotocin (STZ) is widely used to induce diabetes in experimental animals by causing selective destruction of pancreatic beta-cells that secret insulin[5].

Vitamin C (ascorbic acid) is also a well-known natural antioxidant[6]. Vitamin C can recycle the lipid-soluble vitamin E by reducing alpha-tocopheroxyl radicals in membranes[7]. Besides its ability to scavenge various kinds of free radicals, synergistic antioxidant effects are also present in the combinations of vitamin C with other phenolic antioxidants[8].

Vitamin E is a lipid soluble group of compounds with similar biological activity to (RRR)-atocopherol. Both vitamin E and C includes number of stereoisomers. The tocopherol is lipid soluble vitamin E. Ingested vitamin E is absorbed with lipids, packaged into chylomicrons, and transferred to the liver, after which it appears in plasma due to the expression of atocopherol transfer protein in the liver[9]. Vitamin E resides in the cell membrane of the kidney tissues where it primarily serves as a chain-breaking antioxidant to prevent lipid peroxidation. With its potent antioxidant properties, high serum vitamin E concentrations have been associated also with reduced risk of diseases like cardiovascular disease and cancer[10].

Aim of The Work

The present study was aimed to Study histological and ultra structural changes in the kidney of diabetic rat, and to clarify the protective effect of vitamin E,C on kidney of diabetic rat and also in combination with insulin therapy.

Material and Methods

A) Materials :

I. Experimental Animals:

sixty adult male albino rats (Sprague Dawly), weighting 150-200gm aging 8-10 weeks were purchased from Mansoura faculty of Pharmacy and from Mansoura experimental research center (MERC), Egypt. They were housed in stainless steel mesh cages under control condition of temperature (23ºC±3), and relative humidity throughout acclimatization and experimental periods, with ad libitum access to food and water and fixed 12:12-hours light/dark cycle.

Groups of the animals:

After 1 week of acclimatization, the rats were randomly divided into 5 groups (one control and 4 experimental groups), 12 rats each:

1) Group I (control non diabetic group) (12 rats): received single intra-peritoneal (IP) injection of an equal volume of 0.9 % NaCl (normal saline) (pH: 7.4) and olive oil daily throughout the duration of the study (8 weeks).

2) Group 2 (diabetic group) (12 rats): diabetic animals didn't receive any form of treatment throughout the duration of the study (8 weeks).

3) Group 3 (Diabetic rat received insulin) (12 rats): diabetic animals received subcutaneous injection of 2U/Kg/day of insulin mixtard (Egyptian Drug Trading Company) in 2 divided doses at 8 am and 8pm[11], throughout the duration of the study (8 weeks). The dose was modified to keep blood glucose level round (170_200) mg/dl.

4) Group 4 (Diabetic rat received vitamin E and C) (12 rats): diabetic animals received vitamin E in a dose of 250 mg/kg/day[12] and vitamin C in a dose of 1mg/gm/day[13]. Vitamin E dissolved in olive oil and injected i.p wheres vitamin C dissolved in water and given orally.

5) Group 5 (Diabetic rat received vitamin E and C with insulin) (12 rats): diabetic animals received SC injection of 2U/Kg/day of regular insulin in 2 divided doses at 8 am and 6pm, vitamin E in a dose of 250mg/kg/day and vitamin C in a dose of 1mg/gm/day. Vitamin E was dissolved in olive oil and injected i.p wheres vitamin C was dissolved in water and given orally.

At the end of the experiment, the rats were sacrified. The animals were fed on laboratory food and water, the rats were anaesthetized by using ether inhalation. In each rat, right and left kidney were dissected. The right kidney were used for light microscopic examination, while left kidney was used for electron microscopic examination to study renal cortex.

II. Staining techniques

After sacrification, fresh small pieces were obtained from kidney of each animal and fixed in a number of fixative; 10% neutral formalin or in 4%gluteraldehyde. Tissue specimens, fixed in 10% neutral formalin, were processed for preparation of paraffin sections (5-6 μm) to be stained with Hematoxylin and Eosin stains(H&E) for general histological structure[14].

Kidney specimens, fixed in 2.5% gluteraldehyde, were processed for preparation of semithin sections 60-70 nm), stained with toluidine blue and examined with electron microscope, Ultrathin sections (500_800 A) from selected areas in semithin sections were contrasted with uranyl acetate, examined by transmission electron microscope unit in Tanta university using Zeiss EM 100 S transmission electron microscope at 60 KV[15].

Results

I. Light Microscopic examination:

Hematoxylin and Eosin stains (H&E):

General histological examination of the renal cortex of control rats (group I) showed proximal convoluted tubules with narrow lumen. They were lined with cuboidal cells having acidophilic granular cytoplasm and apical brush border. The glomerulus was surrounded by Bowman's capsule having two layers separated by Bowman's space. The outer parietal layer was lined by simple squamous epithelium and inner visceral layer was lined by podocyte . The distal convoluted tubule (DCT) and collecting tubules were also shown(Fig1).

The renal cortex of STZ treated rats for (2month) (group II) revealed congested blood vessels. Glomerului revealed irregularly dilated corpuscular space. Most of proximal convoluted tubule showed degenerated sloughed lining cells and large casts in their widened lumina. The brush border was lost in most lumen of tubules (fig&2).

The renal cortex of STZ treated rats received insulin for (2month) (group III) revealed irregular widening of capsular space and few proximal convoluted tubule

showed degeneration of their lining epithelium (fig.3) these changes were less deteriorated than in diabetic group.

The renal cortex of STZ treated rats received vitamin E and C) for (2month) (group IV) revealed moderately widened capsular space. Some of proximal convoluted tubules appeared more or less normal, while others showed degeneration of their lining epithelium (fig.4) these pathological changes were more than in insulin group (group III) but less than in those of diabetic non treated rat (group II).

The renal cortex of STZ treated rats received insulin with vitamin E and C) for (2month) (group V) revealed renal corpuscle were more or less of normal appearance. Most of proximal convoluted tubules appeared normal (fig.5).

II. Electron Microscopic Examination:

Renal cortex of control rat showed renal corpuscle containing glomerular capillaries with normal basement membrane surrounded by podocytes having major and minor processes in subpodocytic space (fig.6). Also cells of proximal convoluted tubule appeared resting on abasement membrane with rounded and euchromatic nuclei, scattered mitochondria, few lysosomes, longitudinal oriented basal mitochondria, basal infolding and its luminal border showing numerous microvilli (fig.7).

The renal cortex of the rat (2month) after STZ injection (group II) showed marked thickening of glomerular basement membrane, widening of subpodocytic space. The podocyte exhibited and disruption of minor process, irregular nuclear membrane of podocyte and its cytoplasm showed many vacuoles (fig.8). Proximal convoluted tubule showed detachment of apical part of lining cells, marked cytoplasmic vacuolation and loss of microvilli on the apical surface. The lumen contained also necrotic tissue and cast (figs.9).

The renal cortex of the diabetic rats (2month) after insulin treatment (group III) showed area of thickened basement membrane. Podocytes showed lysosome and few degenerated mitochondria

(fig.10). The cells of proximal convoluted tubule showed few vacuoles (V) and lysosomes (L) in their cytoplasm (fig.11). These changes were deteriorated less in than diabetic group.

The renal cortex of the diabetic rats (2month) after vitamin E and C treatment (group IV) showed focal thickening of basement membrane with widening of subpodocytic space (fig.12). The cell lining proximal convoluted tubule showed cytoplasmic vacuolations, lysosomes. While the lumen contained cast (fig.13). These changes are more than insulin group.

The renal cortex of the diabetic rats (2month) after insulin treatment with vitamin E and C (group V) showed glomeruli retaining its normal pattern with normally appeared basement membrane and subpodocytic space(fig.14). Moreover, the proximal convoluted tubule had its normal cell lining with more or less of regular microvilli (figs.15).

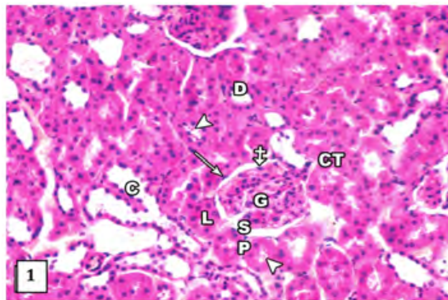

Fig. 1: A photomicrograph of a paraffin section in the renal cortex of control rat showing proximal convoluted tubules (P). With narrow lumen (L). They lined with cuboidal cells having acidophilic granular cytoplasm (CT) and apical brush border (arrow head). The glomerulus (G) is surrounded by Bowman's capsule having two layers separated by bowman's space (s). The outer parietal layer is lined by simple squamous epithelium (arrow) and inner visceral layer (crossed arrow) distal convoluted tubule (D) and collecting tubule (C) also shown. (H&E×400).

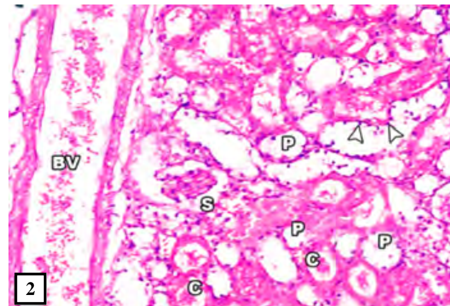

Fig. 2: A photomicrograph of a paraffin section of the renal cortex of a rat (2 month) after STZ injection showing congested blood vessels (BV). A glomerulus reveals irregularly dilated capsular space (S),while most of proximal convoluted tubule become irregular with markedly degenerated sloughed lining cells (arrows head) and large casts (C) in their widened Lumina, while brush border is lost in most lumen of tubules (P)(H&E×400).

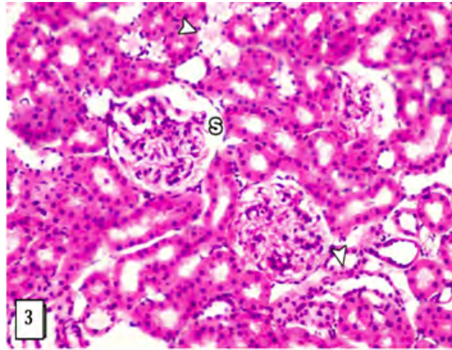

Fig. 3: A photomicrograph of a paraffin section in the renal cortex of a rat (2 months) after STZ injection receiving insulin treatment showing some glomeruli have irregular widening of capsular space (S). Few proximal convoluted tubules show degeneration of their lining epithelium (arrows head) (H&E×400).

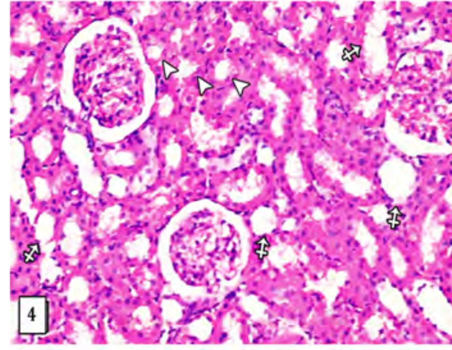

Fig. 4: A photomicrograph of a paraffin section in the renal cortex of a rat (2 month) after STZ injection receiving vitamin E and C showed The glomeruli with markedly widened capsular space (S). Some of proximal convoluted tubules (arrows head) appear more or less normal while others (crossed arrows) show degeneration of their lining epithelium (H&E×400).

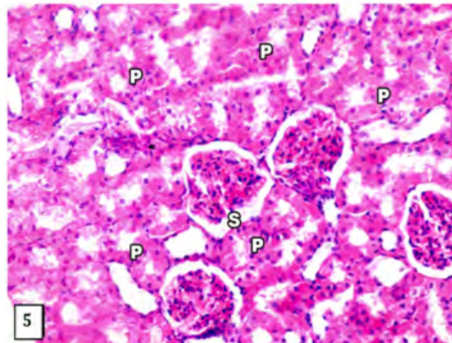

Fig. 5: A photomicrograph of a paraffin section in the renal cortex of a rat (2 month) after STZ injection receiving insulin and vitamin E and C. Showed renal corpuscle (S) are more or less of normal appearance, most of proximal convoluted tubules (P) appear normal . Compare versus fig (1) (H&E 400).

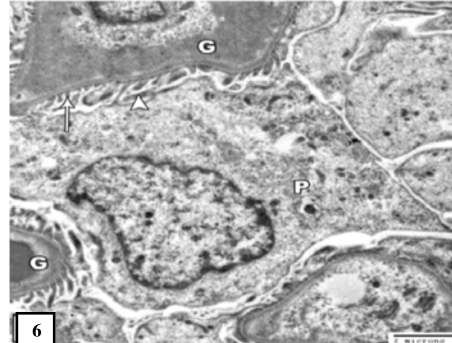

Fig. 6: An electron micrograph of ultra thin section of renal cortex of control rat showing renal corpuscle containing the glomerular capillaries (G) surrounded by normally appeard podocyte (P) with major process (arrow head) and minor process (arrow)(EM×2000).

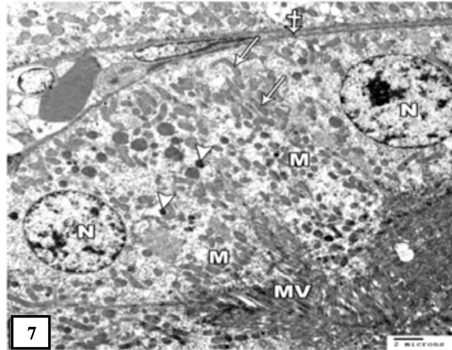

Fig. 7: An electron micrograph of ultra thin section of renal cortex of control rat. The cells of proximal convoluted tubule rest on abasement membrane (crossed arrow).They have rounded and euchromatic nuclei (N) scattered mitochondria (M) and few lysosomes (L). longitudinal oriented basal mitochondria (arrow)The luminal border shows numerous microvilli (MV) (EM×1000).

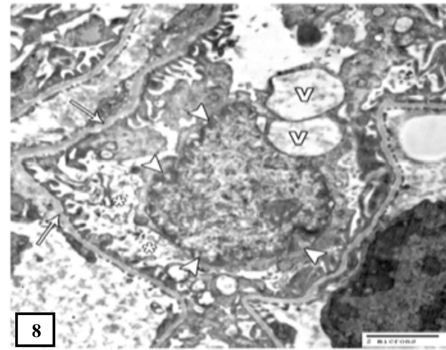

Fig. 8: An electron micrograph of ultra thin section of the renal cortex of a rat (2 month) after STZ reaction showing thickening of glomerular basement membrane (arrows), widening of subpodocytic space and disruption of minor processes (asterix). The nuclear membrane of podocyte is irregular (arrows head) and its cytoplasm shows many vacuoles (V). Compar versus fig(6) (EM×2500).

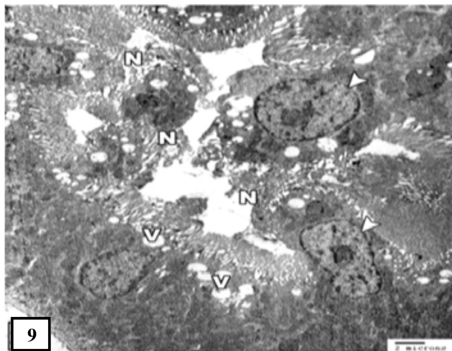

Fig. 9: An electron micrograph of ultra thin section of the renal cortex of a rat (2 month) after STZ injection. The cell of proximal convoluted tubule with detachment of apical part of lining cells (arrows head) and cytoplasmic vacuolation (V). The lumen contains also necrotic tissue (N) ((EM×1000).

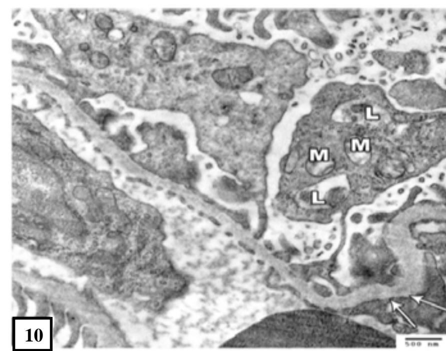

Fig. 10: An electron micrograph of ultra thin section of the renal cortex of a rat (2month) after STZ injection receiving insulin treatment showing area of thickened glomerular basement membrane (arrows). Podocyte show lysosome (L) and few degenerated mitochondria(M) (EM×5000).

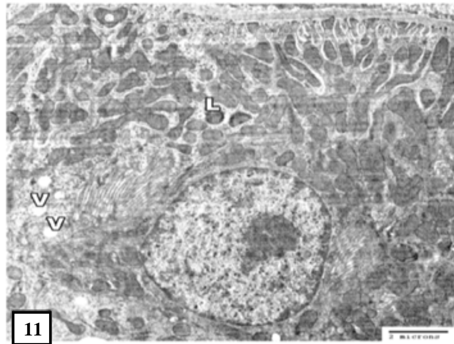

Fig. 11: An electron micrograph of ultra thin section of the renal cortex of a rat (2month) after STZ injection receiving insulin treatment showing the cell of proximal convoluted tubule exhibited few vacuoles (V) and lysosomes (L) in their cytoplasm (EMx2000).

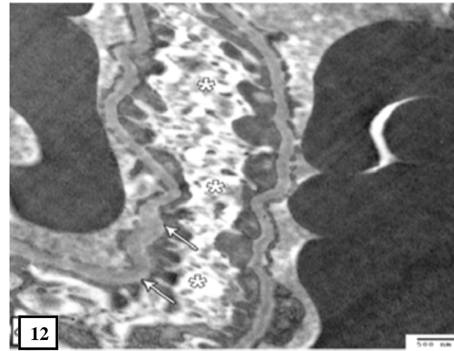

Fig. 12: An electron micrograph of ultra thin section of the renal cortex of a rat (2month) after STZ injection receiving vitamin E and C showing a focal thickening of glomerular basement membrane (arrows) with widening of subpodocytic space (asterix) (TEM&2500).

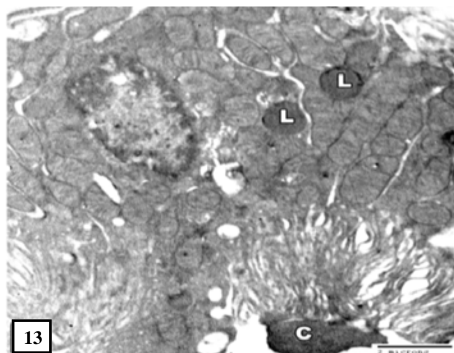

Fig. 13: An electron micrograph of ultra thin section of the renal cortex of a rat (2month) after STZ injection receiving the vitamin E and C. The cells lining proximal convoluted tubule show lysosomes (L), while lumen contain cast (C) (EMx2500).

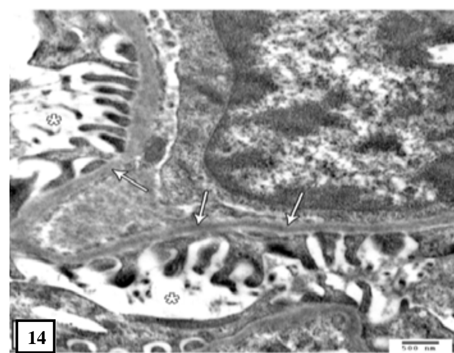

Fig. 14: An electron micrograph of ultra thin section of the renal cortex of a rat (2month) after STZ injection receiving insulin treatment with vitamin E and C. Glomerular basement membrane (arrows) and subpodocytic space (asterix) appear more or less normal. (EMx5000).

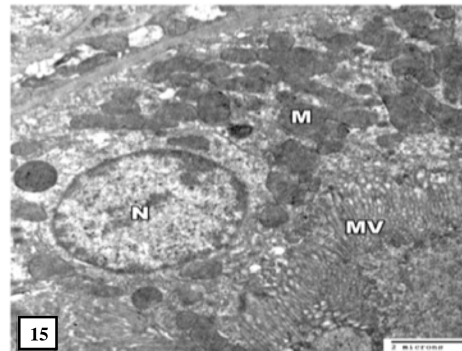

Fig. 15: An electron micrograph of ultra thin section of the renal cortex of a rat (2month) after STZ injection receiving insulin treatment with vitamin E and C. The cell lining of proximal convoluted tubule reveal more or less of normal appearance with euchromatic nuclei (N) scattered mitochondria (M) and apical microvilli (MV).Compare versus fig (8) (EM×1500).

Disscution

Diabetic nephropathy is the most common cause of chronic renal disease[16]. Many agents used to inhibit these damage have been tried in the treatment of nephropathy antioxidants are frequently used for diabetes and its complications such as vitamin E and vitamin C which used in our study in agreement with[17]. As Vitamin C plays a central role in the antioxidant protective system, protecting all lipids undergoing oxidation and diminishing the number of apoptotic cells[18&19]. Furthermore, Vitamin-C regenerates the oxidized Vitamin-E[20]. Vitamin-E, on the other hand, acts as a non-enzymatic antioxidant and reduces lipid peroxidation[21]. Vitamin-E is very effective in glycemic control, lowering the HbA1c levels[22]. This is in contrast to the results of some studies which showed that vitamin E was not beneficial in glycemic control and lipid metabolism[23]. In another study, the researchers demonstrated that a combination of vitamins C and E improved the glomerular functions but did not have any effect on the tubular functions[24].

In this study. we have five groups of rats control non treated rats, STZ induced diabetic rats, STZ induced diabetic rats received vitamin E&C, STZ induced diabetic rats received insulin, and STZ induced diabetic rats received insulin and vitamin E&C. as we study its renal cortex after 2nd month by light and electron microscopes.

In the present work, the histo-

logical structure of the renal cortex of adult control albino rats were examined with light and electron microscopes, revealed similar structures as those mentioned by many authors[25].

Examination of frozen sections of control rats 8weeks after STZ injection in The current study demonstrate effect of diabetes on proximal convoluted tubules cells and podocytes of the glomeruli[26]. As glomeruli show widening of capsular space due to shrunken glomeruli while proximal convoluted tubule showed wide lumen due to damage of brush border, lining cell revealing pyknotic nuclei, degenerated desquamated cells detected with heamatoxylin and eosin stain. The result of present study in agreement with[27] As they stated that there was widened capsular space, degeneration and necrosis of renal tubular epithelia. As Oxidative stress and free oxygen radicals, which develop during nephropathy, trigger apoptosis of the tubular epithelial cells and podocytes of the glomeruli[28].

Electron microscopic examination of the renal cortex reveal thickened glomerular basement membrane surrounded by podocytes with disrupted minor processes in subpodocytic space while the cells of proximal convoluted tubules showed disturbed microvilli, vacuolation in the cytoplasm, primary and secondary lysosomes and degenerated mitochondria with sheded apical cells and large casts in their lumen.

The result of this study consistent with that of[29] as they reported increase in GBM thickness in diabetic nephropathy, due to increase in collagen type IV deposition and impairment of excess extracellular matrix degradation, and injury of podocyte with minor process degeneration due to oxidative stress, which increase intracellular (reactive oxygen species) ROS which mediate apoptosis of podocytes. In addition that some proximal tubular cells die and slough into the tubular lumen and contributes to cast formation with vacuolation in its cytoplasm.

The present work demonstrated that the renal cortex of experimen-

tal rats treated with vitamin E and C for (group 4) 1 month without glycemic control with insulin treatment results in apoptosis of glomeruli and necrosis of proximal tubular cells but these changes are less than STZ induced diabetic rats (group 2).

The result of this study disagree with[30]. Which stated that neither apoptosis of the podocytes nor thickening of the basement membrane was observed after treatment with vitamin E and C for 3weeks, as vitamin E and C helped alleviation of the renal degeneration by protecting the glomerular structures from oxidative injury, with no effect on tubular damage.

Treatment of STZ induced diabetic rats with insulin and glycemic control without treatment with antioxidants (vitamin E and C) (group 3) showing histological, ultrastructural and changes, however these changes less than STZ induced diabetic group (group 2) and STZ induced diabetic group received vitamin E&C (group 4)but more than STZ induced diabetic group received insulin and vitamin E&C (group 5) as some glomeruli show improvement and some proximal convoluted tubule show preserved brush border and small number of vacuolations and lysosomes in its cytoplasm.

The result of this study agree with[11]. Which stated that, continuous subcutaneous insulin therapy improving but did not normalize the hyperglycemia, delaying progression of diabetic nephropathy arresting proteinuria and glomerular basement membrane thickening in diabetic rats with reducing tubular epithelial cells apoptosis.

In the present study treatment of STZ induced diabetic rats with insulin and glycemic control treated with antioxidants (vitamin E and C) (group 5) showing improvement in renal corpuscles and its capsular space near normal with near normal thickness of basement membrane while most of proximal convoluted tubules show preserved brush border and its cells cytoplasm showing normal euchromatic nuclei and scattered mitochondria with apical microvilli like normal.

The result of this study agree with[31]. Which stated that, combination of vitamins (C and E) has advantage over insulin therapy on lipid peroxidation, antioxidant activity, histological changes in the liver of diabetic rats. Also the enzymatic activities of glutathione (GSH), superoxide dismutase (SOD), and the lipid peroxidation product, thiobarbituric acid-reacting substances (TBARS) which measured in liver and pancreas as indicators of antioxidation in these tissues showing improvement.

Conclusion and recommendation

From the present study we conclude that the structure of the renal cortex was affected by diabetes. Also, these study proved that vitamin E and C with glycemic control were more effective in prevention of these structural changes.

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BENHA MEDICAL JOURNAL

COMBINED EFFECT OF VITAMIN E AND C (ANTIOXIDANT) ON KIDNEY OF DIABETIC RATS: HISTOLOGICAL AND ULTRA STRUCTURAL STUDY

Fathy A. Ibrahim Ph.D, Samira L. Abd Elhady Ph.D, Amany M. Shams El Din Ph.D and Medhat F.A. Taha M.Sc

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A LIGHT AND SCANNING ELECTRON MICROSCOPE STUDY OF THE EFFECT OF PNEUMOPERITONEUM ON THE ALBINO RAT JEJUNUM

Gamal A. Mohamed Ph.D, Wael M. Elsaed Ph.D and Ahmed M. Desouky Ph.D*

Departments of Anatomy, Faculties of Medicine,

Mansoura and Ain Shams Universities, Egypt*

Abstract

Background: *Laparoscopic surgical techniques have been increasingly preferred to classic laparotomy. These procedures offer a range of advantages compared with conventional open techniques but require a pneumoperitoneum (Pp) for adequate visualization and exposure of the of intra-abdominal organs to be operated upon.*

Aim of the work: *The goal of the current investigation was to shed light on the histological, morphometric and immunohistochemical alterations in the albino rat jejunum after induced CO2 Pp utilizing both the light and scanning electron microscopes.*

Materials and Methods: *Thirty adult male albino rats (240–250g) were utilized in the existing study, allocated into two equal groups: control and experimental. All the rats were anesthetized by xylazine hydrochloride, combined with ketamine hydrochloride, intramuscularly. The rats of experimental group were placed in a supine position. A Veress needle was placed supraumbilically into the peritoneal cavity to induce Pp by insufflating CO2 manually up to 20 mm Hg and maintained constant for 60 minutes using an 18F Abocath connected to a mercury pressure gauge. The rats of the other group served as controls. They were subjected to sham operation. After 7 days postoperatively, under the same form of anaesthesia, the rats of the two groups were fixed in supine position, the skin was incised along the ventral midline. A jejunal segment from each rat was cleared of the residual contents. Jejunal*

paraffin sections (6 microns) were prepared and stained with Hx&E . For the detection of p53 expression in parrafin sections, p53 Clone sp5 for primary antibody and a horse radish peroxidase (HRP) kit for secondary antibody and DAB (diamino benzidine)) for chromogene were used. Other fine fragments were prepared for examination with scanning electron microscope.

Results: *The apical parts of some jejunal villi of the experimental animals ended with small knobs or took various shapes: tapering, broad or round. Villi got short or even fused with adjacent ones with areas of loss of the covering epithelium. The brush border and basement membrane of some columnar epithelium were distorted. The goblet cell number apparently increased. Extensive epithelial separations from the lamina propria down the sides of the villi. The villous core (lamina propria) lodged a considerable cellular infiltration but near the villous tips, remarkable diminution in connective tissue elements together with appearance of apparently wide spaces. Noticeable cellular infiltration existed also in the submucosa. The musculosa ,more or less, exhibited insignificant decrease in thickness as compared to that of the control group. Significant jejunal morphometric changes were encountered in the experimental group compared to the control one. The scanning electron microscopic examination of the mucosal surface of the jejuum of the experimental albino rats, in our study, showed manifest degenerative alterations. A positive immune reaction to p53 protein mainly in the lamina propria and epithelium of jejunal villi and crypts was detected in the same group.*

Conclusion: *Based on the previous data, it can be concluded that Pp imparted its hazardous afflictions on the rat jejunum. Further studies with different Pp pressure regimens on a larger number of animals over longer durations are recommended.*

Key words: *Jejunum, pneumoperitoneum, rat, light and scanning electron microscope.*

Introduction

Jejunum, as a part of the small intestine, is the site of terminal food digestion. It is also constituted for absorption of nutrients. This function is enhanced by several structural devices responsible for increasing the total intestinal surface area compared to the simple flat intestinal surface, i.e. without these structural devices. These are the plicae circulares, the myriad villi and the microvilli on the epithelial cells[1,2]. Another important function of the small intestine is the transport of its luminal content to the colon. Cells in the epithelial lining produce mucus for lubrication and protection.[3]

Laparoscopic surgical techniques have been increasingly preferred in many therapeutic and diagnostic procedure to classic laparotomy by surgeons since 1987. These procedures have gained wide acceptance in recent years because they offer a range of advantages compared with the conventional open techniques. The laparoscopic method allows the performance of procedures with less pain, a shorter hospital stay, and avoidance of larger abdominal incisions as well as extensive intra-abdominal dissections and minimization of the postoperative complications, such as wound infections, postoperative gastrointestinal atony, wound dehiscence, and intra-peritoneal abscesses. Additionally, the patients who accept laparoscopic operation can recover with a shorter healing time and less operative scars [4,5,6,7,8]. This minimally invasive procedure generally requires Pp for adequate visualization and exposure of the of intra-abdominal organs to be operated upon and to lift the abdominal wall. Many gases have been used for the creation of Pp [9,10]. Currently, CO2 is the most widely used gas for insufflation due to its easy availability, low cost, nonflammabity, chemical stability, and high diffusion capacity with subsequent rapid absorption and excretion, therefore, poses a lower risk of gas embolism [11,12,13]. However, during Pp exposure of organs is always not enough. Also, with the growing number of minimally invasive procedures, concern arises about the potential detrimental effects, adverse alterations and metabolic,

inflammatory, infectious as well as ischemic consequences of Pp on body organs[14,15]. The process, according to some researchers, is analogous to, although less marked than, abdominal compartment syndrome. Despite much research, the consequences of Pp and the mechanisms implicated are not fully elucidated or understood[16,17,18].

Reviewing the available literature, a relative deficiency of comprehensive studies of the influence of Pp on the rat jejunum was observed. Consequently, the notion of the current investigation arose and it was conducted with an intent to shed light on the histological, morphometric and immunohistochemical alterations in the albino rat jejunum after induced CO2-Pp utilizing both the light and scanning electron microscopes.

Materials and Methods

Animals:

Thirty adult male albino rats (240–250g) were obtained from Hellwan Breeding Farm, Ministry of Health, Egypt, to be utilized in the current investigation.

Experimental design:

The rats were randomly alloted into two equal groups: control and experimental. The animals were handled in accordance with guidelines of animal welfare prior to the experiment. They were fed on standard rat chow and water ad-libitum. They were maintained in their respective groups for 20 days before the commencement of the experimental procedure. They were housed in cages with good ventilation at a controlled ambient temperature of 25±2°C with 50±10% relative humidity as well as a 12 h light/12 h dark cycle. The animal had been denied access to food for 12 h, while potable water was still made available ad libitum. Body weight was measured as a mirror of well being and of bowel function. All the rats were anesthetized with xylazine hydrochloride at a dose of 10 mg/kg of body weight, combined with ketamine hydrochloride (Ketalar; Parke Davis, Morris Plains, NJ) at a dose of 75 mg/kg of body weight, intramuscularly. Adequate anesthesia was verified by the absence of withdrawal reflex to painful pinch stimuli. During the procedure, additional doses were

administered if necessary[19]. The rats of experimental group were placed in a supine position.A Veress needle was placed supraumbilically into the peritoneal cavity to induce Pp under sterile conditions using an 18F Abocath connected to a mercury pressure gauge (Insufflator Duo Lab; Carl Zeiss, Oberkochen, Germany). The intra-abdominal pressure was elevated by insufflating CO2 manually up to 20 mm Hg and maintained constant for 60 minutes. All connections in the system were carefully checked. Also, the site of peritoneal cavity puncture was supported by histoacryl tissue glue to prevent air leakage, documented by abdominal pressure monitoring. After 60 minutes, CO2 insufflation was ceased and desufflation was applied. The rats of the control group served as controls. They were subjected to sham operation ie, similarly instrumented, but no Pp was created. The rats of the two groups were then left to recover from the anesthesia. They were allowed food and water and kept alive for the ensuing 7 days postoperatively[20,21]. Before surgical intervention, animals were weighed and under the same form of anaesthesia the rats of the two groups were fixed in supine position, with adhesive tape, at their thoracic and abdominal limbs on a sterile operation field. Shaving of the ventral body wall was performed, and the abdomen was cleaned with 70% alcohol and dried with gauze for skin antisepsis. Using sterile instruments, the skin was incised along the ventral midline and the chest was exposed. Through the wall of the left ventricle, a cannula was inserted, the right auricle was cut to allow drainage and vascular perfusion was begun. About 100 ml of 100mM phosphate-buffered saline (PBS) (pH 7.3) were injected via syringe and under manual pressure, through the cannula into the ascending aorta. The abdominal cavity was then opened with a midline incision and the small intestine was excised and immersed in PBS. A 10 cm long jejunal segment from just distal the duodeno-jejunal flexure of the experimental and control rats were cleared of their residual contents by injection of PBS into the lumen then draining it. Each segment was cut into two equal loops, which were isolated with cotton

thread ligature at both ends and distended by injecting fluid into the lumen through a very fine needle piercing the wall at an acute angle[22].

Histological study:

Light microscopy: Small pieces of the jejunum of the control and experimental animals were fixed in10% neutral formalin, dehydrated, cleared and embedded in paraffin wax. Paraffin sections (6 microns) were prepared and stained with haematoxylin and eosin (Hx&E)[23]. Sections were viewed by a light microscope (Nikon Eclipse N600, Japan) without knowing the group to which each animal belonged. Mucosal lesions were evaluated and scored in grades (G) as described by Chiu et al.[24]. G0:normal epithelium and lamina propria, G1: occasional epithelial injury and subepithelial space at villus tip, G2: the majority of villus tip epithelium is injured together with a subepethelial space, G3: massive lifting of villus and crypt of epithelium, G4: denuded villi and G5: ulceration, disintegration and haemorrhage in the lamina propria.

Scanning Electron Microscopy (SEM): Rectangular specimens of jejunum about 1mm in thickness and 3-8 mm in dimensions were fixed with 2.5% (wt/vol) sodium cacodylate-buffered glutaraldehyde, pH 7.2 at 4°C for 2 h. Samples also post-fixed in 1% sodium cacodylate-buffered osmium tetroxide, pH 7.2 at 4°C for 2 h. After washing and dehydration in ascending grades of ethanol, critical-point drying was performed using the Emitech-K850 critical-point drying unit. The samples were mounted on aluminum stubs with double sided tape and silver glue and then sputter coated with gold by Boc Edwards Scancoat[25]. The specimens were observed using a Jeol field emission scanning electron microscope JSM-6390LV.

Statistical study: Measurement of the villous length (from the tip to base), the width of jejunal villi at the base, height of enterocytes (from the surface to the base passing through the nucleus), the diameter of the intestinal glands (crypt lumen) as well as the thickness of the muscle layer was performed using a Leitz micrometer eye piece, on 10 randomly se-

lected sections for each animal. Density of the jejunal villi/mm^2 and also the height of the micro-villi and their density/μm^2 were estimated on scanning electron micrographs. The measurements obtained from the experimental and control animals were expressed as mean +SEM. Data were collected, tabulated and statistically analyzed using "Statistical Package for the Social Sciences (SPSS) version 15.0 program (SSPS, Inc., Chicago). The measurements were subjected to the test for significance. The paired Student's t test was used for the statistical evaluation. A P-value <0.05 was accepted as statistically significant according to the formula of Daniel[26].

Immunohistochemical study: For detecting apoptosis on histologic sections, the most common and reliable method is called TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling). The biotin-labeled cleavage sites are then detected by reaction with horse radish peroxidase (HRP)-conjugated streptavidin and visualized by DAB (diamino benzidine) showing brown color. We used a TUNEL-based apoptosis kit (Fragel DNA fragmentation kit; Calbiochem, Darmstadt, Germany). The prepared 3 Poly-L-lysine-coated slides were stained with Fragel. For the detection of p53 expression in parafin jejunal sections, p53 Clone sp5 (Labvision, Biogen) for primary antibody and a HRP kit (Labvision, Biogen) for secondary antibody and DAB (diamino benzidine)) for chromogene were used. The positive staining of P53 protein is expressed as brown granules[27].

Results

All utilized rats survived throughout the entire period of the present study. The mean body weight of the control rats and that of the experimental ones at the end of the 7th postoperative day was 246±0.92 and 225.9±0.95 g, respectively. Thus a significant diminution of body weight was noticed (P<0.05) (Table 1).

Haematoxylin and eosin stained paraffin sections of the jejunum of the control albino rats showed the normal architecture being formed of the traditional

four layers of intestine: mucosa, submucosa, musculosa and serosa. The mucous membrane showed long slender intestinal villi that contained a connective tissue core and were covered with columnar epithelium which possessed oval basal nuclei, rested on intact basement membrane and had a vivid apical (luminal) brush border and goblet cells; G0 according to Chiu et al. scoring (Plate A: Figs.1,2,).

The jejunum of the experimental animals, in the current investigation, after the 7th postoperative day showed that apical parts of some villi ended with small knobs or took various shapes: tapering, broad, round, short or even fused with adjacent villi with areas of loss of the covering epithelium entirely or leaving the ghosts of cells. The brush border and basement membrane of some columnar epithelium were distorted. The goblet cell number in the villous and crypt epithelium apparently increased with extensive epithelial separations from the lamina propria down the sides of the villi. The villous core (lamina propria) lodged a considerable cellular infiltration but near the villous tips, remarkable diminution in cellularity and connective tissue elements together with appearance of apparently wide empty spaces, G1, 2 or 3 according to Chiu et al. scoring. Noticeable cellular infiltration existed also in the submucosa. The musculosa, exhibited insignificant decrease in thickness as compared to that of the control group (Plate B: Figs.3,4,5,6 and Plate C: Figs. 7,8,9). The mean villous length and basal width of the control rats, in the present work, were 457.3±1.25 and 96.71±0.58 µm, respectively, while their respective values in the experimental animals became 307.3±1.25 and 80.17±0.65 µm. Thus significant changes were achieved ($P<0.05$). The mean height of the jejunal columnar absorptive cells of the control and experimental groups recorded a significant variation ($P<0.05$) and were 27.08± 0.12 and 26.21±0.10 respectively. The mean diameter of the crypts and the mean thickness of the muscle layer of the control group were 63.93±0.13and 53.73±0.14 µm respectively; in turn, their corresponding values in the experimental group were 50.19±0.19 and

53.61±0.14 μm recording significant alterations ($P<0.05$) (Table 1).

The scanning electron microscopic examination of the mucosal surface of the jejunum of the control rat revealed that it is thrown into numerous, more or less, similarly-looking and regularly-arranged villi separated by deep grooves. The villous surface possessed an even smooth and intact epithelial surface. Mucous plugs could be seen at the top of some cells (representing goblet cell craters) (Plate D: Figs.10,11,12). The mean density of the jejunal villi and microvilli were 13.89± 0.09/mm2 and 76.17±0.25/μm2, respectively. The mean height of these microvilli was1.099±0.01 μm (Table1).

The scanning electron microscopic examination of the mucosal surface of the jejuum of the experimental albino rat after the 7th postoperative day, in our study, showed a more pebbled mucosal surface which was more frequently broken by small orifices of goblet cells that might or mostly might not extrude mucus as well as intestinal debris. Focal detachment of some apical epithelial cells was detected .A fractured columnar absorptive epithelial cell offered a side view of the microvilli which showed focal irregular orientation (Plate E:Figs.13,14,15,16). The mean density of the jejunal villi and microvilli of this experimental group showed significant alterations ($P<0.05$) eliciting 18.23±0.34/μm2 and 73.59±0.29 /μm2, respectively. The height of these microvilli measured 1.095± 0.01 μm (Table1).

The jejunum of rats of the control group showed a negative immune reaction to p53 protein. However, The jejunum of rats of the experimental group showed a positive immune reaction to p53 protein mainly in the lamina propria and epithelium of intestinal villi and crypts detected by peroxidase DAB as brown color reaction (Plate F:Figs.17,18,19).

Table (1): Comparison of data from the control(sham-operated) and experimental animals.

	Control	Experimental
Body weight (g)		
Mean	246	225.9
SEM	0.92	0.95
P		<0.05
Length of jejunal villi (μm)		
Mean	457.3	307.3
SEM	1.25	1.25
P		<0.05
Width of the base of the jejunal villi (μm)		
Mean	96.71	80.17
SEM	0.58	0.65
P		<0.05
Density of the jejunal villi (/mm^2)		
Mean	13.89	18.23
SEM	0.09	0.34
P		<0.05
Height of the columnar cells (μm)		
Mean	27.08	26.21
SEM	0.12	0.10
P		<0.05
Height of the microvilli (μm)		
Mean	1.099	1.095
SEM	0.01	0.01
P		>0.05
Density of the microvilli (/μm^2)		
Mean	76.17	73.59
SEM	0.25	0.29
P		<0.05
Diameter of the crypts (μm)		
Mean	63.93	50.19
SEM	0.13	0.19
P		<0.05
Thickness of the muscle layer (μm)		
Mean	53.73	53.61
SEM	0.14	0.14
P		>0.05

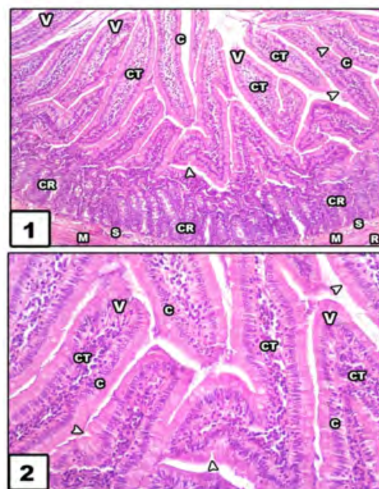


Plate A:Fig.1: A photomicrograph of a paraffin section of the jejuum of the control albino rat showing long slender intestinal villi (V) that contain a connective tissue core (CT) and are covered with columnar epithelium (C) and goblet cells (head arrow). The submucosa (S), the musculosa (M) and the seosa (R) are seen (Hx,E x40). **Fig.2:** A higher magnification of Fig.(1)showing the intestinal villi (V) that contain connective tissue core (CT) and are covered with columnar epithelium(C) with oval basal nuclei and a vivid apical (luminal) brush border. Goblet cells (head arrow)could be seen interposed amongist the columnar cells (Hx,E x100).

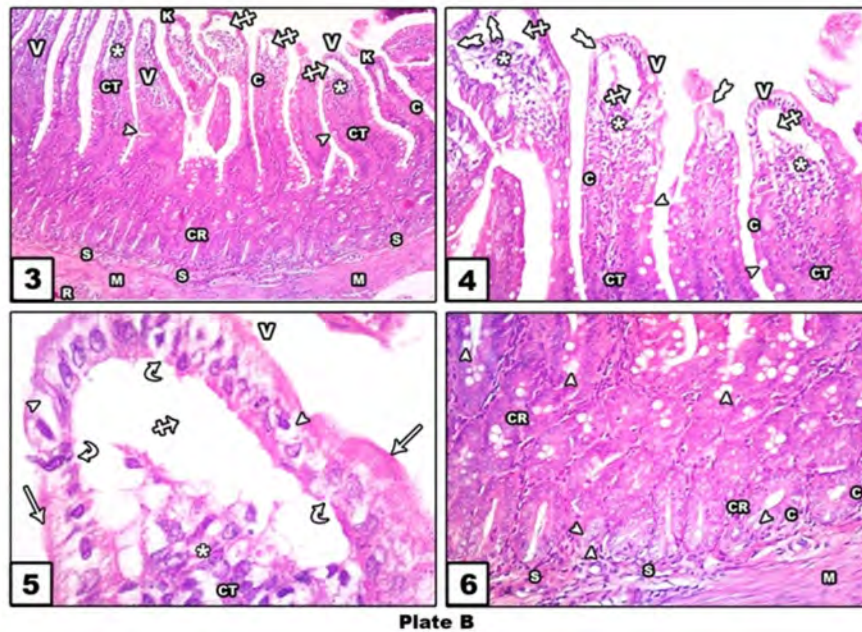


Plate B

Plate B: Fig.3: A photomicrograph of a paraffin section of the jejunum of the experimental albino rat showing slender intestinal villi (V) that contain degenerated connective tissue core (CT) and are covered with columnar epithelium (C) and goblet cells (head arrow). The apical parts of some villi end with small knobs (K). Goblet cell number apparently increases in the villous and crypt (CR) epithelium with appearance of small subepithelial spaces (crossed arrows). The lamina propria lodges considerable cellular infiltration (asterix) but exhibits diminished cellularity and reduced connective tissue elements and empty spaces near the villous tips. The submucosa (S), themusculosa (M) and the seosa (R) are seen (Hx&E, x40). **Fig.4:** A higher magnification of Fig.(3) showing the apical parts ofjejunal villi.Focal loss of the epithelium is encountered (tailed arrow) (Hx&E, x100). **Fig.5:** A further higher magnification of Fig.(3)demonstrating the apical part of a jejunal villus (V). The brush border (arrow) and basement membrane (curved arrow)of some columnar cells aredistorted. The villus core lodges cellular infiltration (asterix) and apparently wide empty spaces (crossed arrow) (Hx,Ex250). **Fig.6:** A high magnification of Fig.(3) showing that the intestinal crypts (CR) are lined with columnar epithelium (C) and goblet cells (head arrow). Noticeable cellular infiltration existed also in the submucosa (S) (Hx,E x100).

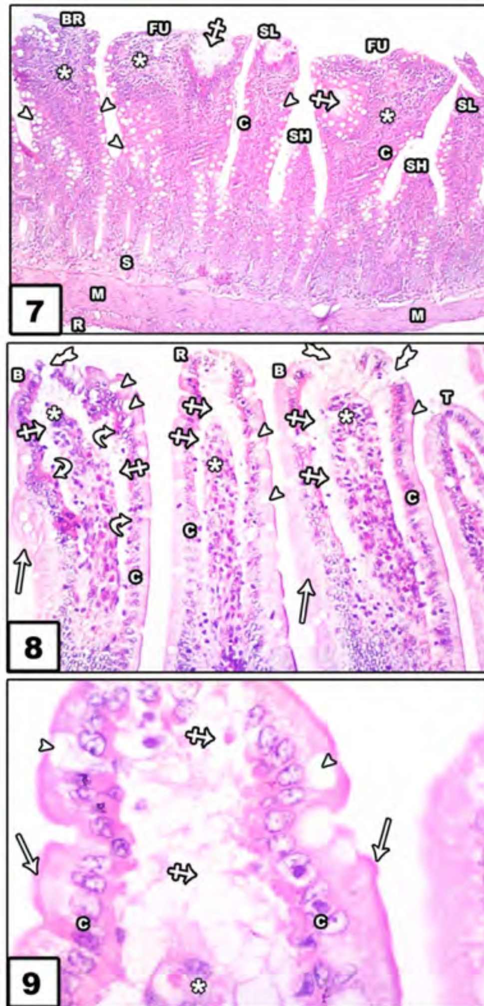


Plate C

Plate C :Fig.7: A photomicrograph of a paraffin section of the jejunum of the experimental albino rat showing variation in the shape of intestinal villi (V): slender (SL), broad(BR),short (SH)or fusedwith adjacent villi (FU). Goblet cell (head arrow) number apparently increases in the villous and crypt epithelium . The lamina propria lodges considerable cellular infiltration (asterix). The submucosa (S), the musculosa (M) and the seosa (R) are seen (Hx&E, x40). **Fig.8:** A photomicrograph of a paraffin section of the jejunum of the experimental albino rat showing the apical parts of some jejunal villi which look tapering(T), broad (B) or round (R). Extensive epithelial separations (crossed arrows) from the lamina propria (asterix) down the sides of the villi and focal loss of the epithelium at the tips are encountered (tailed arrow) entirely or leaving the ghosts of cells. The remaining parts of the villi show intact basement membrane (arrow) with overlying columnar epithelium (C) and apparently numerous goblet cells (head arrow) in between. (Hx&E, x100). **Fig.9:** A higher magnification of Fig.(8) showing the apical part of a villus with subepithelial empty spaces (crossed arrow) (Hx&E, x250).

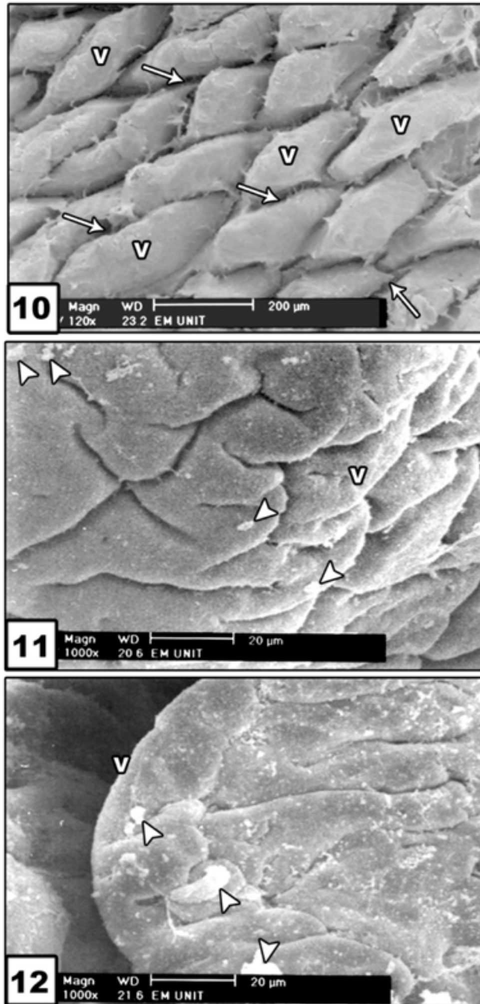


Plate D

Plate D: Fig.10: A scanning electron micrograph of the mucosal surface of an albino rat jejunum of the control group showing numerous, more or less,similarly-looking and regularly-arranged villi (V) separated by deep grooves (arrows) (scale bar 20 µm). **Figs.11,12:** Scanning electron micrographs of the mucosal surface of the jejunum of an albino rat of the control group showing the villus surface (V) with a smooth and intact epithelial surface. Mucous plugs seen at the top of some cells (representing goblet cell craters) (head arrow) (scale bar 20 µm).

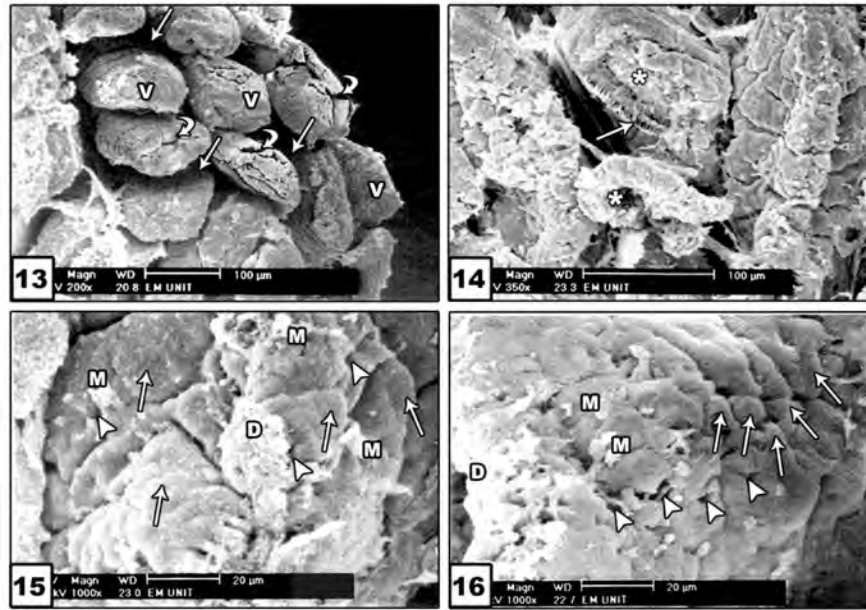
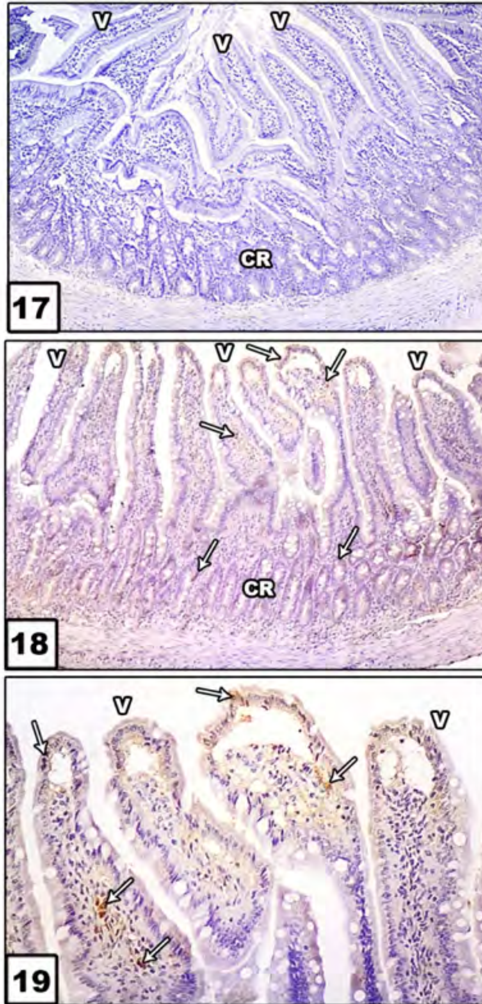


Plate E: Fig.13: A scanning electron micrograph of the mucosal surface of the of the jejunum of the experimental albino rat showing irregulaly arranged villi(V), with detachment of parts of the covering epithelium (curved arrow), separated by grooves (arrow) (scale bar 100 µm). **Fig.14:** A scanning electron micrograph of the mucosal surface of the of the jejunum of the experimental albino rat showing focal detachment of some apical epithelial cells offering a side view of the mircovilli (arrows) and leaving the core of the villus exposed (asterix) (scale bar 100 µm). **Figs.15,16:** Scanning electron micrographs of the mucosal surface of the of the jejunum of the experimental albino rat showing a more pebbled mucosal surface (arrows) which is more frequently broken by small orifices of several goblet cells (head arrow) that may be seen extruding mucus or mostly not - as well as intestinal debris(D) (scale bar 20 µm).



Pate F:Fig.17: A photomicrograph of the jejunum of a rat of the control group showing a negative immune reaction to p53 (Peroxidase DAB method x100). **Fig.18:** A photomicrograph of anti-p53 stained section of the jejunum of a rat of the experimental group showing a positive immune reaction (arrow) mainly in the lamina propriaand epithelium of intestinal villi (V) and crypts (CR) detected by peroxidase DAB positive brown color reaction (Peroxidase DAB methodx100). **Fig.19:** A higher magnification of the previous photomicrograph of anti-p53 stained section of the jejunum of a rat of the experimental group showing a positive immune reaction (arrow) mainly in the lamina propria and epithelial covering of intestinal villi (V) as brown color reaction (Peroxidase DAB method x250).

Discussion

New diagnostic tools have been developed after the advent of laparoscopy. This attractive approach offers many advantages when compared with open surgery and substantially increased the number of minimally invasive techniques. In addition to the benefits of this new approach, several questions have been raised about the possible secondary consequences of gas insufflation and the resulting higher intra-peritoneal pressures, as it is usually performed in a different environment, the pneumoperitoneum (Pp) most commonly created by carbon dioxide (CO2)[28]. The current study has been performed to evaluate the histological, morphometric and immuno-histochemical alterations in the albino rat jejunum after induction of Pp utilizing both the light and scanning electron microscopes.

The mean body weight of the experimental rats, at the end of the 7th postoperative day, in the existing study, significantly decreased as compared to that of the control animals. This could be attributed the possible impaired bowel function following the induction of Pp since the mucosal affection could result in defective absorption and excessive loss of nutrients. Our findings came in accord with those of Farias et al.[29] but contradicted those obtained by Bouvy et al.[30] who denied any significant change in the body weight of the rats of different groups. This controversy could be attributed to differences in the experimental animals utilized and/ or in the technique employed.

Pp, in the current investigation, induced jejunal damage revealed by the altered histoarchitecture. The apical parts of some villi terminated with small knobs or attained various shapes. In addition, villi became short or even fused with areas of loss of the covering epithelium either entirely or leaving the ghosts of cells. The brush border and basement membrane of some columnar epithelium were distorted. Extensive epithelial separations from the lamina propria with appearance of wide spaces near villous tip extending down to the sides of the villi were detected. The lamina propria and submucosa lodged a

considerable cellular infiltration.

The over-all scene of the jejunal ultrastructure, in our study, documented that there were evident degenerative changes confirming those detected by the light microscope. These alterations could be owed to introduction of CO2 under pressure into the peritoneal cavity which might cause reduction in visceral perfusion and an affection of the microvascular blood flow with its consequent deleterious cyto-toxic effects. Grabowski et al.[31] offered a similar interpretation. Other authors [32,33,34] alleged that, the haemodynamic changes result from mechanical distension of the abdominal cavity and from liberation of systemically active vasoconstrictor substances. Intra-abdominal (IAP) pressure affects the splanchnic macro- and microcirculation. It has a direct influence on abdominal vessels and viscera. In the venous system, the increased IAP compresses the splanchnic veins, reducing the blood flow by elevating vascular resistance. Compression of the portal vein, which represents the major outflow tract of visceral organs, leads to blood flow stasis in the splanchnic circulation. This stasis impairs intestinal perfusion at the mucosal and submucosal layers, leading to a reduction in tissue oxygen tension, anaerobic cell metabolism, acidosis, and production of reactive oxygen species (ROS). Overproduction of ROS related to CO2-Pp has been addressed in experimental and clinical trials with conflicting results. Moreover, Mallick et al.[33] and Stallion et al.[35] added that, paradoxically, restoration of blood flow to the ischemic tissue initiates a cascade of events that may lead to additional cell injury known as reperfusion injury. Among the internal organs, the intestine is probably the most sensitive to I/R injury. Unsal et al.[13] announced that unexpected desufflation - insufflation even at normal IAP levels during laparoscopy leads to significant oxidative stress (OS)-induced damage in the terminal ileum. In addition, Guven et al.(36) postulated that Pp even at normal IAP levels, leads to significant OS-induced biochemical and histological damage to the ovaries. Both Gutt et al.[11] and Pross et al.[37] hypthesized that during reperfu-

sion, free oxygen radicals, which are the most important mediators of oxidative tissue damage and consequential organ dysfunction, are generated as a result of I/R induced by the inflation and deflation of the Pp. In general, the most likely causes of OS as a consequence of CO2 Pp are I/R injury due to changes in the abdominal pressure, inflammation associated with tissue trauma, and diaphragmatic dysfunction. Karapolat et al.[18] stressed on that OS damages cellular components, causing microvascular leakage and lipid peroxidation of cellular membranes. This in turn generates more free radicals, with a self-propagating cycle leading to pathological changes ranging from edema and cell injury to cell death by necrosis. Moreover, Pp can affect several homeostatic systems, leading to alterations in the acid base balance, blood gases, hepatic perfusion, and cardiovascular and pulmonary physiology. Although deflation restores visceral perfusion, it does not necessarily relieve OS in the tissues. Other researchers[31,38] postulated that OS caused by ROS induced after the restoration of blood flow is one of the most important mechanisms contributing to organ dysfunction. Therefore, organ suffering following Pp is caused not only by splanchnic or visceral ischaemia, but also by OS seen after this insult. In addition, Brandt[39]; Bulbuloglu et al.[40] and Takizawa et al.[41] emphasized that the small intestine is particularly susceptible to injury from I/R induced by the restoration of blood flow after diverse events.

The mean values of the length of jejunal villi, their basal width, the height of columnar cells and the diameter of crypt, in the current study, showed significant decrease in the animals of the experimental group in comparison with their respective values of the control rats. These observations supported the detected histological findings denoting the occurrence of mucosal atrophy and could be a reflection of the injurious impact of the induced Pp. This was supported by Arumugam et al.[42] and Collard et al.[43] who confirmed that I/R triggers an intense inflammatory response. If severe enough, this response may result not only in local but also in dis-

tant organ upset. It was proposed [44,45] that OS mediators such as activated polymorphonuclear leukocytes and ROS, which cause lipid peroxidation and protein oxidation, are suggested to play a crucial role in I/R damage. The mean height of the microvilli, in the present study, exhibited insignificant variation between the control and experimental animals. Meanwhile, the mean density of the jejunal villi and microvilli of the experimental group showed significant increase compared to those of the control animals. Again, such alterations in these numerical data could be owed to the degenerative distress of Pp. The obtained mean microvillous height ,more or less, simulated that reported by Mayhew and Middleton and Köhler et al.[46,47] but contradicted that announced by Bertoni and Gabella[22]. Variations amongst these studies could be owed to differences in strain, age and weight of the experimental animals and also in the methodology applied. Several studies [48,49,50,51,52] revealed that during intestinal I/R, the expression level of nitric oxide (NO) is also changed, causing vermiculation and dysfunction of mucosal tissue. NO is a transient product of inflammatory processes and is generated from L-arginine by NO synthase. The presence of a large amount of NO as a free radical has been implicated as a cytotoxic factor in a variety of pathophysiological processes, including various forms of inflammation and circulatory shock.

In the present work, the mean thickness of the jejunal muscle layer showed insignificant decrease in the experimental rats compared to the control ones. This, partly, contradicted the findings of Unsal et al.[13] who recorded a significant diminution in its thickness. They explained this by the less effort exerted to move the luminal contents following Pp due to possible vascular affliction which may inhibit contractile responses and bring about structural alterations. On the contrary, Mahdy et al.[53] detected a three-fold increment in the ileal muscle layer proximal to the induced partial obstruction site compared to the control because of the extra attempt done to push the luminal contents via that narrow part.

The rat jejunum of the experimental group, in the existing study, showed a positive immune reaction to p53 protein mainly in the lamina propria and epithelium of intestinal villi and crypts. This could signify the occurrence of apoptosis. Arikan et al.[17] reported that apoptosis, physiological cell death, is a protective mechanism for organisms. It is the most important process to shape the organs during the intra-embryonic period and sustains the physiological balance of the number of cells during life. At the same time, if the cells are damaged by chemical or radioactive agents, in the organs that are not repaired by anti-oncogens, the apoptotic process is stimulated by mediators as p53, and damaged cells are eliminated to prevent malignant transformation. The number of apoptotic cells in intra-abdominal organs increases in proportion to the CO_2 pressure level. Several authors [54,55,56] stressed on that apoptosis has been established as a major mode of intestinal mucosal cell death caused by I/R injury. They ascribed such changes to oxygen free radical-induced peroxidation. I/R can damage the intestinal barrier, which in turn causes the release of some pro-inflammatory cytokines such as tumor necrosis factor and interleukin-6.

In light of the present results ,it could be concluded that Pp imparted its hazardous imprints on the rat jejunum. Further studies with different Pp pressure regimens on a larger number of animals over longer durations are recommended. Our hypothesis is that a better understanding of these influences might lead to safer minimally invasive surgery in the future.

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A LIGHT AND SCANNING ELECTRON MICROSCOPE STUDY OF THE EFFECT OF PNEUMOPERITONEUM ON THE ALBINO RAT JEJUNUM

Gamal A. Mohamed Ph.D, Wael M. Elsaed Ph.D and Ahmed M. Desouky Ph.D

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LAPAROSCOPIC ASSISTED TRANSANAL ENDORECTAL PULL-THROUGH PROCEDURE FOR HIRSCHSPRUNG'S DISEASE

El-Sherbiny Mohamed MD, El-Ghazaly W. Mohammed MD, Badrawy A. Tarek MD, Fathi Ahmad MD and Saed Basem

Departments of Pediatric Surgery, Mansoura University Children Hospital,

Pediatric Gastroenterology, Mansoura University, Children Hospital, Mansoura-Egypt.

Abstract

Purpose/ Background: *The first laparoscopic assisted pull-through procedure for HD was done by Georgeson et al in 1995. The development of this technique has changed the surgical management of HD considerably during the last decade and it is starting to dominate the modern treatment of HD today. Several issues have been raised concerning the indications, feasibility, safety, and the functional results following laparoscopic assisted transanal endorectal pull-through procedure. In this work, we tried to study the technique of laparoscopic assisted transanal endorectal pull-through in the treatment of Hirschsprung's disease from the operative technical point of view, immediate post-operative course, early postoperative course and short term functional outcome to evaluate its feasibility and safety.*

Patients/Methods: *Between January 2009 to November 2012, 25 patients underwent definitive treatment for HD in the pediatric surgery unit in Mansoura university children's hospital. All patients were treated using laparoscopic assisted transanal endorectal pull-through technique.*

Results: *In this study we had 20 patients were males, 5 patients were females. No patients were found to have associated congenital anomalies. The age at the time of pull-through ranged from 2.5 months to 13 years. By contrast enema, we had 11 patients with rectosigmoid transition zone,9 patients was long segment transition zone, 3 patients was ultra-short segment transition zone and two patients had inconclu-*

sive transition zone. Rectal punch biopsy was taken to all patients and was diagnostic in 23 patients. Anorectal manometry was done for 2 patients. Intraoperatively, the transition zone was found at rectosigmoid junction in 11 patients, long segment in 11 patients and ultra-short segment in 3 patients. The mean time for laparoscopic dissection in our study was 92 minutes with minimal time 45 minutes and maximum time 180 minutes .The mucosal dissection mean time was 96 minutes with minimal time 60 minutes and maximum time 180 minutes. The mean time for total procedure was 183 minutes while the minimum time 135 minutes and maximum time 300 minutes. We had one patient was converted to open technique due to thick mesentery, One patient had right ureteric injury due to anatomical abnormities and one patient explored in the second postoperative day and a tear was found at the descending colon at the site of Proline counter-traction suture. Postoperative soiling was decreased from12 patients at the first month postoperative to only3 patients after 2-3 months postoperative.

Conclusion: *Laparoscopic assisted transanal endorectal pull-though procedure is a very good choice for management of long segment cases of HD and cases with difficult transanal dissection and needs laparotomy assistance. We found good results and early regain of continence in ordinary segment cases done by laparoscopic assistance, but we recommend more evaluation for long term results for these cases.*

Keywords: *Transanal endorectal pull-through, Laparoscopy, Hirsch-*

Introduction

Congenital megacolon is one of the major problems in pediatric surgery. Different surgical techniques were done for its treatment with the most recent is transanal endorectal pull-through procedure[1].

Laparoscopic assisted pull-through was first described by Georgeson et al in 1995[2]. Sigmoid colon and proximal rectum were mobilized laparoscopically. Submucosal sleeve was developed transanally to meet dissection from above. The colon was pulled-through down in continuity, divided above the transition zone and secured to the anal mucosa 5 to

10 mm above the pectinate line[3].

There is some debate about the indications of this technique. Patients who may need laparotomy assistance for mobilization of the colon, laparoscopic assisted technique is alternative method to the open technique. Some surgeons used this technique in ordinary segment cases and even ultra-short segment cases with dilated rectum and sigmoid colon to decrease the time of sphincter stretch and improve the postoperative continence [4].

There are a few reports comparing the outcome of open procedure versus the laparoscopic one showing the superiority of the laparoscopic technique in certain aspects[5]. It is less invasive and it could provide better clinical outcome compared with the open technique. It is associated with less intraoperative contamination and less physical trauma leading to minimize adhesion formation [6]. Moreover, the laparoscopic procedure has less postoperative ileus, early hospital discharge and good cosmetic result[7].

Patients and Methods

Between January 2009 to November 2012, 25 patients underwent definitive treatment for HD in the pediatric surgery unit in Mansoura university children's hospital. All patients were treated using laparoscopic assisted transanal endorectal pull-through technique.

Inclusion criteria:

All the patients who were diagnosed as HD and accepted to have laparoscopic assisted transanal endorectal pullthrought procedure.

Exclusion criteria:

Parents refusing to share in the study, patients having colostomy before the pull-through procedure and patients in poor general health or associated with major congenital anomalies.

All patients were subjected to:

Full history taking, complete clinical examination, Routine laboratory investigations, Gasrotgraffin enema, rectal biopsy and Anorectal manometry in doubtful cases.

Operative methodology:

All patients subjected to laparoscopic assisted transanal endorectal Pull-through technique:

I- pre-operative preparation:

Pre-operative preparation starts 3 days before surgery by allowing only clear fluids to the child. Colonic irrigations by 10-20 cc/kg body weight of warm saline every 8 hours in the first two days through a rectal tube passed to a point just above the transition zone then as frequent as 6 hours until the output is clear. The patient is fasting at the night before surgery. Intravenous administration of one dose of 3rd generation cephalosporin 50mg/kg are given on call to the operating room.

II- operative techniques:

Infants were positioned longitudinally at the end of the operating table with their legs partial hanging from the edge of the table. The surgeon performing the laparoscopic portion of the procedure standing at the right side of the patient. then putting the patient in lithotomy position with the pelvis elevated to complete the transanal part of the procedure.

Laparoscopic procedure:

The entire legs and abdomen to the nipples are draped in the regular sterile method.

Suitable Foley's catheter is introduced carefully with complete aseptic technique to empty the bladder. We used it for all patients.

A 5 mm incision is made in the epigastric region midway between the xiphesternum and umbilicus. The incision is deepened in the fascia and an open Hasson's technique is used to introduce the first cannula .In older children we entered thought the umbilicus or small supra-umbilical incision.

A pneumoperitoneum is established. A pressure of 10–12 mm of water is usually well tolerated in all ages. A 5-mm 30° scope is used in all patients. A 5 mm port is then introduced to the right side of the umbilicus at midclavicular line but with a little lower level than the camera port. Another 5 mm port is introduced in the right iliac fossa at a lateral level than the second port. Sometimes the site of these two ports can be

higher or lower levels according to the preference of the surgeon.A fourth 5 mm port is placed on the left iliac fossa for grasping of the colon to facilitate mobilization. In younger infants we used A 3 mm ports instead.

Sometimes we replaced the fourth port by transfixing Proline suture used for counter traction of the colon without the need for the fourth port. We used long Proline needle. The needle introduced through the abdominal wall and under laparoscopic vision, a grasper used to push the left colon against the abdominal wall. The needle transfix the colon wall then caught again outside the abdominal wall. We use two Proline sutures at two levels to facilitate colonic counter-traction.

Colon is inspected for obvious transition zone.

A seromuscular biopsy is taen with laparoscopic scissors for histologic leveling in cases with no obvious transition zone.

Once the proximal resection level has been determined, then the bowel is elevated and mesenteric dissection is started, a window is developed through the rectosigmoid mesocolon adjacent to the colon using monopolar cautery or Legasure electrocoagulation.

Both ureters should be identified and preserved.

The mesenteric window in the sigmoid mesocolon is extended distally all the way to the peritoneal reflection in the pelvis. The dissection should be extended well below the peritoneal reflection down until the presacral space is entered to make the transanal dissection easier. Mesenteric dissection is extended until complete mobilization of the colon is done.

After complete mobilization of the colon we remove the instruments, deflate the abdomen, cover the cannulas by sterile towels and then start trans-anal part of the operation.

Trans-anal part:

Positioning of the patient in lithotomy position with slight elevation of the pelvis.

The Loan Star retractor was used in all cases with fixation of its hooks at mucocatenous junction circumferentially.

Injection of diluted Adrenaline solution with concentration of 1/200,000 submucosally.

An electrocautery incision is made in the mucosa and submucosa 1 cm above the dentate line and the submucosal plane is then developed circumferentially, using combination of cautery, sharp, and blunt dissection creating a tube of submucosa and mucosa along the rectum until the intraperitoneal portion of the upper rectum is reached.

At this point, the dissection was continued for approximately 1 cm cephalad and the anterior wall of the muscularis cuff was opened transversely between two 4/0 silk stay sutures, entering into the peritoneal cavity.

We pullthrough the previously dissected rectum and sigmoid colon transanally. If there still some mesenteric adhesions; we dissect it using the electrocautery.

The muscular cuff is then shortened and split posteriorly to prevent spasms/constriction of the pullthrough bowel segment.

A single layer anastomosis between the full thicknesses of the ganglionated pullthrough colon and the anal mucosa 1 cm above the dentate line is performed circumferentially in quadrants.

After finishing the anastomosis a betadine pack is applied through the anus and left for 24 hours.

The pneumoperitoneum is reintroduced and the pedicle inspected laparoscopically for the potential of internal herniation, twisting or bleeding.

The pneumoperitoneum is evacuated and the ports removed. The port sites are closed with fascial sutures and skin closure.

All patients were evaluated intraoperatively for:

The level of transition zone, time used for Laparoscopic part of the operation, time used for transanal part of the operation, total

time of the surgery, complications during laparoscopic procedure, complications during transanal procedure, amount of blood loss during operation and Length of resected specimen.

Postoperatively, all patients were evaluated in the immediate postoperative period for the following items:

Bleeding, timing of regaining peristalsis, first time of post-operative motion, time of urinary catheter removal, time of starting oral intake, post-operative antibiotic, Post-operative analgesic, wound infection, anastomotic leakage and hospital stays time.

After discharge from the hospital, all patients were evaluated in the early postoperative period (first month postoperatively) for the following items:

Result of operative biopsy, Post-operative distension, attacks of enterocolitis, perineal excoriation, Post-operative soiling and anastomotic stricture.

Late postoperative follow up (after the first month postoperatively) all patients were evaluated for the following items:

Motion frequency, soiling, recurrent abdominal distention, anal stricture, delayed Enterocolitis and cosmetic evaluation of laparoscopic ports wound.

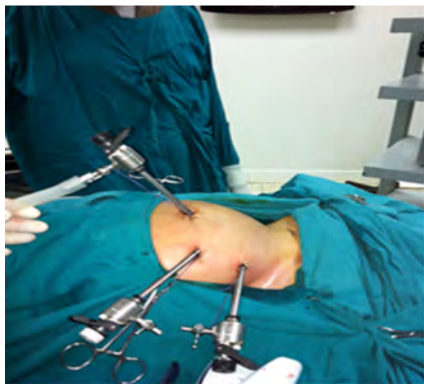
Results

In this study we had 20 patients were males, 5 patients were females. No patients were found to have associated congenital anomalies. The age at the time of pull-through ranged from 2.5 months to 13 years. No family history of Hirschsprung's disease was found in this study. Constipation was the most common presentation for all patients in this study and soiling was the least frequent presenting symptom in this study which was present in only two patients. By contrast enema, we had 11 patients with rectosigmoid transition zone, 9 patients with long segment transition zone, 3 patients with ultra-short segment transition zone and two patients had inconclusive transition zone. Rectal punch biopsy was taken to all patients and was diagnostic in 23 patients. Anorectal manometry was done for only two patients who had inconclusive rectal punch biopsy. Intra-

operatively, the transition zone was found at rectosigmoid junction in 11 patients, long segment in 11 patients and ultra-short segment in 3 patients. The mean time for laparoscopic dissection in our study was 92 minutes with minimal time 45 minutes and maximum time 180 minutes .The mucosal dissection mean time was 96 minutes with minimal time 60 minutes and maximum time 180 minutes. The mean time for total procedure was 183 minutes while the minimum time 135 minutes and maximum time 300 minutes. The mean amount of blood loss was 34 ml and the length of excised segment ranged from 15cm to 70 cm. We had one patient who was converted to open technique due to thick mesentery and inability to complete dissection safely, one patient had right ureteric injury due to anatomical abnormities and one patient explored in the second postoperative day due to peritonitis, a tear was found at the descending colon at the site of Proline counter-traction suture. The mean hospitalization days in our study were 4 days (ranged from 3 days to 8 days). We had few early post-operative complications in this study. We had only one patient with early postoperative abdominal distention and the same patient had early attack of mild EC. One patient showed marked postoperative soiling, eleven patients had minimal soiling and thirteen patients had no soiling. The postoperative biopsy results were diagnostic in twenty four patients with ganglionic proximal cut margins and only one patient had hypoganglionic proximal cut margin of the pulled through colon, This patient had hypoganglionic frozen section intraoperatively and managed by completing the resection at higher level without repeating the frozen biopsy. This patient was the same patient that showed early postoperative distention and EC.

The motion frequency was 2-3 times per day in twenty three patients and one patient had more frequent motion 4-5 times per day and one patient had constipation with motion frequency every 2-3 days. The patient that showed constipation was the same patient with hypoganglionic proximal cut margin. In late post-operative

course we had one patient with recurrent attacks of mild to moderate abdominal distention and EC. This was the same patient that had postoperative hypoganglionic proximal cut margin. Postoperative soiling was decreased from12 patients at the first month postoperative to only 3 patients after 2-3 months postoperative.



Site of ports

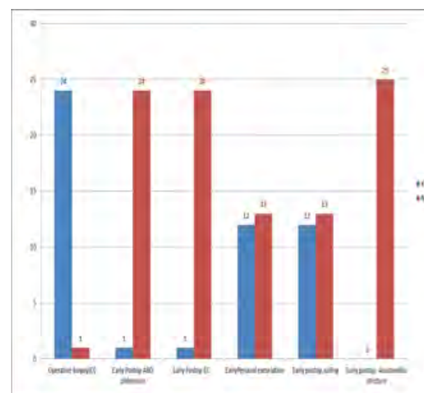


Diagram showing incidence of early postoperative complications (n-25)

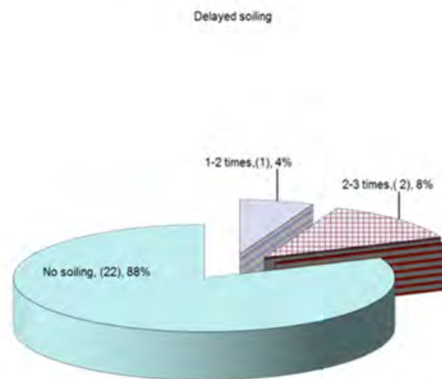


Diagram showing incidence of delayed soiling

Diagram showing incidence of early postoperative

Discussion

The first successful operative management for HD was in 1948 When Swenson and Bill described their pull through procedure by excision of the spastic aganglionic segment followed by colo-anal anastomosis [8].

The first described transanal endorectal pull-through procedure was in 1998 by De La Torre-Mondragon & Orega-Salgado. Since this time this technique have been widely accepted and considered the standard treatment for HD [9].

The first laparoscopic assisted pull-through procedure for HD was done by Georgeson et al in 1995. He described primary laparoscopic pullthrought procedure in 12 patients with very good results. The development of this technique has changed the surgical management of HD considerably during the last decade and it is starting to dominate the modern treatment of HD today [3].

In this study, 30 degree camera was used, a 5 mm port was inserted in the epigastric region midway between the xiphesternum and umbilicus. A 5 mm or 3 mm port is then introduced to the right side of the umbilicus at midclavicular line but with a little lower or higher level than the camera port. Another 5 mm or 3mm port is introduced in the right iliac fossa at a lateral level than the second port. We used two transfixing Proline suture for counter traction of the colon without the need for a fourth port in most of the cases. Georgeson et al., in 2004 used a similar technique to the technique used in this study by using a three trocar technique with the first trocar placed through the umbilicus. A second trocar is placed subcostally in the right upper quadrant and a third trocar is placed in the anterior axillary line half way between the costal margin and the anterior superior iliac spine. A fourth trocar can be placed in the left upper quadrant if needed for traction on the colon[10]. This differs from the first described technique by Georgeson et al., who used a 4-mm 30° scope in infants and a 5-mm 30° scope in older children. The trocar site for the scope was placed just below the liver edge and to the right

of the falciform ligament. Trocars were placed in the right upper quadrant and left upper quadrant of the abdomen. These ports were 4 or 5 mm in diameter, depending on the size of the patient. A suprapubic trocar was used in some patients to provide retraction of pelvic structures and to hold the colon in traction during laparoscopic dissection of the rectum [6]. El Sadat used a 5 mm, 30° scope through a port placed below the liver edge in the right mid-clavicular line. One 5 mm port was placed in the right lower quadrant, another in the suprapubic region and another one sometimes in the left lower quadrant. The exact position of the ports in relation to anterior superior iliac spine varied according to the age of the patient [11].

Mean Operative time for laparoscopic dissection in this study was 92 minutes with minimal time was 45 minutes and maximum time was 180 minutes. The mean time for mucosal dissection was 96 minutes with minimal time was 60 minutes and maximum time was 180 minutes while the mean time for total procedure was 183 minutes while the minimal time was 135 minutes and maximum time was 300 minutes. Georgeson et al., reported that the mean operative time for total procedure was 147 minutes [6]. Liem and Hau reported the mean operative time was 140 minutes [12]. El Sadat reported a significant difference in the operating time according to the length of the aganglionic segment, average time for classic rectosigmoid aganglionosis was 90 minute, and in cases where the funnel was extending higher the average time was 150 minute.

In our study, we had one patient with Diathermy injury to the right ureter because of anatomical variation of its site and one patient was converted to open pull through due to difficult dissection of his thick mesentery. In most of series no recorded injury to ureter which can be explained in our study by anatomical variation of its site and lack of our experience. Conversion to open pull-through occurred in one case in this study due to thick mesentery and we couldn't complete dissection safely in this patient. Laparotomy

assisted transanal endorectal pull-through procedure was done for this patient. Georgeson et al., reported that no required conversion to open laparotomy in their series [3]. The same results were reported by El Sadat in his series[11]. Hau et al., reported conversion to open surgery was required in four patients out of 200 patients due to a high aganglionic segment in 3 patients and in one patient due to heavy intestinal adhesions (Hau et al., 2009).

In our study, we had one patient with difficult mucosal dissection due to megarectum. This patient was 7 years old which explain the occurrence of megarectum and difficult dissection. This result cope with El Halby et al., who reported that mucosal dissection was easier in the younger patients especially in infants and it was difficult in older children who had more thickness of the mesentery, previous attacks of enterocolitis and long standing dilated and hypertrophied colon [14].

The length of excised segment in our study ranged from 15 to 70 cm with a mean length 35 cm. El Sadat reported that the shortest length of resected aganglionic segment was 18 cm while the longest was 38 cm [11].

Starting oral was in the second postoperative day in 23 patients in this study and only two patients were delayed to the 4th postoperative day; the first patient was delayed because of difficult mucosal dissection and difficult colo-anal anastomosis and we preferred to postpone oral to protect the anastomosis; the second patient was explored in the second postoperative day due to peritonitis. We found a tear at the site of counter traction Proline suture which was repaired with covering ileostomy. El Sadat reported more delayed time to start oral; He started oral at 48 hours after the operation in eleven cases (73%), 72 hours in three cases (20%) and after 96 hours in one case (6.7%)[11].

The mean hospital stay in our study was 4 days with minimal time was 3 days and maximum time was 8 days. This similar to 3.7 days reported by

Georgeson[10], 3.4 days reported by Rothenberg[15], 5 days reported by El Sadat[11]. In comparison to series in totally transanal approach, Elhalaby et al., reported mean hospital stay 4.8 days [14]. There is a similarity between the mean hospital stay time in laparoscopic assisted and totally transanal approaches with little superiority of laparoscopic assisted approach.

Enterocolitis has been considered one of the main problems in patients with HD both pre and after definitive treatment[16]. In our study, we had one patient (4%) with early post-operative attack of enterocolitis. He was the same patient who had frequent early post-operative abdominal distension. El Sadat reported enterocolitis in the early post-operative period in 3 cases (20%) that responded to conservative measurement[11]. Georgeson reported 6 patients (7.5%) with early enterocolitis in his series[10]. Elhalaby reported in his multicentric study that postoperative enterocolitis occurred in 26 patients (17.5%) and he recommended short seromuscular cuff, low colo-anal anastomosis, and routine postoperative anal dilatation particularity in neonates and infants to have relative low incidence of post-operative enterocolitis[14].

In our study, we had one patient with marked perianal excoriation (4%); eleven patients with minimal excoriation (44%) and thirteen patients had no excoriation. These were the same results for early postoperative soiling which explain the occurrence and degree of perianal excoriation. Conservative treatment with local protective and anti-inflammatory creams had been used with very good results. Transient perianal excoriation was reported in most of series following transanal endorectal pull-through. Elhalaby reported in his multicentric study perianal excoriation in 48 (32.9%) patients[14]. El Sadat reported excoriation in 4 patients (26.4%)[11]. Post-operative soiling can be explained by the significant stretching of the anal sphincters during mucosectomy with its potential impact on postoperative continence status particularly in older children with marked hypertrophy and dilatation of the colon[17].

Motion frequency in our study was variable. Twenty three patients had frequency 2-3 times per day, one patient had more frequent motion 4-5 day and one patient had constipation with motion every 2-3 days. It looks that fecal continence is better in our series than that of totally transanal approach which can be explained by decrease time of sphincter stretch. One patient in our study had post-operative constipation (4%). Elhalaby reported 6 patients (4%) with recurrent constipation in his multicentric study [14]. This is the same patient that had hypoganglionic postoperative biopsy. This patient is managed conservatively with regular laxative and being considered as a case of residual aganglionosis is put in mind.

We have remarkable improving of soiling condition from early to late postoperative period in this study. In early postoperative period we had thirteen patients (52%) that had no soiling. This number increased to twenty two patients (88%) in late postoperative period. Only three patients still had soiling; one patient (4%) had minimal degree of soiling 1-2 times per day and two patients (8%) had mild degree 2-3 times per day. Elhalaby reported soiling in 7 from 42 patients in his study who continue to show a steady improvement of their continence status [14]. El Sadat reported that continence to stool needs longer period of follow-up especially in younger age group[11]. In our study, we had very good cosmetic results with no ugly scar or port site hernia. Parent's satisfaction from the cosmetic point of view was very good.

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LAPAROSCOPIC ASSISTED TRANSANAL ENDORECTAL PULL-THROUGH PROCEDURE FOR HIRSCHSPRUNG'S DISEASE

El-Sherbiny Mohamed; El-Ghazaly Wali, Mohammed; Badrawy Abd-Elaziz, Tarek; Fathi Ahmad; Saed Basem

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PROGNOSTIC SIGNIFICANCE OF HER-2 NEU AND PROGESTERONE RECEPTOR EXPRESSION IN ENDOMETROID TYPE OF ENDOMETRIAL CARCINOMA

Sylvia A. Ashamallah M.D* and Dalia H. Zayed M.D**

**Pathology and Clinical Oncology and Nuclear Medicine*

***Departments, Faculty of Medicine, Mansoura University, Egypt*

Abstract

Purpose: *The aim of this study was to investigate the expression of Her-2 neu and progesterone receptor (PR) in endometrial carcinoma (EC), to study its correlation to clinicopathologic features and prognostic parameters in EC patients.*

Patients and Methods: *Immunohistochemical (IHC) study of Her-2 neu and PR was performed on archival material of 40 patients diagnosed with EC and treated between January 2004 and December 2007.*

Results: *Her 2-neu was expressed in 15% of cases and was statistically related to higher grade, deep myometrial invasion, and higher stage. PR positivity was determined in 60% of cases and was only significantly related to low tumour grade. On multivariate study, patients' age, expression of PR and Her 2-neu were independent prognostic factors for disease free survival while patients' age and expression of PR were the only prognostic factors for overall survival.*

Conclusion: *Her-2 neu and PR are good prognostic factors for disease free survival in EC and can be reliable parameters in the selection of hormonal or targeted therapy for endometrial carcinoma.*

Introduction

Endometrial carcinoma (EC) is the most common malignancy of the female genital tract in industrialized countries, and occurs predominantly after menopause[1]. Its incidence has increased dramatically over the last decade[2].

Two distinct clinicopathologic types exist; type 1 which is the more common one and it occurs

as a result of excess estrogenic stimulation. The background is usually formed of hyperplasia. This type is usually of low grade and has an indolent course. Endometroid and mucinous variants are the prototype of this category. Type 2 is unrelated to estrogen and usually associated with atrophic endometrium, frequent lack of estrogen and progesterone receptors and older age at presentation. It is usually of high grade and follows an aggressive clinical course. Serous and clear cell variants are the prototype of this category[3,4].

In recent years, the molecular analysis of EC has identified abnormalities in the expression, structure, or activity of oncogene products which can contribute to the development and maintenance of the malignant phenotype[5]. Her-2 neu (human epidermal growth factor receptor-2, also known as c-erbB-2) is one of the most-studied molecular markers in anticancer therapy[6]. Her-2/neu receptor protein is encoded by HER-2/neu gene, which is localized on chromosome 17. Epidermal growth factor (EGF) receptor, related with growth factors, has a regulatory role, particularly by influencing the mitogenic activity[7]. Her-2/neu gene encodes an 185kD trans-membrane cell surface receptor that belongs to the epidermal growth factor receptor family, and has extracellular domain and a cytoplasmic signal-transduction domain with tyrosine kinase activity[8].

Her-2/neu is predominantly activated by forming a heterodimer with other EGFR members, rather than forming a homodimer[9]. Heterodimerisation of Her-2/neu can result in the activation of intracellular signaling cascades, such as the PI3K–AKT and MAPK–ERK pathways[8,10].

Her-2/neu amplification or overexpression has been reported in 4% to 69% of endometrial carcinomas, it[6]. Over-expression of Her-2/neu has been associated with a more aggressive biological behavior and adverse prognostic factors, including advanced stage, higher grade and worsened overall survival[1].

Being a hormone-related can-

cer, estrogen receptor (ER) and progesterone receptor (PR) are also identified as specific prognostic factors for EC[11,12,13]. Estrogen receptors (ERs) and progesterone receptors (PRs) are generally decreased in EC and the loss of receptors is a part of the carcinogenesis of the endometrium. It is postulated that estrogen exposure unopposed by progestins increase the risk of endometrial hyperplasia and cancer[14].

The expression of hormonal receptors in EC is reported to range from 32-77% for ER and 54-72% for PR. The expression of hormonal receptors in EC is associated with other good prognostic factors, such as early stage of disease, less myometrial invasion, low tumor grade, and absence of lymphovascular space invasion (LVSI)[15].

Regarding an impact of ER and PR expression on survival, controversial data exist. Some authors reported PR and/ or ER positivity as the independent good prognostic factors for survival[16] hile others could not demonstrate such findings[17].

The aim of this study was to investigate the expression of Her-2/ neu and PR in endometrial cancer and to study its correlation to clinicopathologic features and established prognostic parameters in EC patients.

Materials and Methods

This is a retrospective study. The archives of the Pathology Department, and Clinical Oncology and Nuclear Medicine department, Faculty of Medicine Mansoura University were searched to identify patients diagnosed with EC and treated between January 2004 and December 2007. Inclusion criteria were patients with EC whose hysterectomy specimen were received at Pathology Department during this period and had been treated at Clinical Oncology and Nuclear Medicine department. Exclusion criteria were patients whose medical records were not available, had no available paraffin blocks or inadequate pathological tumor tissue for immunohistochemical (IHC) processing.

Clinical data abstracted from the patients' files included: age, menopausal status, FIGO stage,

treatment received (surgery with or without adjuvant treatment), date of recurrence and date of last visit or death. Paraffin blocks of specimens were retrieved from the archives and cut at 4-5 microns sections. Slides were stained with Haematoxylin and Eosin stains and then submitted for further immunohistochemical staining procedures.

H & E stained slides were thoroughly examined for tumour type and grade, depth of myometrial invasion, lymphatic or vascular invasion, cervical or adenexial metastasis and lymph node metastasis, Cases were staged according to FIGO 1988 staging system for endometrial carcinoma.

Immunohistochemistry:

Immunohistochemistry study was performed using the avidin-biotin-peroxidase complex method. Antigen retrieval was done in citrate buffer boiling in microwave at pH 6.0. Antibodies against the following antigens were used: rabbit polyclonal antibody to Her-2 (code number A0485) (DakoCytomation, Carpinteria, CA, USA), and PR: rabbit polyclonal antihuman PR antibody ([DakoA0098). Different steps of IHC staining were performed according to the manufacturer instructions for each antibody.

Immunoreactivity of Her-2/neu was observed in the cell membrane and was scored using the Food and Drug Administration (FDA) approved scoring system as: 0, no immunostaining; 1+, incomplete membranous immunostaining of <10% of tumor cells; 2+, weak complete membranous immunostaining of >10% of tumor cells; 3+, strong complete membranous staining of >10% of tumor cells. Scores of 0 or 1+ indicated a negative result, while scores of 2+ and 3+ were regarded as positive Her-2/neu expression [6]. PR examinations were done based on the percentage of the stained cells and the intensity of the nuclear staining. The percentage of positive cells was graded as follows: 1 = 0 to 25% of the nuclei stained; 2 = 26 to 75% of nuclei stained, 3 = more than 76% of the nuclei stained. The staining intensity was scored as follows: 1 = absent or weak, 2 = strong, 3 = very strong. The sum of both parame-

ters determined the IHC score. Tumors were classified to three categories depending on the IHC score. Category I corresponded to a score of 2 and category II to a score of 3 or 4, and category III to a score of 5 or 6. Category-I tumors were considered as immunonegative, whereas category II and III tumors were considered as immunopositive(18).

Treatment modalities and follow up program:

Total abdominal hysterectomy and bilateral salpingo-oophorectomy with pelvic lymph node sampling or dissection in (stages II and III) were done for all patients at that time. Postoperative external beam radiotherapy (EBRT) to whole pelvis was given to patients with Stage I and invasion of more than half of myometrium (except for 3 patients), stage II and stage III. Doses of radiotherapy ranged between 45–50.4 Gy in 25–28 daily fractions of 1.8 Gy given in 5–5.5 weeks.

Low dose rate (LDR) vaginal brachytherapy boost was given in addition to EBRT to improve local control in patients with high grade (grade III) tumours and histological involvement of the cervical stroma aiming at 10–15 Gy at 0.5 cm from the surface of the applicator.

A combination of adriamycin (60 mg/m2) and cisplatin (50 mg/m2) every 3 weeks for 6 cycles, has been used in 4 patients with stage III endometrial carcinoma.

Patients treated for endometrial cancer were followed up for both recurrence and late toxicity. For the first 3 years, patients were seen every 2 months. History, physical and vaginal examination were performed. Further investigations (CT, MRI, blood tests, examination under anaesthesia) were requested if clinically indicated. For the next 2 years and until the completion of 5 years in total, 6-monthly appointments were recommended. During this surveillance the increased risk of cancers of the breast, ovary and colon in patients with endometrial cancer was taken in consideration into account.

Statistical Analysis

The association between the ex-

pression of Her-2/neu and PR and the following clinicopathological factors were studied: age, FIGO stage, tumor grade, and depth of myometrial invasion, cervical involvement, lymph node metastasis, disease-free survival (DFS), and overall survival (OS).

DFS was defined as interval from the end of treatment to the time of recurrence or progression of disease. Patients who were lost to follow up, DFS data were right-censored at the time of the last evaluation or contact when the patients were known to be disease-free. OS was defined as the time from the date of diagnosis to date of death. For patients who were still alive at the time of the study or dead from other causes, OS data were right-censored at the date of last follow-up visit. Data were analyzed using SPSS (Statistical Package for Social Sciences) version 15. Qualitative data was presented as number and percent. Non-parametric data was presented as min – max and median. Mann-Whitney test and Kruskal-Wallis test were used for comparison between groups. Kaplan- Meier survival curve was used to estimate survival. Cox regression and hazard ratio were used to test the effect of different risk factors on survival. P value is considered significant if it is < 0.05.

Results

This retrospective study involved 40 patients, who met the inclusion criteria during the period of study. Patients' age at diagnosis ranged from 40 to 71 years (mean of 55.4±7.83 years).

All had the diagnosis of type 1 endometroid adenocarcinoma; 5 cases showed adenosquamous pattern, 2 had adenoacanthoma and 2 were of villo-glandular pattern. Most cases were grade 1 (16 cases, 40%), and stage I (22 cases, 55%). Myometrial invasion involving more than 50 % of myometrial thickness was encountered in 12 cases (30%); lymph vascular space invasion was seen in 18 cases (45%). Metastatic deposits were detected in the cervix and ovary in 16 (40%) and 6 cases (15%) respectively, while in 2 cases (5%), metastatic deposits in pelvic lymph nodes were detected.

Among patients with available clinical data, 13 (32.5%) had no further treatment after primary surgery while 27 (67.5%) had adjuvant treatment as radiation therapy (n=23), radiation and chemotherapy (n=4). Clinicopathological features of the patients are shown in Table 1.

Among all 40 cases, 6 (15%) had positive staining with Her2-neu (Figure 1). Her-2 positivity was found in 2of G2-patients (22%) and 4 of G3-patients (3%). A significant difference was detected ($p < 0.05$) among different histological grades as regards Her-2 neu expression.

In all cases with positive Her 2-neu expression, the tumour invaded more than $^1/_2$ of the myometrial thickness, while Her 2-neu was not expressed in any tumour invading less than $^1/_2$ of the myometrium. The difference in Her2-neu expression between the 2 groups was statistically significant.

Immunoreactivity with Her-2 neu was detected in 1 case with stage II (8%), in 5 (83%) with stage III and the difference was statistically significant ($P<0.001$). However, Her-2/ neu was negative in all stage I cases. The relation between Her 2-neu expression and menopausal status, infiltration of the cervix, and lymph node metastasis was statistically insignificant (P value >0.05) (Table 2).

PR positivity was proved in 24 cases (60%) (Figure 2). The percentage of positive PR staining among different grades (I-II-III) were 14 cases (87.5%), 5 cases (55.6%) and 5 cases (33%), respectively. There was a statistically significant relation between PR expression and tumour grade ($p< 0.05$).

Immunoreactivity for PR was detected in 16 cases in stage I (72%), in 7 cases (58%) in stage II and in only one case of stage III (17%). The relation between PR expression and tumour stage showed no statistical significance ($P>0.05$). Other factors including menopausal state, depth of myometrial invasion, infiltration of the cervix and lymph node metastases were not significantly related to PR expression ($P > 0.05$) (Table 3).

Survival studies:

Period of follow up ranged from 3-80 months with median value of 49 months. On follow up, 14 patients (40%) developed recurrence.

Survival time ranged from 3 - 80 months, with a median follow-up period of 53 months, 19 patients died and 4 patients had recurrence. Overall 5 -year survival was (75.5%). On univariate analysis (Table 4), younger age (< 60 years) was associated with greater median overall survival than older age (> 60 years) (65 vs. 38 months) and was statistically insignificant (P=0.401). Median OS survival was better in patients with low grade tumours (grade I, II) than those with high grade tumour (grade III) (71 vs. 19 months), it showed statistical significance (p=0.005). Tumours infiltrating less than half of the myometrial thickness showed better median OS (60 months) than those infiltrating more than half of the endometrial thickness (7months), and the difference was statistically significant (p=0.003). The median OS for stage I, II and III were 60, 46, 7 months respectively (p= 0.004). Positive expression of Her 2-neu was associated with reduced median overall survival (7 months) as compared to negative tumours (60 months), with statistically significant difference (P=0.001) (figure 3A). Patients with tumours expressing PR had significantly better OS than those with PR-negative tumours (71 vs. 7 months, P<0.001) (figure 4 A). Patients who had received adjuvant radiotherapy treatment showed shortened median overall survival compared to those who had surgery only (53 vs. 55 months, p= 0.057). Multivariate analysis confirmed the prognostic significance of age < 60 years (P=0.015; HR= 0.234; 95 % CI: 0.073 to 0.756), and PR expression (P<0.0001; HR= 38.228, 95 % CI: 5.153 to 283.585).

Disease free survival (DFS) ranged from 1-80 months with a median value of 32 months. On univariate analysis (Table 5), patients less than 60 years of age were found to have longer median DFS compared to patients older than 60 years, at time of presentation (64 vs. 32 months, P=0.343).

Lower grades of endometrial carcinoma (G 1&2) were significantly associated with a longer median disease free survival than grade 3 tumours (64 vs. 7 months, P=0.005). Tumours infiltrating less than half of the myometrial thickness showed better DSF (median: 58 months) than those infiltrating more than half of the myometrium (median DFS: 6 months), and the difference was statistically significant (p=.003). The median DFS for stage I, II and III were 58, 38, 2 months respectively (p=.003). Positive expression of Her 2-neu was associated with shortened median DSF (2 months) as compared to negative tumours (58 months), with statistically significant difference (P=0.001) (figure 3 B). Patients with tumours expressing PR had significantly better DSF than those with PR-negative tumours (median DFS: 65 vs. 3 months, P<0.001) (figure 4 B). Patients who received post-operative adjuvant treatment had a better DFS than those who were treated by surgery alone, however, the difference was insignificant (52 months, 30 months respectively, p= 0.053). Multivariate analysis confirmed the prognostic significance of age < 60years (P=0.027; HR= 0.252; 95% CI: 0.075 – 0.854), Her 2-neu expression (P<0.0001; HR=0.004; 95% CI: 0.000 – 0.070) as well as PR expression (P<0.0001, HR= 181.465, 95% CI: 15.675 – 2100.759).

Table (1): Clinico-pathological features of patients.

		N	%
Menstrual Status	Premenopausal	6	15
	Postmenopausal	34	85
Histologic Grade	Grade 1	16	40
	Grade 2	9	22.5
	Grade 3	15	37.5
Myometrial invasion	< 1/2	28	70
	> 1/2	12	30
Lymph vascular space invasion	Positive	18	45
	Negative	22	55
Cervix	Positive	16	40
	Negative	24	60
Ovary	Positive	6	15
	Negative	34	85
Lymph Node status	Positive	2	5
	Negative	38	95
FIGO Stage	I	22	55
	II	12	30
	III	6	15
Her 2-neu Expression	Positive	6	15
	Negative	34	85
Progesterone Receptors Expression	Positive	24	60
	Negative	16	40
Adjuvant treatment	Yes	27	67.5
	No	13	32.5

Table (2): Relation of Her2-neu expression and different clinico-pathological parameters

		Her 2 neu expression		
		Positive N %	**Negative N %**	***P value***
Menopausal status	Premenopausal	3 (50%)	3 (50%)	0.221
	Post menopausal	3 (9%)	31 (91%)	
Grade	Grade 1	0	16 (100%)	0.032
	Grade 2	2 (22%)	7 (78%)	
	Grade 3	4 (37%)	11 (63%)	
Stage	I	0	22 (100)	<0.001
	II	1 (8%)	11 (92%)	
	III	5 (83%)	1 (17%)	
Myometrial infiltration	<1/2	0	28 (100%)	<0.001
	> 1/2	6 (50 %)	6 (50 %)	
Cervix	Positive	5 (46%)	11 (54%)	0.19
	Negative	1 (4%)	23 (96%)	
Lymph node	Positive	1 (50%)	1 (50%)	0.155
	Negative	5 (13%)	33 (87%)	

Table (3): Relation of Progesterone receptor expression and different clinicopathological parameters

		PR expression		
		Positive N (%)	**Negative N (%)**	***P value***
Menopausal status	Premenopausal	2 (33%)	4 (67%)	0.865
	Post menopausal	22 (65%)	12 (35%)	
Grade	Grade 1	14 (88%)	2 (12%)	0.008
	Grade 2	5 (56%)	4 (44%)	
	Grade 3	5 (33%)	10 (67%)	
Stage	I	16 (73%)	6 (27%)	0.064
	II	7 (58%)	5 (42%)	
	III	1 (17%)	5 (83%)	
Myometrial infiltration	<1/2	19 (68 %)	9 (32 %)	0.121
	>1/2	5 (42%)	7 (58%)	
Cervix	Positive	7 (44 %)	9 (56 %)	0.087
	Negative	17 (71 %)	7 (29 %)	
Lymph node	Positive	0	2 (100%)	0.076
	Negative	24 (63%)	14 (37%)	

Table (4): Median overall survival in months for endometrial carcinoma as a function of possible prognostic factors.

Prognostic factor	Median OAS (in months)	P value
***Age**		
<60 years	64	0.343
>60 years	32	
***Tumour grade**		
G 1&2		0.005
G 3	19	
***Depth of myometrial invasion**		
<1/2	60	0 .003
>1/2	7	
***Tumour stage**		
I	60	0.004
II	46	
III	7	
***Her 2-neu expression**		
Positive	7	0 .001
Negative	60	
***PR expression**		
Positive	71	<0.001
Negative	7	
Adjuvant treatment		
Yes	53	0.057
No	55	

Table (5): Median disease survival in months for endometrial carcinoma as a function of possible prognostic factors.

Prognostic factor	Median OAS (in months)	P value
***Age**		
<60 years	65	0.401
>60 years	38	
***Tumour grade**		
G 1&2	64	0.005
G 3	7	
***Depth of myometrial invasion**		
<1/2	58	0 .003
>1/2	6	
***Tumour stage**		
I	58	0.003
II	38	
III	2	
***Her 2-neu expression**		
Positive	2	0 .001
Negative	58	
***PR expression**		
Positive	65	<0.001
Negative	3	
Adjuvant treatment		
Yes	52	0.53
No	30	

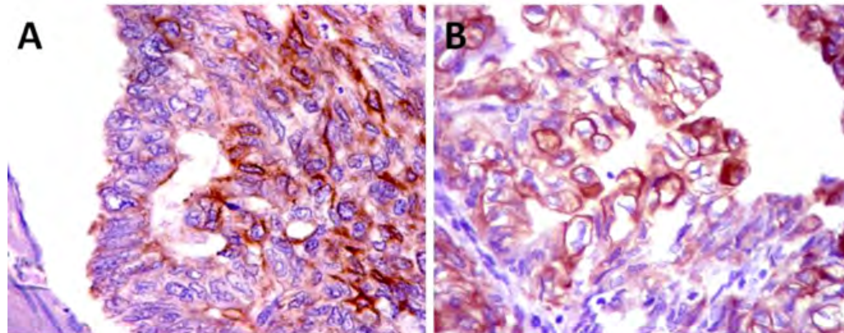

Figure 1: Immunohistochemistry for Her-2/neu in endometroid type of endometrial carcinoma. **A)** Incomplete strong membranous staining of< 10% of neoplastic cells(score 1). **B)** Strong complete membranous staining of > 10% of neoplastic cells (score 3) (IHC, original magnification X400).

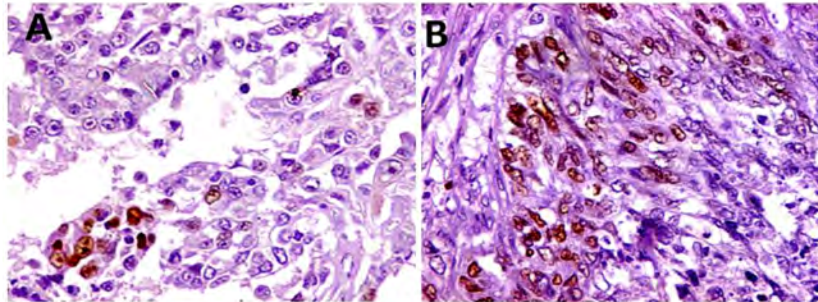

Figure 2: Immunohistochemistry for PR in endometroid type of endometrial carcinoma. **A)** Strong nuclear staining in < 25% of neoplastic cells (score 3). **B)** Strong nuclear staining of > 75% of neoplastic cells (score 5) (IHC, original magnification X 400).

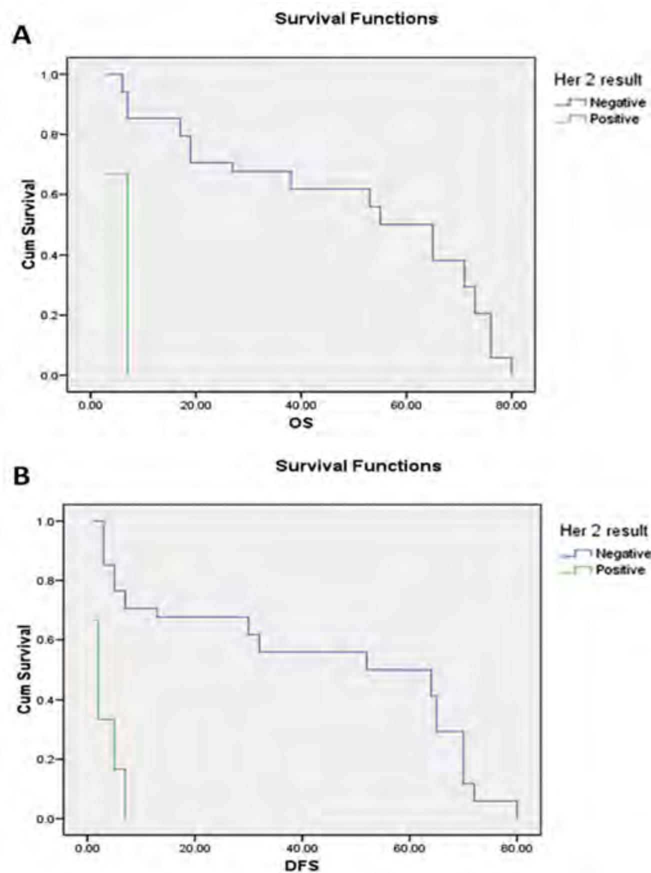

Figure 3: A) Overall survival in relation to Her 2-neu expression. **B)** Disease- free survival in relation to Her 2-neu expression.

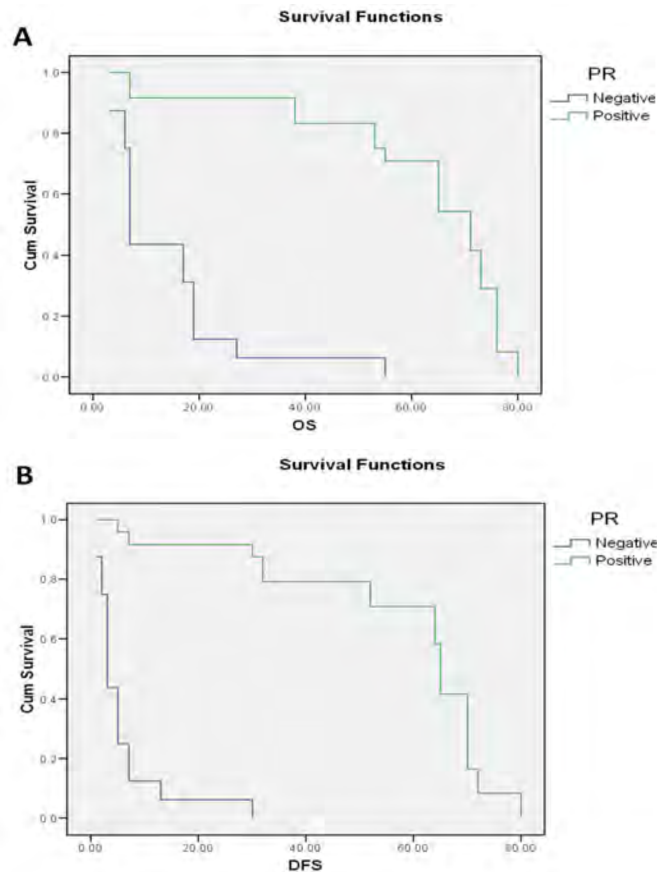

Figure 3: A) Overall survival in relation to PR expression. **B)** Disease- free survival in relation to PR expression.

Discussion

Proto-oncogenes are a group of normal genes that play important roles in the regulation of cell proliferation. Abnormalities in the expression, structure, or activities of proto-oncogene products contribute to the development and maintenance of the cell malignant phenotype. The human erbB-2 gene products, like Her-2/neu, transmembrane receptor protein that plays an important role in coordinating the complex erbB signaling network, are responsible for regulating cell growth and differentiation[19].

When Her-2/neu is normally expressed, it leads to the combi-

nation of a few copies of Her-2/neu heterodimers and the Her-2/neu -mediated signaling is weak, resulting in a normal cell growth . Her-2/neu protein overexpression and Her-2/neu gene amplification are frequently associated with a more aggressive human tumors including breast and ovarian cancer, prostate, bladder, cervical cancer, and EC. Overexpression of the Her-2/neu oncogene occurs in about 10% to 40% of EC. Kohlberger et al., concluded that Her-2/neu oncoprotein is demonstrated in all clinical stages and it did not seem to be a late event in the natural history of EC[20]. Her-2/neu oncoprotein expression has been associated with other adverse prognostic factors, including advanced stage, higher grade and worsened overall survival[5].

This retrospective study was performed on archival material of forty patients diagnosed with endometrial carcinoma. Statistical analysis was performed to determine whether age, tumour grade, Figo stage, Her-2/neu, PR expression or postoperative radiotherapy affected OS or DFS.

In the present series, Her-2/neu was expressed in 6 cases (15 %). A significant correlation was found between Her-2/neu overexpression and tumor grade (p =0.032). The ratio is closely related to that reported by Gates et al. and Gul et al. whose series expressed Her-2/neu in 16.4% and 18.1% respectively of their cases [5,21]. However, in their series no significant relation with tumour grade was found. Gul et al. owed this to the relative abundance of cases in GII and the insufficient number in GIII which is the reverse to the distribution of our cases (GII: 22% and GIII: 37%)[5]. In another study, tumour grade was significantly related to Her-2/neu amplification (tested by FISH technique)[6].

As regards the depth of myometrial invasion, Her-2/neu expression was significantly associated with deep invasion of myometrium (more than $^1/_2$ of myometrial thickness). The same finding was reported by Srijaipracharoen et al. and Khalifa et al.[15,22].

Her-2/neu expression was significantly higher among stage III

cases than stage II cases (83% vs. 8%) and none of stage I cases expressed this marker. In the study conducted by Gul et al., even though relatively high proportion of Her-2/neu expression in stage III tumors (80%), there was not any statistically significant correlation because of the insufficient number of stage III cases[5]. Other researchers reported no significant relation between Her-2/neu expression and tumour stage [2,15]. The difference between their results and ours may be explained on the basis of different sample size and the inclusion of non-endometroid patterns in their cases while our cases were all of the endometroid variety.

In the present study, the expression of Her-2/neu cases had no significant association with menstrual status, invasion of the cervix or LN metastases of the patient. The same finding was observed in the studies done by Mori et al., Gul et al., Gates et al. and Brys et al.[2,5,21,23]

In EC, the main adjuvant therapy after surgery is radiation therapy (with or without chemotherapy) while hormonal therapy has more important role for advanced or recurrent diseases. The response of EC to hormone correlates directly to degree of tumor differentiation, which is in turn linked to hormonal receptor status particularly progesterone receptor levels[24]. Thus, knowing the status of these hormonal receptors would be certainly helpful in selecting treatment options. Immunohistochemistry (IHC) technique is used to evaluate steroid hormonal receptors rather than biochemical method due to the false positive results of the latter technique as a result of contamination by PR-rich normal endometrial tissue. Although some authors also reported inaccuracy of IHC method in quantitation of positive receptor status, this technique is generally used in clinical practice nowadays[15]. Patients with well-differentiated tumours and positive progesterone receptor status show better response to Oral medroxyprogesterone acetate (200 mg/day). A higher response rate may be achieved when Tamoxifen (40 mg/day) is combined with medroxyprogesterone acetate (200mg /day)[25].

Immunohistochemical analysis of PR in our cases revealed positive reaction in 24 out of 40 cases (60%). This finding was near to that reported by Srijaipracharoen et al., whose series expressed PR in 65.7% of cases(15). Other studies showed a higher proportion of PR-positive cases reaching up to 81.9 %(5). Different rates of hormonal receptors expression from various studies certainly depend on many factors, such as, proportion of low and high grade tumor which were reported to have higher and lower degree of hormonal receptors expression, respectively. Thirty-seven percent of our cases were G3 in contrast to 11.1 % of theirs.

Both of the previous studies elucidated a significant association between PR expression and lower grades of EC. This was the same in our work, as the PR expression was significantly higher in G1 and G2 than in G 3 (88%, 56%, 33% respectively, p=0.008).

PR expression was higher in tumours invading less than _ oh the myometrial thickness than those invading more than half of the thickness although this didn't reach a statistical significance. A similar result was reported by Srijaipracharoen et al. and Gates et al.(15,21). However, the researchers could establish a significant association between loss of PR expression and deep myometrial invasion (more than _ of thickness). The lack of significant association in our data can be owed to lack of non-endometroid variants which are known to invade deeper in the myometrium and have an aggressive clinical course(4).

In agreement with Gul et al.(5) no statistically, significant association between PR expression and FIGO stage of patients although PR expression was higher in stage I and II when compared to stage III of our cases. Only 2 of our cases had positive lymph nodes and both of them were PR-negavtive but no statistical significance could be detected. This is attributed to the very small number of lymph node-positive cases. To the contrary, Srijaipracharoen et al. (15) found a significant association between lymph nodes status and PR expression. Noteworthy, 20 cases (8.5%) had lymph node me-

tastases.

Other clinicopathologic factors namely menstrual status and invasion of the cervix didn't have a significant association with PR expression in our studies as well as in many other studies[15,21].

Median OS was 53 months (range 3-80 months). The 5 years survival rate was 75.5% which conforms to that reported by Srijaipracharoen et al.[15], in their series the 5 years survival rate was 80.8%.

Many prognostic factors were reported to impact both DFS and OS in EC patients such as histopathological type, stage of disease, tumor grade, and depth of myometrial invasion, cervical invasion, and lymph node (LN) status[17]. In our study, we found that stage of disease, tumor grade, depth of myometrial invasion, and cervical invasion were prognostic factors to DFS and OS.

Kaplan Meier analysis revealed a significant impact of Her-2/neu and loss of PR expression on both OS and DFS. These results come in agreement with study of Gates et al., Brys et al.[21,23]. Mori et al. [2], as well concluded that high Her-2/neu expression was a factor that negatively influenced DFS and OS rates by univariate analysis. In our study, after adjusting other known prognostic factors in multivariate analysis, Her-2/neu expression failed to retain a statistical significant impact on OS. Same results were reported by Gul et al. and Grushko et al.[5,6]. However, Her-2/neu was found to be an independent predictive factor for DSF. This finding is supported by the study of Mori et al. [2]. Other studies confirmed the independent predictive role of Her-2/neu on both OS and DFS in multivariate analysis[15]. On the other side some researchers failed to detect any prognostic significant effect of Her-2/neu. The variability in results in the literature is probably related both to a different mix of patients in different series, and the wide variety of antibodies and staining conditions used[5].

In both univariate and multivariate analysis, loss of PR expression was the only variable that

maintained a significant impact on both OS and DFS. Similarly, Gates et al.[21] found a significant relation between loss of PR expression and shortened OS in both uni- and multivariate analyses.

In another study conducted by Jongen et al. absence of PR expression appeared to be an independent prognostic factor for relapse of disease in multivariate analysis[26].

In the current study, Patients who had received adjuvant radiotherapy (RT) treatment showed shortened median overall survival compared to those who had surgery only and this could be attributed to the fact that radiotherapy was decided only for tumours with a more advanced stages in addition to that data from randomized studies such as PORTEC-1(Postoperative Radiation Therapy in Endometrial Carcinoma) and the GOG-99 (Gynecologic Oncology Group) have shown a reduction in locoregional disease recurrence but not benefit in overall survival in early stages of endometrial carcinoma namely stage I. Similar findings were reported by others. Those studies have shown that the majority of the initial recurrences for patients with disease limited to the uterus were limited to the vagina, suggesting that vaginal vault brachytherapy alone could be used as an adjuvant treatment. To compare adjuvant pelvic RT with vaginal BT alone in uterine-confined disease, the PORTEC-2 study randomized patients between those two modalities and showed very satisfactory vaginal and pelvic control rates and equal survival with both modalities[25].

Attempts to elucidate the mechanisms of signal transduction via receptors of the erbB family, and especially the sequence of events which occurs during neoplastic transformation, seem to be enormously important from the therapeutic point of view. For example, the efficacy of trastuzumab therapy (as a single agent or combined with chemotherapy) and the assessment of Her-2/neu have been proposed for treatment of recurrent or metastatic endometrial carcinoma with controversial results[7,27]. Moreover, the absence of PR expression in EC, which is known as endocrine related neo-

plasm, could be a reliable parameter in the selection of specific hormonal treatment models.

In conclusion, our study showed 15% of Her-2/neu and 60% of PR expression in endometroid type of endometrial carcinoma. Her-2/neu expression was associated with bad prognostic factors as high grade, deep myometrial invasion and advanced stage. Meanwhile, PR was found to be significantly associated with grade I-II tumor. In multivariate analysis, Her-2/neu appeared to be a poor indicator for disease-free survival and PR expression tended to show favorable influence for both overall and disease-free survivals. These results provide additional evidence of the potential prognostic role of Her-2/neu and PR expression in endometroid endometrial carcinoma.

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PROGNOSTIC SIGNIFICANCE OF HER-2 NEU AND PROGESTERONE RECEPTOR EXPRESSION IN ENDOMETROID TYPE OF ENDOMETRIAL CARCINOMA

Sylvia A. Ashamallah M.D and Dalia H. Zayed M.D

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VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN DIAGNOSIS OF COMPLICATED LIVER CIRRHOSIS

Maha Ragab Habeeb MD, Mohamed Abd El-Maksoud MD* and Lamiaa Farouk Arafa MD**

Departments of Internal Medicine, Tropical Medicine, Medical Biochemistry**, Mansoura Faculty of Medicine, Mansoura, Egypt*

Abstract

Aim: *The study aimed at evaluating the measurement of plasma levels of vascular endothelial growth factor (VEGF) in cirrhotic patients complicated with oesophageal varices (OV) and/or hepatocellular carcinoma (HCC) as a diagnostic tool in such patients as well as to compare between serum α-fetoprotein and plasma VEGF as a marker for HCC.*

Subjects and methods: *Plasma levels of VEGF and serum levels of α-fetoprotein were determined in 70 patients with liver cirrhosis divided into three groups: group 1 (G1) included 20 patients with uncomplicated cirrhosis, group 2 (G2) included 25 patients with liver cirrhosis complicated with OV and group 3 (G3) included 25 patients with liver cirrhosis complicated with OV and non-metastatic HCC. Twenty five age and sex matched healthy volunteers were selected as a control group.*

Results: *Comparison of plasma level of VEGF in different groups revealed a statistically significant increase in G2 and G3 than the control. Although the differences between patient's groups were statistically significant, the lowest values in G2 overlapped those in G1. VEGF mean plasma levels were significantly higher in patients with risky variceal signs in G2 and G3 patients. α-fetoprotein serum levels were significantly higher in all patients groups than the control. The difference was highly significant in G3 when compared with G1 or G2 values. There was no correlation between VEGF plasma level and α-fetoprotein serum levels in all patients' groups.*

Conclusion: *α-fetoprotein is a useful follow up marker for detection of HCC in patients with cirrhosis. Although VEGF cannot be used to di-*

agnose OV in patients with cirrhosis, it may be useful in suspecting appearance of risky variceal signs in cirrhotic patients.

Introduction

Cirrhosis is a chronic liver disease with a multifactorial etiology (eg, chronic hepatitis viral infection, alcoholic liver disease, and nonalcoholic fatty liver disease) that results in liver damage[1]. Liver cirrhosis constitutes a major health problem in Egypt. The primary goals of therapy for cirrhosis are to manage patients' symptoms and prevent the occurrence of cirrhosis-related complications until liver transplantation can be performed[2].

Variceal hemorrhage is perhaps the most devastating portal hypertension-related complication in patients with cirrhosis, occurring in up to 30% of such individuals during the course of their illness. Varices form at a rate of 8% per year in patients with cirrhosis and are associated with a hepatic venous pressure gradient >10 mmHg. Although mortality rates of variceal hemorrhage have been falling over the last few decades due to the implementation of effective treatments and improvements in general medical care, it still carries a mortality rate of up to 20% within 6 weeks of the bleeding episode[3].

Liver cirrhosis (LC) of divergent etiologies is at high risk of developing into hepatocellular carcinoma (HCC)[4]. About 5–15% of patients with cirrhosis can have HCC at the time of initial diagnosis of liver disease. The incidence of hepatocellular carcinoma (HCC) is rising worldwide being currently the fifth most common cancer and third cause of cancer-related mortality[5]. In fact, HCC is now the first cause of death among cirrhotic patients[6]. In Egypt, HCC was reported to account for about 4.7% of chronic liver disease (CLD) patients[7]. HCC is difficult to diagnose at early stage and has a very poor survival rate when diagnosed at a late stage[8].

Vascular endothelial growth factor (VEGF) is a potent and unique angiogenic protein that induces endothelial cell proliferation

and migration and acts as a crucial survival factor for endothelial cells[9]. Blood level of VEGF in patients with liver cirrhosis remains controversial. In recent years, vascular endothelial growth factor (VEGF) has been recognized as one of the key molecules involved in the pathophysiology of portal hypertension[10,11]. Yin et al. (2005) found increased VEGF expression in oesophageal varices of portal hypertensive rats[12].

Several studies have also suggested a relationship between the progression of chronic liver disease and hepatocarcinogenesis. Angiogenesis is essential for carcinogenesis and is induced directly by vascular endothelial growth factor (VEGF), leading to tumor growth and metastasis[13]. Although elevated circulating VEGF levels have been measured in patients with various tumours, few data are available with regard to plasma VEGF levels in patient with HCC[14].

Alfa-fetoprotein (AFP) is a commonly used tumour marker in the detection of HCC. Generally, AFP shows acceptable sensitivity; however, in some cases, AFP has poor specificity in the detection of HCC [15]. Therefore, novel biomarkers are needed to be used for early detection of cases with HCC. With the advance of cellular and biological techniques, many molecular markers have been studied such as angiogenic factors[16].

The objective of the present study was to evaluate the value of measuring VEGF plasma levels in patients with liver cirrhosis complicated with OV and/or HCC as well as to compare between serum AFP and plasma VEGF as markers in detecting HCC.

Subjects and Methods

Patients with liver cirrhosis child B and C who were admitted to Mansoura University Gastroenterology Unit and Tropical Medicine Unit were recruited in the study in the period between June 2010 and July 2012. All patients were subjected to detailed history and thorough clinical examination, routine laboratory investigation (complete blood count, liver function tests, prothrombin time kidney function tests), serum α-fetoprotein level (AFP), and viral

markers: HBs antigen by ELISA and HCV antibody by 3rd generation ELISA .

The diagnosis of cirrhosis was based on clinical findings, abdominal sonography and characteristic laboratory findings. The diagnosis of HCC was performed using clinical criteria and the findings obtained by ultrasonography (US), computed tomography (CT) or magnetic resonance imaging (MRI) [17,18]. Chest x-ray and bone scan were done to detect metastasis.

Each cirrhotic patient underwent an upper GIT endoscopy to detect oesophageal varices (OV). Patients with extra-hepatic metastasis of HCC, hypertension, D.M, uraemia and peripheral vascular occlusive diseases were excluded from the study. The selected seventy patients were divided into three groups:

- **Group (Gl):** included 20 cirrhotic patients without OV nor HCC.
- **Group2 (G2):** included 25 cirrhotic patients with OV only.
- **Group3 (G3):** included 25 cirrhotic patients with OV and HCC.

In addition, twenty five age and sex matched healthy subjects were selected as a control group (C).

Samples and assays:

Eight ml fasting venous blood samples were withdrawn from patients and control groups. Each blood sample was divided into two parts: a) 3 ml blood sample was collected in EDTA containing tube then centrifuged at 3000rpm for 5 minutes at 4°C and the separated plasma was rapidly frozen at -70°C for storage until the time of assay of VEGF. The concentration of plasma VEGF was measured using the enzyme linked immunosorbant assay (ELISA) kits according to Hyodo et al. (1998)[19]. b) 5 ml of blood sample were collected in dry tube without anticoagulant, allowed to clot at room temperature for 30 minutes, centrifuged at 3000rpm for 10 minutes and the separated serum was stored at -20°C until the assay of serum albumin, GPT, GOT, creatinineand α-fetoprotein. SGOT and SGPT were estimated by colorimetric method according to Reitman (1957)[20]. Serum albumin was estimated by colorimetric as-

say according to Webster (1977) [21]. Serum creatinine was estimated by colorImetric assay according to Henry (1974)[22]. Alfafetoprotein was estimated by enzyme linked immunosorbant assay (ELISA) kits according to Rose (1986)[23].

Statistics:

Results were expressed as mean±SD for normally distributed data. For comparison of quantitative variables in two groups the Student's t-test was used. To study the relationship between two quantitative variables Spearman's Correlation Coefficient was calculated. For comparison of qualitative data the Chi-square test was used. All tests were two tailed and considered statistically significant at $P<0.05$. These tests were done on an IBM compatible personal computer using the statistical package for social scientists (SPSS version 13).

Ethical approval:

An ethical approval was obtained from our local Ethics Committee (Medical Ethics Committee, Faculty of Medicine, Mansoura University). Patients were enrolled after written informed consent was obtained.

Results

Seventy patients (Child B and C) were included in the study with matched age and sex. All patients were suffering from liver cirrhosis and were HCV +ve. Moreover, 10%, 12%, 20% of Gl, G2, G3 groups were HBs-Ag +ve respectively. Bleeding per orifices and encephalopathy were the most important presentation in patients groups (Table 1).

Oesophegeal varices were ranging from grade II to grade IV in G2 and G3. Their number ranged from one to four varices. Risky signs were detected in 40% of G2 and 44% of G3 patient’s groups (Table 2).

Table (1): Clinical characteristics of the studied groups.

	C (n=25)	G1 (n=20)	G2 (n=25)	G3 (n=25)
Age (mean± SD)	47.6 ± 7.4	52.2 ± 9.6	49.6 ± 9	526 ± 67
Gender:				
Male	15 (60%)	8 (40%)	18 (72%)	20(80%)
Female	10 (40%)	12 (60%	7 (28%)	5(20%)
Child-Pugh score:				
B	0 (0%)	13 (65 %)	10 (40%)	14 (56%)
C	0 (0%)	7 (35 %)	15 (60%)	11 (44%)
Clinical presentation:				
*** Edema lower limb**	0 (0%)	8 (40%)	8 (32%)	12 (48%)
*** Bleeding tendency**	0 (0%)	9 (45%)	18 (72%)	20 (80%)
*** Upper GIT Hge**	0 (0%)	2 (10%)	10 (40%)	12 (48%)
*** Encepalopathy**		3 (15 %)	7 (28%)	10 (40%)
HCV+ve	0 (0%)	20 (100%)	25 (100%)	25 (100 %)
HBS Ag +ve	0 (0%)	2 (10 %)	3 (12%)	5 (20%)

Table (2): Endoscopic findings of OV in G2 and G3.

Grade	Clinical group	
	G2	**G3**
I	-	-
II	30%	20%
III	30%	40%
IV	40%	40%
Risky signs	40%	44%

Table (3): Plasma levels of VEGF (pg/ml) in different studied groups.

Data	VEGF (Pg/ml) mean±SD
Control	32.24 ± 13.55
G1	38.18 ± 6.15
G2	59.47 ± 11.29
G3	113.46 ± 25.46
Pl : G_1Vs C	0.23
P2 : G_2Vs C	< 0.0001
P3 : G_3Vs C	0.0001
P4 : G_1Vs G_2	0.002
P5 : G_1Vs G_3	0.0001
P6 : G_2Vs G_3	< 0.0001

Table (4): Serum levels of α-fetoprotein (ng/ml) in different studied groups.

Data	α-fetoprotein (ng/ml) mean±SD
Control	5.9 ± 1.8
G1	69.65± 11.20
G2	97.37±27.59
G3	511.58±131.17
Pl : G_1Vs C	< 0.0001
P2 : G_2Vs C	< 0.0001
P3 : G_3Vs C	0.0001
P4 : G_1Vs G_2	<0.01
P5 : G_1Vs G_3	0.0001
P6 : G_2Vs G_3	< 0.0001

Table (5): Correlations between α-fetoprotein and VEGF in different studied groups.

Group		α-fetoprotein	
		R	P
C	VEGF	-0.061	0.885
G1	VEGF	-0.433	0.284
G2	VEGF	-0.037	0.931
G3	VEGF	-0.531	0.175

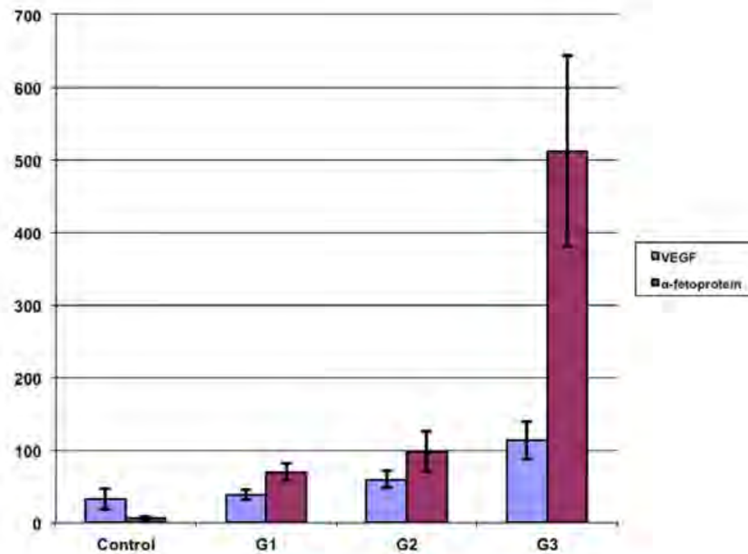

Fig. (1): Mean for different groups of VEGF and alpha-fetoprotein.

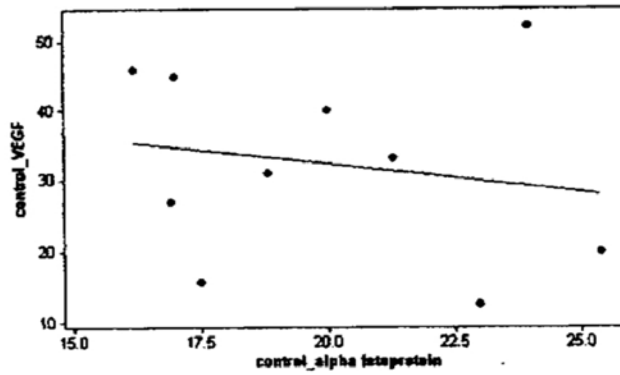

Fig. (2): Correlation between alpha-fetoprotein and VEGF in control group.

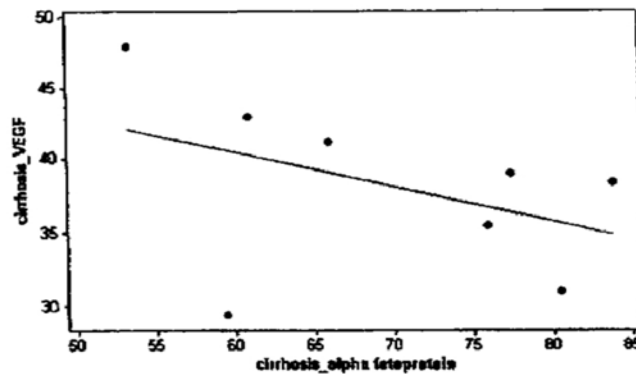

Fig. (3): Correlation between alpha-fetoprotein and VEGF in G1patients

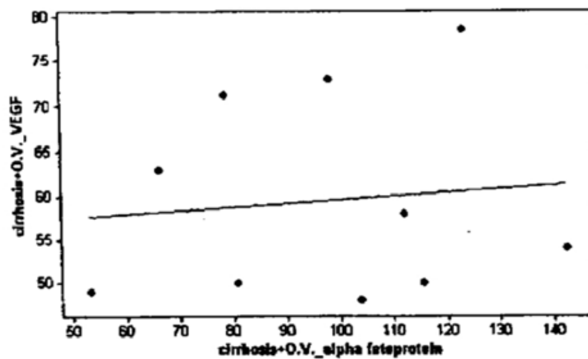

Fig. (4): Correlation between alpha-fetoprotein and VEGF in G2 patients.

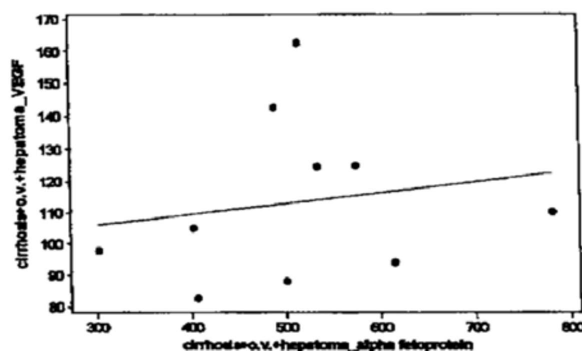

Fig. (5): Correlation between alpha-fetoprotein and VEGF in G3 patients.

Discussion

Vascular endothelial growth factor (VEGF) is a well-known angiogenic mediator and has a role in vascular permeability[24]. There are conflicting results concerning the serum VEGF in liver cirrhosis. In the present study, serum VEGF was found to be increased in all patients groups when compared with the control (Table 3). Similar results were reported by Abdel Haleemet al. (2007) and Abdelmoatyet al. (2009)[25,26]. Moreover, Jerzy et al., (2008) recorded elevated circulating levels of VEGF and its receptors in patients with liver cirrhosis[27]. In our study, the increase in plasma VEGF was insignificant in G 1, while it was significantly higher in G2 and G3 when compared to the control group (Table 3). The increased levels with cirrhosis may be attributed to the fact that chronic inflammation with hepatic fibrosis causes remodeling of the blood vessels and capillarization of sinusoids in the liver. The hepatocytes of the cirrhotic liver are therefore under sustained mechanically reduced blood flow and oxygen pressure, which are strong stimuli for angiogenesis[28]. Jerzy et al suggested that VEGF serum and receptor levels reflect the degree of impairment of hepatic function in liver cirrhosis[29]. This may indicate that the rise in plasma levels of VEGF is the end of honeymoon and the beginning of complication rather than repair.

As shown in Table 3, VEGF

mean value was significantly higher in G2 when compared with G1. However, the lowest values in G2 were very near to that in G1 which may limit the use of plasma VEGF in suspecting the occurrence of OV in patients with cirrhosis. Yin et al. (2005) investigated the expression of TNF-α and VEGF in the oesophagus of portal hypertensive rats and concluded that increased VEGF may not be an early event in development of OV and propably play a role in weakening the oesophageal wall and the rupture of OV[12]. The late stage increased VEGF can probably cause oedema of submucosal layer and lead varices losing their surrounding support, consequently resulting in the weakness of the oesophageal wall and predisposes varices to rupture by inducing vascular hyperpermeability. Li et al. (2003) demonstrated that age and plasma VEGF and basic fibroblast growth factor (bFGF) are the most significant predictors of spider angiomas in cirrhotic patients[30]. The presence of spider angiomashas been reported to be associated with oesophegeal bleeding[31].

Interestingly, our finding showed that the VEGF plasma levels were significantly higher in patients with risky variceal signs (mean±SD= 111.67±32.41 Pg/ml) than those without (mean±SD= 78.07±20.54 Pg/ml) in G2 and G3 patients groups ($p<0.0001$). This noteworthy observation suggests that plasma VEGF plasma level may be used as a biological follow up marker suggesting risky varices in patients with cirrhosis after exclusion of HCC.

VEGF has been examined for its possible relationship with liver fibrosis and portal-systemic collateral vessel formation in portal hypertension[10]. Experimental studies have shown that intestinal VEGF levels are elevated in cirrhosis[32,33]. Increased intestinal VEGF levels can exacerbate portal hypertension at least in two ways: 1) by increasing eNOS-derived NO production and subsequent vasodilation, thereby increasing the flow of blood to the portal vein and 2) by increasing angiogenesis in the splanchnic circulation, which may also contribute to increased blood flow to the portal vein. Both processes are important for the development of the hyperdynamic cir-

culatory syndrome associated with portal hypertension in cirrhosis [34]. However, it is not known what triggers the up-regulation of intestinal VEGF levels during the development of cirrhosis.

Hypervascular tumours are a key to the hypothesis that VEGF is overexpressed relative to the stage of liver disease and hepato-carcinogensis. In the present study, our finding showed a significantly higher plasma levels of VEGF in patients with HCC (G3) when compared to the control, G1 and G2 (Table 3). This may be due to their production by HCC cells for the formation of tumour vessels[35]. Ourfinding agreed those reported by Zhao et al., 2003 who measured the plasma levels of VEGF and found a markedly elevated value in the majority of patients with HCC and was closely related to more advanced stage of diseases[36]. Further studies have examined the role of VEGF in the progression of chronic liver disease and carcinogenesis[37,38]. With respect to molecular mechanism, Uematsu suggested hypoxia-induced angiogenesis through transcriptional activation of VEGF during hepatocarcinogenesis and cirrhotic change[39]. But exact mechanism remained unclear so far.

AFP is considered the most useful tumor marker in screening HCC patients,[40,41] and greater than 70% of HCC patients have high serum AFP concentration because of tumor excretion[41]. The biological and pathophysiological roles of the association of AFP with an increased risk of HCC development remain unclear. Tateyamaet al reported that AFP levels were elevated in parallel with advanced fibrosis stages and correlated well with the fibrosis stage [42]. In consistent, our study found significant increase in serum AFP concentration in HCC group when compared with control, Gl and G2 ($P<0.0001$) (Table 4). However the significant increase in AFP serum level was less prominent ($P=0.01$) between G2 and G 1 patients groups.

As shown in figure 5, VEGF showed a stepwise increase in patients groups. On the other hand, α-fetoprotein showed a very high activity in HCC group than the

other patients groups. α- fetoprotein may thus be considered more valuable in detecting HCC. There was no correlation between plasma levels of VEGF and serum levels of α-fetoprotein in each of the investigated groups (Table 5 and Figures 1-4). Thus, each marker provides independently different information and therefore it is expected to increase diagnosis accuracy if the two markers are used especially in cases with non-conclusive α-fetoprotein results.

In conclusion, our study could point to the usefulness of serum VEGF as potential prognostic factor in liver cirrhosis as it may reflect the hepatic function impairment and seems to be associated with portal hypertension symptoms. VEGF plasma level may be used as a biological follow up marker suggesting risky varices in patients with cirrhosis after exclusion of HCC. VEGF are significantly higher in HCC patient than liver cirrhosis patients without HCC. The clinical benefit of using VEGF as a biomarker in HCC diagnosis is still doubtful because their sensitivity is not more than that of AFP.

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VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN DIAGNOSIS OF COMPLICATED LIVER CIRRHOSIS

Maha Ragab Habeeb MD, Mohamed Abd El-Maksoud MD and Lamiaa Farouk Arafa MD

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CYTOKERATIN 18 AS A POTENTIAL MARKER FOR EARLY RECURRENCE OF HEPATOCELLULAR CARCINOMA AFTER SUCCESSFUL LOCAL ABLATION

Hatem Elalfy MD, Tarek Besheer MD, Mona Abo-Bakr El-Hussiny MD*, Sahar Alsayed Mohamed Alsayed MD* and Galal Alhaware MD**

Departments of Tropical Medicine, Clinical pathology, Radiology***

Mansoura Faculty of Medicine, Mansoura, Egypt

Abstract

Introduction and aim: *Evidence has now accrued that hepatocellular apoptosis plays a central role in chronic liver disease including hepatocellular carcinoma (HCC). Cytokeratin 18 (CK18) fragments have been examined as potential tumor markers in many different types of epithelial cell carcinomas, including HCC. Therefore we evaluated serum level of CK18 as a prognostic marker predicts both early recurrence of HCC after curative local ablation and macroscopic vascular invasion.* ***Methods:*** *A case control study was done on 108 patients with HCC of various Barcelona Clinic Liver Cancer (BCLC) Staging underwent percutaneous local ablation and followed up for periods 3-39 months from June 2009 till September 2012 for local recurrence, compared to 110 Control subjects classified into 80 cirrhotic patients proved by liver biopsy and 30 healthy control. We excluded conditions with high apoptosis. Serum level of CK18-M30 apoptosense analyzed in all subjects provided written informed consent.* ***Results:*** *study showed that CK18 was significantly elevated in HCC patients versus control groups (p< 0.05). There was no significant difference in CK18 pre and post successful local ablation (percutanous ethanol injection or radiofrequency ablation) (p=0.15), whereas there was significant increased basal CK18 level in patients with recurrent local lesion versus nonrecurrent (p< 0.001). Interestingly there was marked significant increase in serum CK 18 with macroscopic vascular invasion level versus those without ((p< 0.001).*

Conclusion: *CK18 M30 could be used as a prognostic marker predicts early local recurrence of HCC and macroscopic vascular invasion but it was of no value in detection successful ablation.*

Introduction

Cytokeratins are a highly complex subclass of the intermediate filaments gene family which represent a stable composition in each type of epithelial cell and has been used in identification of different epithelial tissues and their neoplasms[1-4]. In the normal human liver, hepatocytes have a very simple keratin composition: CK 8 and 18 which required for the maintenance of hepatocyte integrity[5-6], and altered expression of keratin gene is known to be related to liver diseases, including chronic hepatitis, increased hepatocyte fragility and decreased bile secretion[7]. CK 7 and 19 are useful markers of bile ducts[8-9].

Keratin molecules of cancer cells have investigated for many years which identified modulation of cytokeratin 18 (CK18) during tumor transformation in human hepatocellular carcinoma (HCC)[7]. Most of the hepatocellular adenomas and carcinomas observed in HE-stained sections were positive for CK8/18 that overexpression may drive neoplastic transformation of glutathione S-transferase placental-form positive liver cell foci during rat hepatocarcinogenesis[10-11].

Evidence has now accrued that hepatocellular apoptosis plays a central role in chronic liver disease including hepatocellular carcinoma, viral hepatitis, alcoholic hepatitis, nonalcoholic steatohepatitis, cholestatic liver disease and hepatic fibrosis and cirrhosis. CK18 fragments have been examined as potential tumour markers in many different types of epithelial cell carcinomas, including hepatocellular carcinoma[11-13].

Hepatocellular carcinoma (HCC) is a primary liver cancer and is the fifth most common cancer worldwide[14]. HCC is difficult to diagnose at early stage, and has a very poor survival rate when diagnosed at a late stage[15].

In most patients with cirrhosis, successful percutaneous ablation

or surgical resection of HCC is followed by recurrence. Intrahepatic recurrence found in patients with HCC may be either local tumor progression or intrahepatic distant recurrence. Local tumor progression (LTP) occurs along the peripheral margin of the ablative lesion and intrahepatic distant recurrence (IDR) is a new HCC tumor remote from the margin of the ablative lesion[16].

When surgery is not possible, there are several minimally invasive options for chemical or thermal tumor ablation[17-18-19]. The most frequent event observed during the follow up of curatively treated HCC patients is intrahepatic recurrence[20-22]. In cases of liver cirrhosis, many HCC recurrences develop in a multicentric fashion, it is estimated that approximately 50% of HCC is already multicentric in the early stage; but frequently HCC recurrence also shows intrahepatic metastasis, even at a relatively early stage[23]. Therefore we evaluated the apoptosis marker (Ck-18 caspase cleaved serum level M30) in HCC patients with different Barcelona Clinic Liver Cancer (BCLC) stages, its correlation for recurrence detection and analyzed the risk factors that predict HCC recurrence and vascular invasion.

Material and Methods

This hospital-based case-control study included 108 HCC patients recruited prospectively from out and inpatient clinic of Mansoura University hospital during the period from June 2009 till September 2012. 110 subjects were also included as control group and classified into 30 healthy volunteers and 80 cirrhotic patients without HCC (by biopsy were 6/6 evaluated in the context of interferon therapy). The study was approved by the Institutional Review Board of our university and an informed consent was obtained form all subjects.

The study patients with HCC were HCV infected diagnosed and classified according Barcelona Clinic Liver Cancer (BCLC)[24] staging into 59 patients BCLC-A, 32 patients BCLC-B and 17 patients BCLC-C and all with portal hypertension.

Exclusion criteria for study pa-

tients underwent to local ablation were patients with multifocal >3 lesions, large size >5, Child-C, patients with diagnostic AFP levels in cirrhotic (>200 ng/ml), haemostatic disorders, patients failed for successful ablation, HCC secondary to other liver diseases than HCV and conditions with high apoptosis e.g. extra hepatic malignancy, severe infections and any systemic disorders.

Pretreatment Studies:

The pretreatment assessment of each patient included complete history, physical examination, complete blood count (CBC), renal and liver function tests, upper endoscope, abdominal US, and triphasic multislices computed tomography (CT) of the abdomen. Cirrhosis diagnosed based on histology in cirrhotic control or clinical, laboratory, and US with child -Pugh assessment of liver function status. Portal hypertension was diagnosed in the presence of esophageal varices or splenomegaly with platelets count <100 x109/l.

Tumor ablation:

For radiofrequency ablation (RFA): under guidance using the commercial RFA system valley lab cool tip model (manufactured in USA) with needle of 15-25 cm long creating thermal ablation of zone up to 5 cm in diameter.

Percutanous ethanol injection (PEI): used in lesions less than 3 cm, a 22-gauge needle was introduced percutanously after local xylocaine infiltration, the needle advanced into the tumor or its marginal area under ultrasonographic guidance. Absolute ethanol was slowly injected with careful attention to avoid passage of ethanol into the vein adjacent to the lesion. The amount of ethanol injected each time was 5-10 ml, depending on ethanol diffusion, which was checked in real time on ultrasound. The injection was done once a week for four to six sessions[25].

In both techniques successful ablation denoted by absence of characteristic enhanced pattern in contrast enhanced CT imaging done four weeks after the procedure, PEI restricted to lesions less than 3 cm in nearby vessels or viscous or near to liver capsule or common bile duct .

Follow-up Studies:

The protocol included abdominal US, AFP assays, and Child-Pugh related tests every 4 months (more frequently when needed) and CT every 6 months in the first year after treatment and yearly thereafter (more frequently if US, or AFP suggested recurrence). All ablated patients cannot be followed up by alph-fetoprotein as it isnot elevated. Recurrence was diagnosed when enhancement reappeared within the ablation zone or 2.0 cm from its margins or appearance of new lesions[26].

Biochemical Assessment:

Anti-HCV and HCV-RNA detection will be performed locally at baseline for recruitment by standard routine Elisa and RT-PCR tests respectively. Routine laboratory tests include liver function tests (albumin, total bilirubin, ALT, AST) and serum creatinine (Dimension RXL MAX DADE BEHRING. Inc., Newark, DE, 19714-6101-USA.).

Special investigations:

Measurement of Caspase-Generated CK-18 Fragments in the Blood. Serum CK18 M30-Apoptosense [Glory Science Co., Ltd, Del Rio, TX78840, USA. ELISA assay kit]. A blood sample was obtained from each patient at the time of liver biopsy and serum stored frozen at -80°C. Serum was subsequently used for quantitative measurement of the apoptosis- associated neo-epitope in the C-terminal domain of CK-18 using the M30-Apoptosense ELISA kit. Ck-18 assay investigated in pre-ablation and post-ablation in 17 BCLC-A and 13 BCLC-B and it was found no difference in between the two levels so the study continue in preablation levels only.

Analysis of Risk Factors for Tumor Recurrence:

Recurrence rate was compared by the following possible prognostic factors: patient age, sex, serum levels of ALT, AST, albumin, bilirubin, modified Child-Pugh classification, number of tumors, size of tumor nodule, serum alpha-fetoprotein level, platelet count, in addition to Ck-18 level and BCLC stages.

Statistical analysis:

Statistical analysis of the data

was done by using Statistical Package for Social Science (SPSS) version 20.0 Qualitative variables were presented as number and percent. Quantitative variable were presented as mean ± SD. The Kolmogorov-Smirnov (K-S) Test was done to test the normality of data distribution. Comparisons for parametric data were carried out by unpaired t test for two different groups and analysis of variance (ANOVA) for more than two groups. Comparisons for non parametric data were carried out by Mann-Whitney for two different groups and kruskal-wallis H test for more than two groups. The x2-test and Fisher's exact probability test were used to compare independence. Cumulative disease-free survival was estimated using the Kaplan-Meier method and the significance of the hazard ratio for recurrence was evaluated with univariate analysis using the log-rank test. If multiple hazard ratios were proven to be significant by this test, we performed multivariate analysis using a stepwise Cox proportional hazard regression model to search for independently significant risk factors. The results were reported as ratios with 95% CI. A P-value,0.05 was considered statistically significant.

Results

Table 1 showed the baseline characteristics of the 108 patients which classified into 91 (84.26%) patients without macroscopic vascular invasion and 17 (15.74%) patients with macroscopic vascular invasion. The etiology of cirrhosis was HCV. Patients with HCC classified according to Child Pugh into 72 Child A and 36 Child B and according to BCLC staging into 59 (54.63%) patients with BCLC A, 32 (29.63%) with BCLC B and 17 (15.74%) patients with BCLC C. Among 91 patients without radiological evidence of vascular invasion 30 (33%) underwent PEI and 61 (67%) underwent to RFA and the range of followed up period for 3-39 months with end point recurrence occurance. All patients without macroscopic vascular invasion demonstrate mean of alpha fetoprotein (22.15 ±11.1) whereas patients with macroscopic vascular invasion demonstrate a range of alpha fetoprotein (1650±200.12). Ck-18 level showed a wide variability between different groups as it was mild elevated

in cirrhotic control group (92.3±13.5), moderately elevated in patients without macroscopic vascular invasion (196.6±135.5) and marked elevation in macro-vacsular invasion (538.18±229.21) Ck-18 analysis was done basally before ablation and after ablation and both group show no difference.

Apoptosis were compared between different groups including healthy control and patient groups (table 2) and this analysis revealed highly significant Ck-18 level in macrovacsular invasion (BCLC-C) (538.18±229.21) versus BCLC-A (226.1±197.8) and BCLC-B (167.71±74.3) p< 0.001. Ck-18 level showed highly significant difference as regard size of the lesion in lesion < 3cm (137.6±94.7), 3-5cm (265.5±207.5) and in lesion >5cm (576.5±280.9) p<0.001.

Analysis of Ck18 in HCC group without vascular invasion (table3) demonstrated that Ck-18 was highly significant difference in recurrent HCC (284.03±187.8) versus non-recurrent (105.6±29.1) (p<0.001). The recurrence free period demonstrated higher levels of Ck18 in earlier recurrence <12 months (306.3±212.4) versus late recurrence (12 and more than 24 months) with p value <0.001. Ck-18 level in single focal hepatic lesion (198.5±166.9) revealed elevated significant difference than multiple lesions (238.7±168.8) (p<0.04).

Table 4 summarized the results univariate analysis for prognostic risk factors associated with tumor recurrence of HCC after local ablation. It demonstrated that 8 variable were independent risk factor included increasing age, sex with female more than males by 3 times, ALT and AST levels, maximal tumor diameter with lesions between 3-5 cm more than 3 times risky than lesion < 3 cm, multiplicity of lesions with 2 lesion more risky by 2.5 times than single lesion, Ck-18 serum levels (p<0.05). A multivariate analysis using a Cox proportional hazard model and eight parameters selected based on the results of the univariate analysis revealed that only increasing age, female sex, increasing maximal tumor diameter, high Ck-18 serum levels were the prognostic factors that independently statistically significant

for prediction of HCC recurrence ($p<0.05$).

Ck-18 showed (table 5) significant positive correlation to serum bilirubin and AFP levels and significant negative correlation to ALT, AST and platelet counts with no significant correlation to other parameters.

Analysis of the data in figure 1 using kaplan-meier estimates of the survival probability for patients with Ck-18 caspase cleaved levels in which the censored cases were non-recurrent cases. Ck-18 level <180 over time 1 and 2 years; survival probability were 70.9% (95% confidence interval 59.6-82.2 months) and 64.5% (95% confidence interval 52.6-76.4 months); whereas the level >180 survival probability were 34.4% (95% confidence interval 17.18-51.7 months) for one year and all cases showing recurrence at less than 2 years .

Table (1): Clinical, biochemical and demographic characteristics of study population.

	Controls		PATIENTS	
	Healthy	**Cirrhotic**	**HCC without macro vascular invasion**	**HCC with macro vascular invasion**
Number	30 (27.27%)	80 (72.73%)	91 (84.26%)	17 (15.74%)
Age (years)	48.47 ± 6.43	50± 6.7	52.66 ± 5.67	51.25 ± 5.3
Sex male female	17 (56.6%) 13 (43.4%)	45 35	42 (46.1%) 49 (43.9%)	11 (64.7%) 6 (35.3%)
Child Class. A B	- -		63 (69.2%) 28 (30.8%)	9 (52.9%) 8(47.1%)
BCLC A B C	- - -	- - -	59 (64.8%) 32 (35.2%) -	- - 17
ALT (IU/l)	16±3.7	64 ±17.2	62.46±40.15	120.2 ±48
AST (IU/l)	19±4.5	78 ±15.6	69.7±43	115.2±37.7
AFP (ng/ml)	5.14 ± 2.34	21±9.3	22.15 ± 11.1	1650±200.12
INR	0.92 ± 0.12	1.2±0.37	1.12 ± 0.14	1.3 ± 0.07
S. Albumin (g/dl)	4.12 ± 0.52	3.9±0.4	3.25 ± 0.36	3.2 ± 0.36
S. Bilirubin (mg/dl)	0.61 ± 0.42	0.7±0.2	1.19 ± 0.36	1.48 ± 0.28
Platelet counts ($\times 10^9$/l)	195.56±36.41	175.23±56.5	80.05 ± 28.95	85.6 ± 11.7
Ascites	-	-	24 (26.3 %)	15 (88.2%)
CK18 (ng/ml)	65.0 ± 10.17	92.3 ± 13.5	196.6 ± 135.5	538.18±229.21

Table (2): Analysis of Ck-18 in HCC with and without macro vascular invasion.

	No	Ck18 (ng/ml)	P value
BCLC- A BCLC- B BCLC-C	59 (54.6%) 32 (29.6%) 17 (15.8%)	226.1 ± 197.8 167.71 ± 74.3 538.18 ± 229.21	< 0.001
Sex Male Female	53 (49.07%) 55 (50.93	256.9 ± 237.01 258.8 ± 193.1	0.976
Child class A B	72 (66.7%) 36 (33.3%)	230.8 ± 186.7 312.2 ± 256.3	0.09
Maximal tumor diameter < 3cm 3-5 cm >5cm	21 (19.4%) 81 (75%) 6 (13.6%)	137.6 ± 94.7 265.5 ± 207.5 576.5± 280.9	< 0.001

Table (3): Analysis of ck-18 in HCC without macro vascular invasion.

	No	Ck18 (ng/ml)	P value
Recurrent free period < 12m 12-24m > 24m	38(41.7%) 13(14.3%) 40(44%)	306.3 ± 212.4 218.9 ± 42.06 105.6 ± 29.1	< 0.001
Recurrent Non-recurrent	51 (56%) 40 (44%)	284.03 ± 187.8 105.6 ± 29.1	< 0.001
Ascites Present Absent	24(26.3%) 67(73.7%)	192.6 ± 68.2 210.2 ± 190.6	0.51
Multiplicity Single lesion Double lesion	79 (86.8%) 12 (13.2%)	198.5 ± 166.9 238.7 ± 168.8	0.04

Table (4): Univariate and Multivariate analysis Showing Predictors of First Recurrence.

		Univariate			Multivariate		
		P value	95%CI	HR	P value	95%CI	HR
Age (years)		0.009	1.04-1.1	1.05	0.000	1.06-1.3	1.19
Sex	Male Female	0.000	0.16-0.5	1 3.3	0.01	0.1-0.8	1 3.3
ALT (IU/l)		0.005	0.97-0.99	0.98	0.6	0.98-1.01	0.9
AST (IU/l)		0.001	0.96-0.97	0.97	0.11	0.95-1.006	0.9
Maximal tumor diameter		0.000	1.7-4.03	2.7	0.002	1.5-7.1	3.3
Ck18 (ng/ml)		0.000	1.005-1.009	1.007	0.00	1.002-1.007	1.004
Size subclass	< 3cm 3-5 cm	0.008	0.11-0.7	1 3.57	0.09	0.09-1.2	1 3.3
Multiplicity	1 lesion 2 lesions	0.005	0.2-0.7	1 2.5	0.8	0.4-1.8	1 1.1

Table (5): Correlation of Ck-18 level with demographic and laboratory parameters.

	CK18 (ng/ml)	
	R	P
Age (years)	0.03	0.7
ALT (IU/l)	-0.3	0.000
AST (IU/l)	-0.37	0.000
Alb (g/dl)	0.09	0.38
Bili (mg/dl)	0.3	0.002
PT (seconds)	-0.07	0.4
Platelet (×109/l)	-0.56	0.000
AFP (ng/ml)	0.2	0.02

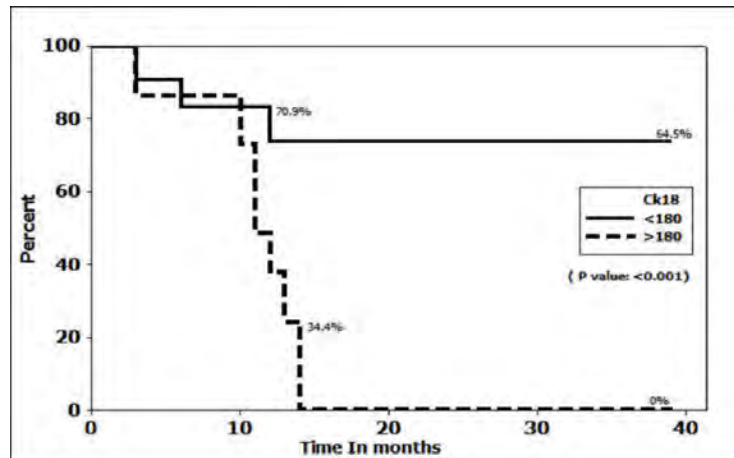

Fig. (1): Survival probability for patients with Ck-18 caspase cleaved levels in which the censored cases were non-recurrent cases.

Discussion

HCC is multi-factor process that acts in combinations including viral infection, oncogen activation, apoptosis, cirrhosis development and inactivation of tumor suppressor genes. This study evaluated the apoptosis in HCC patients with different BCLC stages and follow up for recurrence detection and analyzed prognostic risk factors for predicting the HCC recurrence with stress on the association of apoptosis marker (Ck-18 caspase cleaved serum level M 30) to the recurrence rate.

Many studies on Ck-18 and its relation to hepatocellular carcinoma show an evidence in playing an important role in tumorigenesis of hepatocellular carcinoma.

kawai et. al. 2009[10] showed that Ck-18 may become a useful immunohistochemical marker for detecting hepatocellular proliferative lesions while Gonzalez-Quintela and co-workers[27] found a fragment of Ck-18 with highest level in patients with hepatocellular carcinoma, over expression of Ck8/18 in hepatocellular carcinomas has been previously illustrated by immunohistochemistry. The results of experimental studies conducted by kawai and coworkers[10] suggest that Ck8/18 immunohistochemistry can be used as marker for detecting liver preneoplastic and neoplastic lesions in mice. In accordance with these studies our results indicate a highly significant Ck18-m30 levels in the metastatic group with major macrovascular invasion with significant elevation of alpha fetoproteins >400 ng/ml while the other patients groups either BCLC-A or B have significant elevation in comparisons to both control groups either healthy control or cirrhotic control (fibrosis score 6/6 by liver biopsy).

Results of the present study demonstrated highly significant Ck-18-m30 levels in recurrent lesions versus non recurrent (p value<0.001); also in follow up the earlier recurrent lesions <12 months have a higher levels than other recurrent lesions.

A previous studies denotes increases in Ck-18 fragments in serum from patients with breast, liver and lung cancers, interpreted to reflect spontaneous apoptosis of tumor cells[28-29], this is consistent with the finding of the present study which denotes a significant increase in Ck-18-m30 fragments in HCC patients in comparison to HCV related liver cirrhosis. In this study not all HCC stages were similar, the macrovascular invasion group demonstrate marked elevation. This could be explained by increase in the spontaneous apoptosis of HCC cells released into blood vessels. It will be interested to study Ck-18 serum levels changes in vascular invasion of HCC in larger sample sizes.

This study demonstrated a significant association between Ck-18 levels and tumor size, this could be explained in the light of

increase the microvascular invasion; mchugh and co-workers found microvascular invasion is strongly associated with tumor size and AFP >100 ng/ml and greatly increase the risk of recurrence after transplantation for HCC[30].

Microscopic vascular invasion was an independent risk factor for both recurrence and poor survival even after liver transplantation [31-32]. Many studies have demonstrated that microvascular is risk factor for early tumor recurrence after resection[33-34]. Chen and co-workers found three independent factors associated with residual tumor and early recurrence after resection were high preoperative serum alpha-fetoprotein level, larger tumor size and micro vascular invasion[35].

It is important for hepatologist to assess patients prognosis for the purpose of determining the treatment options and improving the long term outcome, thus knowledge the risk factors of recurrence could be helpful for hepatic diseased patients which detected in our study by cox regression model: increasing age, female sex, increasing maximal tumor diameter, higher Ck-18 serum levels were independent risk factors that predict HCC recurrence. Lai et al[36] showed strong correlation detected between AFP values, tumor dimensions, and microvascular invasion, all well-known predictors of HCC recurrence. In contrast to this study Ng et al[37] reported that the age and sex of patients had no independent influence on the risk of recurrence. Women with HCC appear to have a better prognosis than men.

Limitations of the study include relatively small number of patients so results obtained from this study need further studies with larger sample size to be disseminated to try to get cutoff value for CK-18 serum measurement in HCC recurrence. However patients express high levels of this marker require carful surveillance.

In conclusion serum CK18 M30 could be used as a prognostic marker Predicts early recurrence of HCC and vascular invasion but it was of no value in detection successful ablation.

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CYTOKERATIN 18 AS A POTENTIAL MARKER FOR EARLY RECURRENCE OF HEPATOCELLULAR CARCINOMA AFTER SUCCESSFUL LOCAL ABLATION

Hatem Elalfy MD, Tarek Besheer MD, Mona Abo-Bakr El-Hussiny MD, Sahar Alsayed Mohamed Alsayed MD and Galal Alhaware MD

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FUROSEMIDE VS. ACETYLCYSTEINE FOR MANAGEMENT OF POSTOPERATIVE PULMONARY COMPLICATIONS; A CONTINUOUS CHALLENGE IN ICU

Ghada El Rahmawy MD, Doaa G. Diab MD, Reem Abd El Raouf MD and Aida M. Yousef MD*

*Department of Anesthesiology and * Department of chest, Faculty of Medicine, Mansoura Univresity, Egypt*

Abstract

Management of postoperative pulmonary complications is a continuous challenging work. ***Aim:*** *comparing the effect of nebulized furosemide versus oral acetylcysteine on the postoperative pulmonary complications after major abdominal surgery.* ***Patients and methods:*** *After approval of Ethics committees protocol, written informed consent was obtained from all patients. This randomized prospective study was conducted on 58 patients ASA I-II aged from (20-60 years), admitted postoperative to the surgical ICU following open major abdominal surgeries under general anesthesia (GA) and suitable size ryle tube was inserted for each patient of the studied groups. Postoperatively, oxygen through an air-entrainment mask was given for all patients with FIO2of 0.5. Eligible patients were randomly allocated to receive one of the two study protocols via sealed envelope assignment. Patient either involved in the furosemide (F) group n=29 where the patient is treated by 20mg furosemide diluted in 10 ml normal saline. Nebulization setting is done for 10 min. While in acetylcysteine (A) group n=29 the patient is treated by oral acetylcysteine sachets 600 mg in 75ml of water through ryle tube then the tube is closed for 10 minutes. Both studied drugs were given every 8 hours for the first 3 days postoperative. Arterial blood gases, Spirometric values includes FEV1, FVC and FEV1/FVC ratio were recorded pre and postoperatively after drugs administrations at 6hours, 24 hours and 48 hours. Postoperative, modified Borg rating scale for dyspnea and duration of ICU stay were also recorded. Any patient need mechanical ventilation either non Invasive or invasive was exclud-*

ed from the study. ***Results:*** *There was no significant statistical differences among both groups as regarded age, weight, height, sex, duration of surgery and anesthesia. But, there was a significant decreased in the duration of ICU staye in (F) group than (A) group. There was insignificant differences in between both groups in the arterial blood gases (Ph, PaO2, and PaCO2) preoperatively and postoperatively for 48 hours while, there was a statistically significant increased in pulmonary function tests includes FEV1,FVC and FEV1/FVC ratio at 6 hours and 24 hours postoperatively in group(F) in compared with group (A). There was statistically significant decreased in modified borg rating scale for dyspnea in (F) group than (A) group at 6hours, 24 hours and 48 hours postoperatively. Conclusion: nebulized furosemide improve the postoperative pulmonary outcome and reduce the ICU stay compared to oral acetylcysteine after major abdominal surgery.*

Keywords: *Furosemide, Acetylesystine, postoperative pulmonary complications.*

Introduction

Postoperative pulmonary complications as (bronchospasm, pneumonia, excessive bronchial secretions with productive cough or hypoxemia up to respiratory failure...) that are related to anesthesia and surgery considered as life threatening factor for postoperative mortality and morbidity[1]. Although aggressive management is mandatory to reduce this mortality, prophylactic and supportive measures would be more better that is could be achieved by good choice of anesthetic technique, postoperative analgesia and physiotherapy[2]. Mucolytics like acetylcysteine has been used to reduce sputum viscosity making expectoration easier and improving oxygenation[3]. It also has vasodilator properties by increasing cyclic GMP levels and by contributing to the regeneration of endothelial-derived relaxing factor beside its antioxidant[3,4]. Moreover, along with the conventional bronchodilatators, there is so called "extraordinary bronchodilatators" have appeared as furosemide[5].

Many studies have proven and concluded that aerosolized fruosemide prevents bronchospasm, and may ameliorate dyspnea because of its bronchodilatory effects[6].

Inhaler therapy has several advantages over other routes when treating lung disease; Drug delivery via the lung allows direct delivery to the site of action, for small quantity of drug to be enough for an adequate response, an onset of action that is usually faster, compared to injectable dosage forms: inhaled drug therapy is painless and the systemic bioavailablity has less variability in side effects and efficacy than other drug delivery methods[7].

Current study was designed to compare the effect of nebulized furosemide versus oral acetylcysteine on the postoperative pulmonary complications after open major abdominal surgeries.

Patients and Methods

After approval of Ethics committees protocol, explanation of the study to the patient, written informed consent was obtained from all patients. This randomized prospective study was conducted on 58 patients ASA I-II aged from (20-60 years), admitted postoperative to the surgical ICU following open major abdominal surgeries e.g. (open cholcystectom, splenectomy, colectomy, hernioraphy with mesh repair ....etc.). This study was conducted at surgical ICU of Mansoura University Principal hospital from January -till June 2012. Patients with history of smoking, hypersensitivity to acetylcysteine, or morbid obesity were excluded from the study. Routine monitors were applied for recording heart rate (HR), mean arterial blood pressure (MBP), respiratory rate (RR) and peripheral oxygen saturation (SpO2) as a (Baseline values) before surgery and drugs administration.

General anesthesia (GA) standardized for all study groups was induced with fentanyl (1ug/kg) and thiopentone sodium (3-5 mg/kg), succinyl choline (1mg/kg) was used to facilitate tracheal intubation. After induction of anesthesia, suitable size ryle tube was inserted for each patient of the studied groups. Anesthesia was maintained with end tidal isoflurane (0.5-1 MAC) and muscle relaxation achieved by atracurium (0.5mg/kg) with IV morphine (0.1mg/kg) for analgesia. Postoperatively, oxygen through an air-entrainment mask was given for

all patients with FIO2of 0.5. Eligible patients were randomly allocated to receive one of the two study protocols via sealed envelope assignment. Patient either involved in the Furosemide (F) group n=29 where the patient is treated by 20mg furosemide diluted in 10ml normal saline. Nebulisation setting is done for 10 min. While in acetylcysteine (A) group n=29 the patient is treated by oral acetylcysteine sachets 600mg dissolved in 75ml of water through ryle tube and is closed for 10 minutes. Both studied drugs were given every 8 hours for the first 3 days postoperative. Arterial blood gases, Spirometric values includes FEV1, FVC, FEVI/FVC are recorded by (Smart PFT, Medical Equipment Europe (MEE) Spirometry, GmbH) preoperatively and postoperatively after drugs administration at 6hours, 24 hours and 48 hours. Postoperative, modified Borg dyspnea rating scale(8) which includes: (0)= nothing at all, (0.5)= very, very slight (just noticeable), (1)= very slight, (2)= slight (light), (3)= moderate, (4)= somewhat sever, (5)= sever (heavy), (7)= very sever, (10)= very, very sever, maximal. Also, duration of ICU stay were recorded. Any patient needs mechanical ventilation non invasive or invasive was excluded from the study.

Statistical analysis: Sample size was calculated by using t test for mean in G *power 3.1.5 program. According to pilot study (5 patients in each group) we calculated that 29 patients per group were sufficient to give $p<0.05$ significant with confidence interval of 95% with actual power of 96% when mean value of FEV1/FVC ratio at the 6 hours postoperative in group (A) was 0.75 and in group (F) was 0.79.

Statistical analysis was carried out using the Statistical Package for Social Sciences 16 (SPSS Inc., Chicago, IL, USA). Data was presented as number, percentage, means and standard deviations. Parametric data were analyzed using Student t test. Non parametric data were analyzed by Mann-Whitney test. chi-square test was used for comparison between percentages and frequencies. Significance level was established at a P value ≤ 0.05.

Results

The patients characteristics of both groups are shown in (Table-1), with no significant statistical differences among both group as regarded age, weight, height, sex, duration of surgery and anesthesia. But, there was a significant decreased in the duration of ICU stay in (F) group than (A) group (p 0.004). No patients excluded from the study.

There was insignificant differences in between both groups in the arterial blood gases (Ph ,Pao2, and PaCo2) preoperatively and postoperatively for 48 hours ($p>0.05$). (table 2). There was a statistically significant increased in pulmonary function tests includes FEV1, FVC and FEV1/FVC ratio at 6 hours and 24 hours postoperatively in group (F) in compared with group (A). FEV1 was significantly high at 6 hours postoperative (p0.001) and at 24 hours postoperative (p 0.02) in group (F) in compared with group (A). FVC was significantly high at 6 hours postoperative (p 0.02) and at 24 hours postoperative (p 0.04) in group(F) in compared with group (A). FEV1/FVC was significantly high at 6 hours postoperative (p 0.04) and at 24 hours postoperative (p 0.003) in group (F) in compared with group (A) (table- 2).

There was statistically significant decreased in modified borg dyspnea rating scale in (F) group than (A) group $p<0.05$ at 6hours (0.8 ± 0.5) in (F) group vs. (2.1 ± 0.8) in (A) group, at 24 hours (0.4 ± 0.3) in (F) group vs. (1.2 ± 1.1) in (A) group and at 48 hours(0.2 ± 0.4) in (F) group vs. (1.1 ± 0.9) in (A) group (figure 1).

Table (1): Patients characteristics.

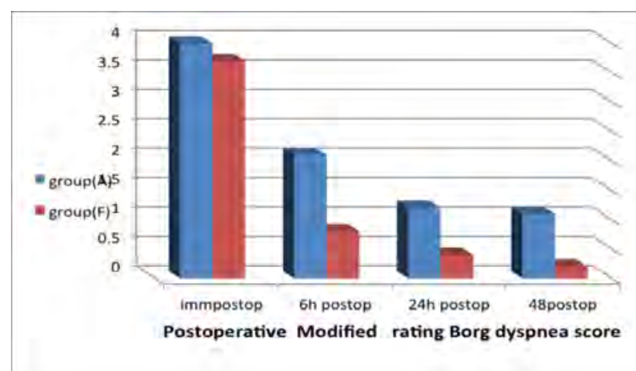
	Group (A)	Group(F)	P value
Age(year)	51(24-62)	53(26-63)	
Sex male/female	11/18	9/20	
Weight(kg)	78(52-105)	75(50-110)	
Height (cm)	165(145-170)	162(150-175)	
Operative time(min)	108±23.6	115± 19.2	
Anesthesia time(min)	120±18.5	125±15.6	
Duration of ICU stay	3.8±1.8	2.1±1.1*	0.004

Group (A): N acetyl cystine group Group (F): Furosemide group
Values are presented as median (range) or mean ±SD
ICU, intensive care unit. * significant when p≤0.05

Table(2):Pulmonary function tests and ABG preoperative and postoperative for 48 hours

	Group(A)				Group(F)			
	preop	**6h postop**	**24h postop**	**48h postop**	**preop**	**6h postop**	**24h postop**	**48h postop**
FEV1	2.8 (2.5-2.9)	1.9 (2.3-2.9)	2.6 (2.1-2.7)	2.8 (2.6-2.9)	2.9 (2.6-2.9)	2.4 (2.3-2.5)*	2.9 (2.5-3.1)*	2.9 (2.7-3)
FVC	3.2 (2.9-3.4)	2.7 (2.3-2.9)	3(2.8-3.2)	3.2 (2.9-3.4)	3.4 (2.9-3.6)	2.9 (2.3-2.5)*	3.3 (2.9-3.6)*	3.3 (3.1-3.6)
FEV1 /FVC	0.83 (0.77-0.93)	0.77 (0.69-0.85)	0.84 (0.77-0.88)	0.85 (0.81-0.93)	0.84 (0.78-.92)	0.82 (0.78-0.89)*	0.84 (0.86-.93)*	0.85 (0.79-0.92)
Ph	7.38± 0.03	7.33± 0.02	7.36± 0.05	7.35± 0.03	7.36± 0.04	7.32± 0.03	7.35± 0.05	7.37± 0.02
PaO2	86.6± 8.2	85.6± 6.5	87.5± 5.3	89.8± 3.4	88.2± 6.4	89.4± 4.2	90.4± 3.2	90.4± 6.4
PaCO2	36.6± 1.7	38.7±1.9	36.8±1.6	37.1±1.3	35.2±1.8	34.2±1.4	34.7±1.5	36.2±1.7

Group (A): N acetyl cystine group Group (F): fusamide
Values as median (range) or mean± SD *significant when p<0.05



Group (A): N acetyl cystine group **Group (F):** fusamide
Fig. (1): Mean values of Modified borg' rating dyspnea scale in the studied groups for 48 hours postoperative.

Discussion

Previous studies reported that acetylcysteine has been used in various respiratory diseases as it has mucolytic and antioxidant effect lowering the incidence of post-operative atelectasis[3,4]. However, it has proven that it has broncho-constrictor effect on the tracheo-bronchial tree because of its supposed irritant effect which may regress its benefit[9].

Present study demonstrated that nebulised Furosemide improved the pulmonary functions significantly and mostly (FEV1& FVC) & (FEV1/FVC) that is in agreement with Raj Kumar et al, 2003[10] as he concluded that nebulized Furosemide improved the spirometric values, explaining that by the bronchodilator effect of Furosemide nebulization but this study was conducted on asthmatic patients with the dose of (40mg) Furosemide in 1ml normal saline.

Current work reported that modified borg' dyspnea rating scale was significantly lower in Furosemide group in compareson to acetylcystien group. In the same line Kohara and coworkers [11] proved the role of aerolized Furosemide (20mg) in reducing the dyspnea in terminal cancer patients with reduction of the respiratory rate and use of accessory respiratory muscles with no change in arterial blood gases or heart rate. The possible mechanism of action of inhaled Furosemide is not clear. However, the reduction of dyspnea by nebulized furosemide may be attributed to suppression of pulmonary C- fibers in bronchial epithelium[12]. It also inhibits cough and bronchospasm as the result of lung exposure to aerosolized low chloride solutions of Furosemide, it induces ionic changes in the cellular environment which prevents stimulation of irritant receptors[13]. Furosemide also stimulates pulmonary stretch receptors and may relieve dyspnea by the effects of large tidal volume through sensitization of lung inflation receptors [14] and release of the bronchodilator prostaglandins from the airway epithelium.

Moreover, Previous study reported that Furosemide reduces the epithelial potential difference

and short the circuit current in airway, that may has direct effect on airway epithelial cells[15]. Also, its diuretic effect cause reduction in pulmonary oedma that decrease respiratory effort[16].

So, the conclusion of the present study is that nebulized Furosemide improve the postoperative pulmonary outcome and reduce the duration of postoperative ICU stay compared to oral acetylcysteine after major abdominal surgery.

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FUROSEMIDE VS. ACETYLCYSTEINE FOR MANAGEMENT OF POSTOPERATIVE PULMONARY COMPLICATIONS; A CONTINUOUS CHALLENGE IN ICU

Ghada El Rahmawy MD, Doaa G. Diab MD, Reem Abd El Raouf MD and Aida M. Yousef MD

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MECHANISM OF ERYTHROPOIETIN HORMONE IN JEJUNAL MOTILITY IN EXPERIMENTAL ANIMALS

Ahmed A. Elgendy MD and Abeer F. Mostafa MD

Department of Medical Physiology, Faculty of Medicine, Mansoura University. Egypt

Abstract

Background: *Erythropoietin (Epo) receptors are present on the epithelium of the gastrointestinal tract (GIT) but the role of Epo in the GIT is not known.*

Aim: *The aim of the present work was to study whether erythropoietin hormone alters the jejunal motility in experimental rat. Another aim was to clarify the postulated mechanisms of erythropoietin in jejunal motility.*

Study design: *This work was done on isolated segment jejunal strips. These specimens obtained from albino rats. The jejunal strips were dissected and isolated in aerated kreb's solution. The specimens were washed, prepared for application for recording its motility by Bridge Amp connected to a Power Lab recording unit with its isotonic transducer. The dose dependant effects of Epo (10,20 and 30 IU/mL) were studied in this study which classified into four groups; Group I experiments: were carried out to study the effect of erythropoietin (10,20 and 30 IU/mL)on the basal motility of jejunum, Group II experiments were carried out to study the effect of erythropoietin (10and 30 IU/mL) on the motility of jejunum after adding Ach (10^{-5}M), atropine sulphate (10^{-6}M), norepinephrine ($3x10^{-6}$M), phentolamine (10^{-6}M), or propranolol hydrochloride (10^{-6}M), Group III experiments: were carried out to study the effect of erythropoietin (10and 30 IU/mL)on the motility of jejunum after adding verapamil hydrochloride (10^{-6}M) Flunarizine (50 μM) which block T- calcium channels and caffeine (2 mM) which depletes calcium stores, Group IV experiments were carried out to study the effect of erythropoietin (10 and 30 IU/mL) on the motility of jejunum after adding sodium nitroprosside (10^{-5}M) or L-NAME (10^{-4}M) and lidocaine (3×10^{-3}M).*

Results: *The results showed that erythropoietin produced a signifi-*

cant increase in the contraction of the jejunum. This stimulatory effect of erythropoietin may be mediated through voltage independent calcium channels.

Conclusion: *Erythropoietin induces a stimulatory effect on the motility of jejunum. The stimulatory effects of erythropoietin is mediated mainly through activation of non voltage gated calcium channels not mediated through nitric oxide (NO) release from the nitrergic neurons or from activation of cholinergic neurons so Ca^{+2} is essential for the stimulatory effects of erythropoietin on the motility of jejunum.*

Introduction

Erythropoietin (Epo): A hormone produced by peritubular cells in the kidneys of the adult and in hepatocytes in the fetus[1]. Small amounts of extra-renal Epo are produced by the liver in adult human subjects. Human Epo has a molecular weight of 34,000[2]. The Epo gene has been found on human chromosome 7 (in band 7q21)[3]. Erythropoietin (Epo), a hypoxia-inducible cytokine, is required for survival, proliferation, and differentiation of erythroid progenitor cells. and its receptors are found all over the body[4]. Other effects of Epo include a hematocrit-independent, vasoconstriction-dependent hypertension [5], increased endothelin production[6], upregulation of tissue renin[7], change in vascular tissue prostaglandins production[8], stimulation of angiogenesis[9], and stimulation of endothelial and vascular smooth muscle cell proliferation[10]. Erythropoietin receptors expression has also been observed in the central nervous system and in neuronal cell lines[11]. Recombinant human Epo (rHuEpo) is currently being used to treat patients with anemia associated with chronic renal failure, AIDS patients with anemia due to treatment with zidovudine, nonmyeloid malignancies in patients treated with chemotherapeutic agents, perioperative surgical patients, and autologous blood donation[12].

Erythropoietin (Epo) stimulates a significant increase in the intracellular calcium concentration ($[Ca^{2+}]i$) through activation of the transient receptor potential channel TRPC2[13,14,15]. Since most erythropoietin is produced by the kidney it should make sense that

a damaged kidney cannot produce normal amounts of erythropoietin and anemia results. Erythropoietin injections are very effective and easy to administer by owners at home. Resolution of anemia leads to better appetite, more energy and higher life quality.

Because of the presence of erythropoietin and its receptors in the stomach and small intestine, it would be reasonable to expect its participation to bowel functions. In fact, there are no studies have provided evidence of a close relationship between erythropoietin and small intestinal motility. Erythropoietin (Epo) receptors are widely expressed in the small bowel of neonatal rats and evidence suggests Epo has important trophic effects in developing bowel [16]. Erythropoietin reduces ischemia–reperfusion injury in the small intestine of rats[17]. It is clear that the effects and the mechanism of Erythropoietin induced changes in gastrointestinal motility have not been fully understood.

Aim of the study:

The principal objective of this study was to investigate, clarify the effect of Erythropoietin on the jejunal motility in rat and to study the possible mechanisms of its action.

Material and Methods

The experiments were carried out on 30 Albino Wister rats of both sexes weighing 100-180 gm ,obtained from the Mansoura University animal House, 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. They were classified into 4 groups. This research was approved by the Medical Research Ethics Committee of Mansoura University. Segments from the jejunum were mounted in 5 ml organ bath chambers containing continuously oxygenated kreb's solution[18]. The motility was continuously recorded with a power lab recording unit and further analyzed with chart 7 software (The used apparatus for recording of gut motility in the present study was Bridge Amp

connected to a Power Lab 4/30 recording unit with its High Grade isotonic transducer and further analysis with chart 7 software).

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 kreb's solution (118.1 mM Nacl, 4.69 mM kcl, 2.5 mM Cacl2, 25 mM NaHco3, 1.2 mM KH2 Po4 1.2 mM Mgso4 and 11 mM glucose). Segments from the jejunum were mounted in 5 ml organ bath chambers containing continuously oxygenated kreb's 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 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:

Group I: to study the effect of Erythropoietin (Eprex 10,20,and 30 IU, available in 1 cc injection vials containing 2000 units, 3000 units, 4000 units, 10,000 units, 20,000 units and 40,000 units per vial) on the basal motility of jejunum[19,20].

Group II: to study the effect of Erythropoietin on the motility of after adding acetylcholine (Ach) 10^{-5}M[21], atropine sulphate (10^{-6} M)[19], norepinephrine ($3x10^{-6}$M) [22], propranolol hydrochloride (10^{-6}M)[23], or phentolamine (10^{-6}M)[24].

Group III: were carried out to study the effect of erythropoietin (10, and 30 IU/mL) on the motility of jejunum after adding Verapamil hydrochloride (Isoptin) (10^{-6}M) [25], Flunarizine (50 μM)[26] and caffeine (2 mM)[27].

Group IV: to study the effect of Erythropoietin (10, and 30 IU/mL) on the motility of jejunum after adding sodium nitroprosside (10^{-5}M)[23]. or L-NAME (10^{-4}M) [28] and lidocaine (3×10^{-3}M) [29].

All sacrificed animals had been disposed by safety cabinet in MERC (Medical experimental re-

search center) in Mansoura University.

Statistical Analysis

The ANOVA procedure was used, to test significance of difference among the means of more that two groups of the investigated and control groups. P is significant if < or = 0.05[30].

Results

Table (1-a): Dose dependent effects of Erythropoietin on amplitude and the tone of jejunal motility.

N= 8	Amplitude (mm)		Tone (mm)	
	Basal	Effect	Basal	Effect
Erythropoietin 10 IU/mL **Mean±SD** **P**	0.52±0.11	0.74±0.14 > 0.05	- 21.32±4.54	- 18.47±3.65 > 0.05
Erythropoietin 20 IU/ml **Mean±SD** **P**	0.52±0.11	1.16±0.45 < 0.05	- 21.32±4.54	-16.57±6.8 < 0.05
Erythropoietin 30 IU/mL **Mean±SD** **P**	0.52±0.11	**1.37 ±1.56** < 0.001	- 21.32±4.54	**5.34 ±14.9** < 0.001

N: number of contractions (8).
P: values as compared with the basal contractions.

Table (1-b): Effects of Erythropoietin in calcium free solution on amplitude and the tone of jejunal motility.

N= 8	Amplitude (mm)		Tone (mm)	
	Basal	Effect	Basal	Effect
Erythropoietin10 IU/mL **Mean±SD** **P**	0.21±0.05	0.22±0.03 > 0.05	0.98±0.23	0.97±27 > 0.05
Erythropoietin30 IU/mL **Mean±SD** **P**	0.21±0.05	0.23±0.06 > 0.05	0.98±0.23	0.79±0.25 > 0.05

N: number of contractions (8).
P: values as compared with the basal contractions.

Table (1-c): Effects of Erythropoietin in calcium excess solution on amplitude and the tone of jejunal motility.

N= 8	Amplitude (mm)		Tone (mm)	
	Basal	Effect	Basal	Effect
Erythropoietin10 IU/mL **Mean±SD** **P**	0.11±0.003	0.26±0.10 < 0.05	-0.8±0.001	1.02±0.56 < 0.001
Erythropoietin30 IU/mL **Mean±SD** **P**	0.11±0.003	0.34±0.14 < 0.001	-0.8±0.001	2.07±073 < 0.001

N: number of contractions (8).
P: values as compared with the basal contractions.

Table (2-a): Effects of Erythropoietin on jejunal motility after adding acetylcholine (10^{-5}M).

N=8	Amplitude				Tone			
	Basal	Ach	Epo 10IU/mL	Epo 30IU/mL	Basal	Ach	Epo 10IU/mL	Epo 30IU/mL
Mean±SD	0.15±0.007	3.12±1.8	1.45±0.57	0.11±0.005	-5.00±1.05	10.54±4.32	5.2±1.32	2.3±0.84
P		< 0.001	< 0.05	> 0.05		< 0.001	> 0.05	< 0.001
P1			< 0.001	< 0.001			< 0.001	< 0.001

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding acetylcholine.

Table (2-b): Effects of Erythropoietin on the jejunal motility after adding Atropine sulphate (10^{-6}M).

N=8	Amplitude				Tone			
	Basal	Atropine	Epo 10IU/mL	Epo 30IU/mL	Basal	Atropine	Epo 10IU/mL	Epo 30IU/mL
Mean±SD	0.31±0.005	0.08±0.43	0.22±0.004	0.22±0.004	1.3±0.44	-1.1±0.54	-0.05±0.008	1.80±0.64
P		< 0.001	< 0.05	> 0.05		< 0.001	< 0.05	> 0.05
P1			< 0.001	< 0.001			< 0.05	< 0.001

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding atropine sulphate.

Table (2-c): Effects of Erythropoietin on the jejunal motility after adding nor-adrenaline ($3x10^{-6}$M).

N=8	Amplitude				Tone			
	Basal	nor-adrenaline	Epo 10IU/mL	Epo 30IU/mL	Basal	nor-adrenaline	Epo 10IU/mL	Epo 30IU/mL
Mean±SD	0.83±0.03	0.10±0.002	0.57±0.32	0.86±0.35	1.23±0.06	0.56±0.005	-5.2±1.32	0.11±0.006
P		**< 0.001**	**< 0.001**	**> 0.05**		**< 0.001**	**< 0.001**	**< 0.001**
P1			**< 0.001**	**< 0.001**			**< 0.001**	**< 0.001**

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding nor-adrenaline

Table (2-d): Effects of Erythropoietin on the jejunal motility after adding phentolamine (10^{-6} M).

N=8	Amplitude				Tone			
	Basal	phentolamine	Epo 10IU/mL	Epo 30IU/mL	Basal	phentolamine	Epo 10IU/mL	Epo 30IU/mL
Mean± SD	0.33±0.004	0.16±0.003	0.23±0.002	0.75±0.36	-0.01±0.004	0.01±0.002	0.01±0.002	0.16±0.003
P		> 0.05	< 0.05	< 0.001		> 0.05	> 0.05	< 0.05
P1			> 0.05	< 0.001			> 0.05	< 0.05

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding Erythropoietin.

Table (2-e): Effects of Erythropoietin on the jejunal motility after adding propranolol hydrochloride (10^{-6} M).

N=8	Amplitude				Tone			
	Basal	propranolol	Epo 10IU/mL	Epo 30IU/mL	Basal	propranolol	Epo 10IU/mL	Epo 30IU/mL
Mean±SD	0.31±0.002	0.15±0.001	0.22±0.003	0.75±0.36	0.02±0.003	0.02±0.003	0.02±0.001	0.16±0.003
P		> 0.05	> 0.05	< 0.001		> 0.05	> 0.05	< 0.05
P1			> 0.05	< 0.001			> 0.05	< 0.05

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding Erythropoietin.

Table (3-a): Effects of Erythropoietin on the jejunal motility after adding Flunarizine 50 µM.

N=8	Amplitude				Tone			
	Basal	Flunarizine	Epo 10IU/mL	Epo 30IU/mL	Basal	Flunarizine	Epo 10IU/mL	Epo 30IU/mL
Mean±SD	0.25±0.005	0.08±0.004	0.05±006	0.05±0.006	0.12±0.006	0.01±0.007	0.012±0.003	-0.02±0.003
P		< 0.001	< 0.001	< 0.001		< 0.05	< 0.001	< 0.001
P1			< 0.05	> 0.05			< 0.001	< 0.001

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding Flunarizine.

Table (3-b): Effects of Erythropoietin on the jejunal motility after adding Verapamil hydrochloride (10^{-6} M).

N=8	Amplitude				Tone			
	Basal	Verapamil	Epo 10IU/mL	Epo 30IU/mL	Basal	Verapamil	Epo 10IU/mL	Epo 30IU/mL
Mean±SD	0.33±0.005	0.12±0.09	0.22±0.08	0.34±0.13	0.13±0.003	-0.43±0.22	0.22±0.08	0.22±0.8
P		< 0.001	< 0.001	> 0.05		< 0.001	< 0.001	< 0.05
P1			< 0.05	< 0.001			< 0.001	< 0.001

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding Verapamil hydrochloride.

Table (3-c): Effects of Erythropoietin 10 IU/mL on the jejunal motility after adding Caffeine (2 mM).

N=8	Amplitude				Tone			
	Basal	**Caffeine**	**Epo 10IU/mL**	**Epo 30IU/mL**	**Basal**	**Caffeine**	**Epo 10IU/mL**	**Epo 30IU/mL**
Mean±SD	**0.68±0.22**	**1.43±0.58**	**2.66±0.83**	**3.54±1.22**	**-7.61±2.34**	**-7.12±2.55**	**-6.54±1.33**	**-2.53±0.97**
P		**< 0.05**	**< 0.001**	**> 0.001**		**> 0.05**	**> 0.05**	**< 0.001**
P1			**< 0.001**	**< 0.001**			**> 0.05**	**< 0.001**

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding Erythropoietin.

Table (4-a): Effects of Erythropoietin 10 IU/mL on the jejunal motility after adding L-NAME (10^{-4} M).

N=8	Amplitude				Tone			
	Basal	L-NAME	Epo 10IU/mL	Epo 30IU/mL	Basal	L-NAME	Epo 10IU/mL	Epo 30IU/mL
Mean±SD	0.37±0.007	0.59±0.25	0.65±0.32	0.76±0.34	-0.12±0.005	2.23±0.79	2.74±0.97	4.72±1.87
P		< 0.05	< 0.05	< 0.001		< 0.001	< 0.001	< 0.001
P1			> 0.05	> 0.05			> 0.05	< 0.001

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding Erythropoietin.

Table (4-b): Effects of Erythropoietin 10 IU/mL on the jejunal motility after adding Lidocaine (3 × 10 3M).

N=8	Amplitude				Tone			
	Basal	Lidocaine	Epo 10IU/mL	Epo 30IU/mL	Basal	Lidocaine	Epo 10IU/mL	Epo 30IU/mL
Mean±SD	0.14±0.005	0.02±0.004	0.26±0.006	0.26±0.09	-15.34±2.33	-19.75±5.54	-16.72±4.34	-15.71±2.56
P		< 0.05	< 0.001	< 0.001		< 0.001	> 0.05	> 0.05
P1			> 0.05	< 0.001			< 0.001	< 0.001

N: number of contractions (8).
P: values as compared with the basal contractions.
P1: values as compared with the contractions after adding Erythropoietin.

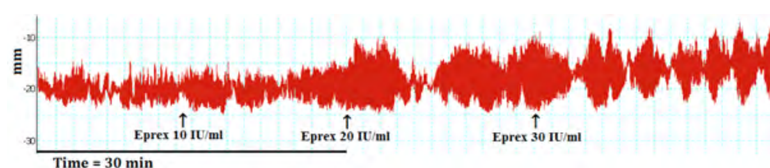

Fig. (1): Effects of Erythropoietin 10, 20 and 30 IU/mL on amplitude and the tone of jejunal motility.

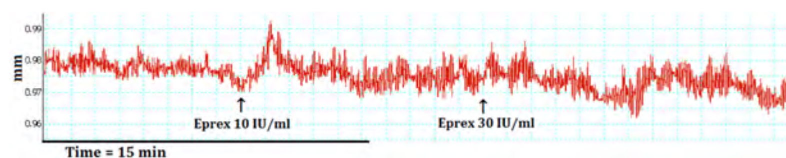

Fig. (2): Effects of Erythropoietin in calcium free solution.

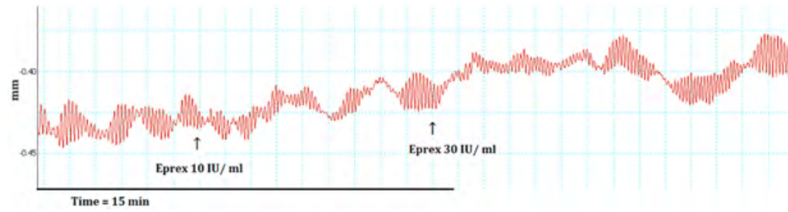

Fig. (3): Effects of Erythropoietin in calcium excess solution.

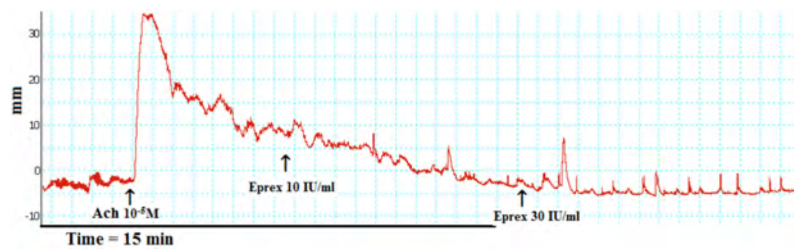

Fig. (4): Effects of Erythropoietin after Ach administration.

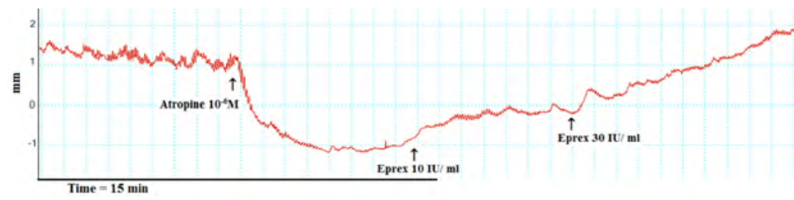

Fig. (5): Effects of Erythropoietin after atropine administration.

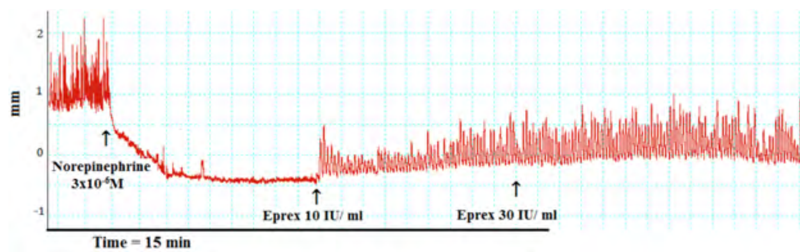

Fig. (6): Effects of Erythropoietin after noradrenaline administration.

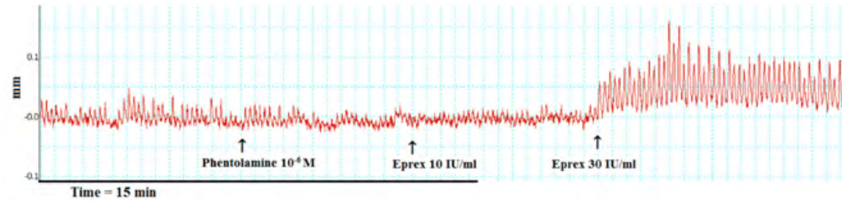

Fig. (7): Effects of Erythropoietin after phentolamine administration.

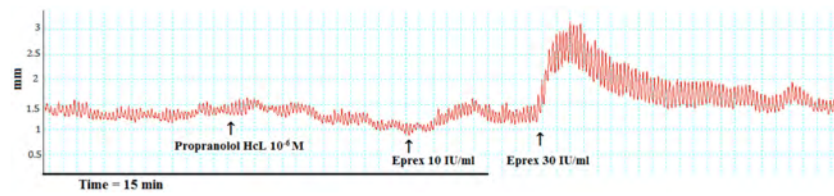

Fig. (8): Effects of Erythropoietin after propranolol hydrochloride administration.

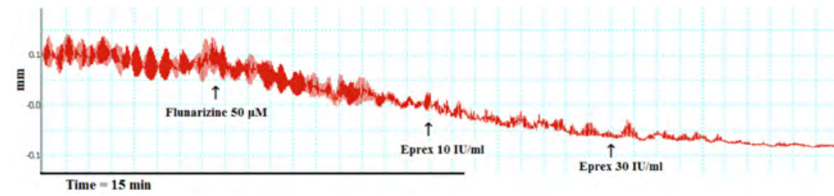

Fig. (9): Effects of Erythropoietin after Flunarizine.

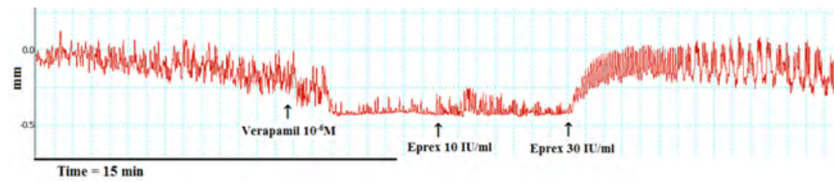

Fig. (10): Effects of Erythropoietin after Verapamil hydrochloride.

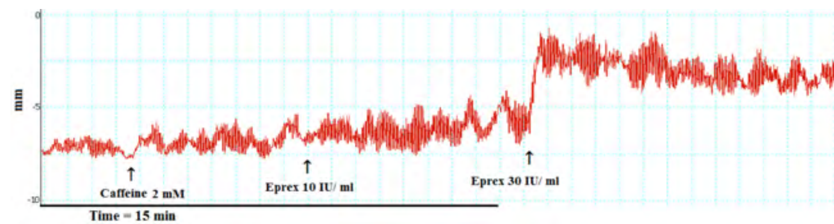

Fig. (11): Effects of Erythropoietin in the jejunum after Caffeine.

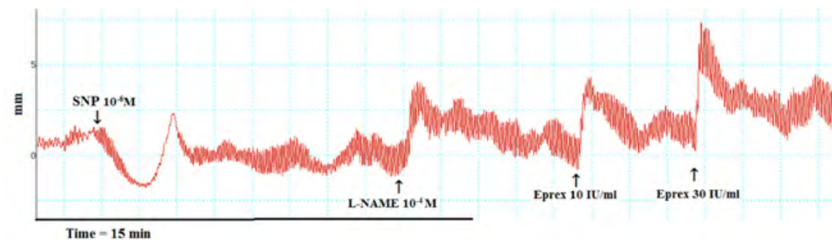

Fig. (12): Effects of Erythropoietin in the jejunum after L-NAME.

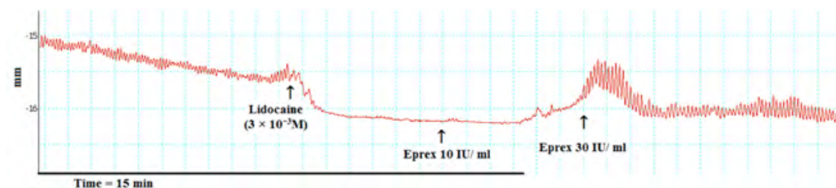

Fig. (13): Effects of Erythropoietin in the jejunum after Lidocaine.

Discussion

Our study showed the effects of erythropoietin (Epo=Eprex) on the motility of jejunum in albino rats of both sexes. The effect of erythropoietin on the gastrointestinal tract motility is not fully investigated by researchers. So we try to investigate the effect of erythropoietin on jejunal motility and its postulated mechanisms.

The aim of this research was to study the effects of erythropoietin on the basal motility of jejunum then its effects after adding acetylcholine (Ach), atropine sulphate, noradrenaline, phentolamine, propranolol hydrochloride, verapamil, caffeine, Flunarizine, L-NAME, sodium nitroprusside and lidocaine on the jejunum. The motility of jejunum was assessed from the following aspects; the amplitude and the tone of contractions.

Erythropoietin has one major use: the treatment of anemia due to chronic renal disease[31]. Erythropoietin has a wide range of actions including vasoconstriction-dependent hypertension[5], stimulating angiogenesis[9], and inducing proliferation of smooth muscle fibers[10]. It has also been shown that erythropoietin can increase iron absorption by suppressing the hormone hepcidin [32].

The study showed the dose response effects of erythropoietin hormone on the motility of jejunal segments in albino rats of both sexes. As demonstrated in table (1-a) and figure (1), Erythropoietin showed significant increase in the amplitude and the tone of jejunal motility ($P<0.05$) with erythropoietin 20 and 30 IU/ml but insignificant change at erythropoietin 10 IU/ml. These results were in agreement with[33] where the maximal increase of peak current was obtained with 30 IU/ml Epo, whereas higher and lower concentrations were not effective, suggesting a bell-shaped dose-response curve for Epo. Furthermore, the same concentration of Epo (30 IU/ml) increased calcium concentrations in endothelial and vascular smooth muscle cells[34].

In our in vitro study erythropoietin stimulates the jejunal motility and this stimulatory effect may be mediated through calcium channels mechanism and Epo stimulation resulted in a significant and dose-dependent increase in $[Ca^{2+}]i$ .This in agreement with Krapf and Hulter[5]. Erythropoietin (Epo) stimulates a significant increase in the intracellular calcium concentration ($[Ca^{2+}]i$) through activation of the transient receptor potential channel TRPC2 [35]. Tong et al., determined the function of individual TRPC channels in erythropoietin modulation of calcium influx, by digital video imaging that was used to measure calcium influx through these TRPCs[35]. Our results are also supported by Chu et al.,[36] who determined that erythropoietin regulates calcium influx through TRPC2. So erythropoietin resulted in a dose-dependent increase in $[Ca^{2+}]i$, which required extracellular calcium influx. Moreover, a review article in Am. J. Physiol. Cell Physiol.[37] suggested that Epo modulates calcium influx through voltage-independent calcium-permeable channel(s). Furthermore; Digicaylioglu[12] demonstrated that the IP_3 is involved in Epo activation of TRPC2; TRPC2 mutants were prepared with substitution or deletion of COOH-terminal IP3 receptor (IP_3R) binding domains. In cells expressing TRPC2 with mutant IP_3R binding sites and Epo receptor, interaction of IP_3R with TRPC2 was abolished, and Epo-modulated in-

crease in [Ca^{2+}]i was reduced.

Moreover, Carlini et al.,[6] explained action of erythropoietin (Epo) by binding of Epo to the erythropoietin receptor (EpoR) on the smooth muscle surface and activates a JAK2 signaling cascade. Also, erythropoietin interacts with its target cells through specific high-affinity receptors and Ca^{2+} may be involved in the receptor-ligand interaction[4]. An increase in RNA synthesis due to activation of transcription is one of the earliest recognized effects of the hormone and appears not to require protein or DNA synthesis. These in agreement with Morakkabati et al.,[34] who demonstrated that Epo modulates TRPC2 activation through a PLCγ-mediated process that requires interaction of PLCγ and IP_3R with TRPC2. They also show that TRPC2 Tyr^{226} is critical in Epo-dependent activation of TRPC2. These data demonstrate a redundancy of TRPC channel activation mechanisms by widely different agonists[34]. This was confirmed by Wilkinson et al., [38] who suggested that a transient increase in intracellular free [Ca^{2+}] in response to externally applied recombinant human Epo (rhEpo) which evident by superfusion of rhEpo leads to an increase in peak Ca^{2+} current. Epo also does not deplete the internal Ca^{2+} stores[39]. Meanwhile , the Epo effect on the whole cell Ca^{2+} current could be explained in terms of an increase either in the conductance of the channels or in the number of available channels[40].

However, the extracellular calcium is needed for the action of Epo and that was proved in our finding which indicated that the response to high Epo concentrations (30 IU/ml) was attenuated than previous response in table 1-a and figure 1, and the response to low Epo concentrations (10 IU/ml) was abolished (Table 1-b and Figure 2) in calcium free solution but a significant increase in the amplitude and the tone of jejunal motility ($P<0.001$) with Epo in calcium excess solution in all doses (10,20 and 30 IU/ml) as demonstrated in table 1-c and figure 3.

Moreover, addition of acetylcholine (Ach) to the incubation media showed a significant increase the jejunal contractions.

Epo after Ach caused more significant increase in the jejunal contraction as compared with the effect of Ach alone. These observations means that Epo potentiates the effect of Ach on the contraction of the jejunum as shown in Table (2-a) and figure (4). Moreover, table (2-b) and figure (5) showed significant decrease in the contraction of the jejunum after addition of atropine sulphate and addition of Epo after atropine causes significant increase in the jejunal contractions in both the amplitude and the tone. These finding agreed with the observations demonstrated for the first time that intravenous administration of Epo increased the number and intensity of contractions in the stomach of anesthetized rats, and that these actions of Epo were not eliminated by pretreatment with atropine, or in totally vagotomized rats[41]. Also MedWatch, 2007[20] demonstrated that eprex stimulated the motility both in vivo and in vitro and that atropine administration not block the effect of Epo. These observations mean that, the peripheral effects of Epo seem to be not mediated via cholinergic neurons within the gut wall. These observations mean that Epo mediating its excitatory effect on the jejunum via non cholinergic mechanism.

Table (2-c) and figure (6) show significant decrease in the contraction of the jejunum after addition of nor-adrenaline and addition of Epo after nor-adrenaline caused significant increase in the jejunal contractions.

In the presence of phentolamine (-adrenergic receptor blocker), Epo -induced contractions were not affected significantly as shown in table (2-d) and figure (7) which denoting an insignificant change in the contraction of the jejunum after addition of phentolamine and furthermore, addition of Epo 30 IU/Ml after phentolamine causes significant increase in the jejunal contractions. Also, propranolol hydrochloride (receptor blocker) as shown in table (2-e) and figure (8) caused insignificant change in the amplitude and the tone of the contractions of the jejunum. Epo 30 IU/ml addition after propranolol hydrochloride caused significant increase in the

amplitude & tone of the jejunum. These observations means that Epo effects on the jejunal motility is not mediated via adrenergic receptors mechanism.

Table (3-a) and figure (9) showed a significant decrease in the contraction of the jejunum after addition of Flunarizine and addition of Epo after Flunarizine causes significant decrease in the jejunal contraction in both doses (10 and 30 IU/ml). The transient response to Epo was dependent on external Ca^{2+} and remained even after depletion of internal Ca^{2+} stores by caffeine. The response to Epo was absent when cells were superfused with the Ca2+ channel blocker Flunarizine. This result in agreement with Epo elevates intracellular Ca^{2+} levels through voltage-independent Ca^{2+} channels[41].

The Ca^{2+} channel antagonist, verapamil hydrochloride showed a significant decrease in the tone of jejunal contractions as shown in table (3-b) and figure (10) and an addition of Epo after verapamil not prevent EPO stimulatory effects on the contractions of the jejunum. 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. These results in agreement with our previous results regarding postulated mechanism of action of Epo as demonstrated in (Tables 1-a,3-a & figures 1 & 9) and this was in contradict for suggestion by (36) who claimed that the Epo-induced $[Ca^{2+}]i$ increase in human erythroblasts is mediated via Ca^{2+} entry through a voltage-independent Ca^{2+} channel.

Furthermore, as demonstrated in table (3-c) and figure (11), caffeine showed significant increase in the tone and the amplitude of the jejunum motility, and addition of Epo after caffeine causes significant increase in the tone and the amplitude of the jejunum motility. In contrast to the electrophysiological measurements, which indicated that the effect of Epo on transmembrane Ca^{2+} flux,[42] could not exclude the possibility

that activation of the EpoR led to Ca^{2+} release from intracellular stores and suggesting that if Epo interacts with the internal stores, it is unable to deplete the internal Ca^{2+} stores. However, when the internal stores were gradually depleted by repeated applications of caffeine, Epo continued to induce a small increase of $[Ca^{2+}]i$ superimposed on the caffeine response. This behavior would be consistent with an Epo-induced Ca^{2+} influx. We superfused cells with 20 mM caffeine before applying Epo as shown in Fig. 11, under these conditions Epo still increased the Ca^{2+} level. Furthermore, the removal of extracellular Ca^{2+} (Fig.2) or the block of the T-type Ca^{2+} channel by 50 μM Flunarizine (figure 9) abolished the Epo-induced increase of $[Ca^{2+}]i$, indicating that an external source of Ca^{2+} was required.

Epo induces an elevation in $[Ca^{2+}]i$ via Ca^{2+} influx. First, increased fluorescence signals were obtained from cells in which the internal stores were depleted by caffeine. Second, after blocking the T-type Ca^{2+} channels with flunarizine or removal of external Ca^{2+}, Epo failed to raise the $[Ca^{2+}]i$ in cells pretreated with caffeine, demonstrating that the Ca^{2+} influx is most likely to occur by the activation of T-type Ca^{2+} channels.

In table (4-a) and figure (12), L-NAME showed significant increase in the tone and the amplitude of the jejunum motility, while addition of Erythropoietin after L-NAME caused significant increase in tone and amplitude with both doses (10 and 30 IU/ml). The addition of the nitric oxide synthase inhibitor, L-NAME before application of erythropoietin causes significant increase in the amplitude and the tone of jejunal contractions and insignificant effect on erythropoietin. These observations means that, the effect of erythropoietin may be most probably not mediated through nitric oxide (NO) release. So, it is suggested that erythropoietin not act on nitrergic neurons and not stimulate nitric oxide release which increases the acetylcholine release from cholinergic neurons and acts on smooth muscles directly leading to its contraction. These finding agreed with the results done by

Nakamura et al, (2009)[43] in guinea pig confirming that the described contractile responses of Epo are nitric oxide independent.

Furthermore, in attempt to study the role of sodium channels in mechanism of action of Epo, administration of lidocaine as a membrane stabilizer, it showed a significant decrease in the tone and the amplitude of the jejunum motility, and addition of Epo 30 IU/ML after Lidocaine caused a significant increase in both tone and amplitude of jejunal contractility (table 4-b and figure 13). These results may raise the concept that lidocaine block voltage gated calcium channels but not ligand gated calcium channels of Epo. Further support for this result comes from electrophysiological findings, which indicated that Epo induces an increase in about 40% of T-type Ca^{2+} channel current[39] after inactivation of voltage gated Ca^{2+} channels by lidocaine.

Conclusion

Our present study demonstrates that Epo stimulates the contractility (tone and amplitude) of the jejunum in a dose dependent manner. The calcium channels, particularly the voltage independent pathway, seem to be critical to explain effect of Epo on the jejunum. Moreover, our findings could not explain the involvement of both cholinergic and nitrergeric neurons in mediating the effects of Epo on jejunal contractions so the receptor mechanism of this action of Epo still needs to be clarified.

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MECHANISM OF ERYTHROPOIETIN HORMONE IN JEJUNAL MOTILITY IN EXPERIMENTAL ANIMALS

Ahmed A. Elgendy MD and Abeer F. Mostafa MD

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ASSOCIATION BETWEEN HCV INDUCED MIXED CRYOGLOBULINEMIA AND PULMONARY AFFECTION: THE ROLE OF TNF-ALPHA IN THE PATHOGENESIS OF PULMONARY CHANGES

Abdel Baset M. Saleh MD, Hatem El-Alfy MD*, Mona M. Arafa MD*, Nashwa Abousamra MD, Adel El-Badrawy MD***, Mie A. Mohamed MD****, Enaase A. M. E. Barakat MD***** and Basem S. El Deek MD******

*Departments of Thoracic Medicine, Tropical Medicine, Clinical Pathology**, Radiology***, Pathology****, Internal Medicine***** and Community Medicine******
Faculty of Medicine, Mansoura University*

Abstract

Background and aim of the work: *Chronic hepatitis C virus (HCV) infection is associated with both pulmonary involvement and cryoglobulinemia. Therefore, this study was designed to investigate the relationship between pulmonary involvement and mixed cryoglobulinemia in chronic HCV infected patients and to investigate the role of TNF-alpha in the pathogenesis of pulmonary changes.*

Subjects and Methods: *After hospital ethics committee approval and formal patient consent were obtained, 100 patients with compensated hepatitis C virus infection as confirmed by PCR were recruited in this cross sectional study. Their demographic and laboratory data, abdominal ultrasound findings, pulmonary function tests (spirometry), arterial blood gas (ABG) parameters, TNF-alpha levels, and data from high-resolution chest CT were collected and analyzed using SPSS version 16, and a serum cryoglobulin assay was performed in all of the studied patients.*

Results: *The prevalence of mixed cryoglobulinemia was 61.7% in the studied HCV patients. Pulmonary symptoms were observed in more than half of these patients. The most common complaint among the symptomatic patients was dyspnea (51.7%), followed by cough (43.3%). Oxygen saturation (Spo2 and Sao2%), and FEV1 and FVC levels, were*

significantly decreased in the cryoglobulin positive patients compared to the cryoglobulin negative patients. A statistically significant correlation was found between the presence of cryoglobulins and FEV1 level, FVC level, serum albumin level, viremia level, thrombocytopenia and arterial blood gas parameters. No correlation was found between cryoglobulinemia and TNF-alpha level.

Conclusions: *The results of this study suggest that pulmonary involvement is common in patients with chronic HCV infection and mixed cryoglobulinemia. Cryoglobulinemia may lead to pulmonary involvement through vascular and interstitial deposition of cryoglobulins, which results in impaired gas exchange and airway affection.*

Key Words: *Chronic hepatitis C virus, Cryoglobulinemia, Gas exchange, pulmonary involvement.*

Introduction

Chronic hepatitis C virus (HCV) infection is a major public health problem worldwide, and more than 170 million people are chronically infected with HCV (approximately 3% of the world's population)[1]. In Egypt, the situation is quite worse because the overall anti-HCV antibody prevalence is 14.7%[2].

HCV predominantly affects the liver; however, it can also produce a number of extra hepatic manifestations, such as mixed cryoglobulinemia (MC), which is the most common and severe extra hepatic manifestation. HCV is a systemic vasculitis involving the small- and medium- sized arteries and veins. MC is characterized by the deposition of immune complexes containing mainly rheumatoid factor, IgG, HCV RNA, and complement on endothelial surfaces, causing vascular inflammation through poorly understood mechanisms [3]. Moreover, many inflammatory diseases are known to be associated with the overproduction of cytokines such as TNF-alpha. TNF-alpha performs several proinflammatory functions including the promotion of leukocyte-endothelium interactions and the activation of the arachidonic acid pathway[4].

The prevalence of mixed cryoglobulinemia is related to the endemic presence of HCV infection,

and its prevalence varies widely between countries, ranging from 10-70%; this geographical heterogeneity may be due to population selection and time lead bias[1]. Chronic HCV infected cryoglobulinemic patients have an apparent duration of HCV infection that is almost twice as long as that in chronic HCV infected non cryoglobulinemic patients, thereby suggesting that MC is associated with an increased duration of HCV infection[5].

Many MC patients are asymptomatic. MC is diagnosed when a patient has typical organ involvement (mainly skin, kidney, or peripheral nerves) in addition to circulating cryoglobulins. Cutaneous purpura is the most common manifestation of cryoglobulinemic vasculitis. The most frequently affected internal organs are the peripheral nerves, kidneys, lungs and joints[6].

Pulmonary involvement in MC is usually mild, although severe pulmonary complications including diffuse alveolar damage, organizing pneumonia and hemoptysis have been reported[7]. However, a few studies have demonstrated pulmonary involvement in chronic HCV infection with and without cryoglobulinemia; the prevalence of pulmonary involvement in MC is not well understood.

Therefore, this study was designed to investigate the relationship between pulmonary involvement and mixed cryoglobulinemia in chronic HCV infected patients and to investigate the role of TNF-alpha in the pathogenesis of pulmonary changes. Understanding the epidemiology of the relationship between pulmonary involvement and mixed cryoglobulinemia in chronic HCV infected patients and the pathogenesis of pulmonary changes will provide novel insights into better management, especially in the present era of liver transplantation, in which the presence of advanced pulmonary affection may be considered as a major hurdle in the treatment of chronic HCV infected patients.

Subjects and Methods

Study design:

This was a cross sectional study including 100 patients with compensated HCV infection (69

males and 31 females) aged 54.63 years.

Sample size and power of the study:

The sample size was calculated using the medcalc program available at www.medcalc.be on 21-2 - 2011. The confidence level for our study was 95% with an alpha error of 0.05. The power of this study was set at 80% with a beta error of 20%. The maximum prevalence of mixed cryoglobulinemia was considered to be 60%. The minimal prevalence of mixed cryoglobulinemia was considered to be 50%. The estimated sample size was 195 patients. Two hundred forty patients with chronic HCV infection were screened for study eligibility, and 100 patients were recruited in the study according to the inclusion and exclusion criteria.

This study was performed at the Tropical Medicine Department, in collaboration with the Thoracic Medicine, Clinical Pathology, Pathology and Radiology Departments, Mansoura University Hospital, between May 2010 and December 2011. One hundred patients with chronic HCV infection as confirmed by PCR and abnormal liver function tests were included.

Patient selection:

This study included a convenient sample of patients such as those attending the inpatient and outpatient clinics of the Tropical Medicine Unit, and all of the patients provided written informed consent. The Institutional Review Board (IRB) of our Faculty of Medicine approved the study.

Inclusion criteria for patient selection were as follows:

1- Adults.

2- Any gender.

3- Egyptian nationality.

4- HCV infection documented by PCR.

5- Abnormal liver function tests.

The patient group was further subdivided into two subgroups according to the presence or absence of cryoglobulinemia (mean age of HCV infected cryonegative patients was 45.5 ? 5.89 years and that of HCV infected cryopositive patients was 47.8 ? 7.98 years).

Exclusion criteria were as follows:

• Patients with decompensated liver disease (i.e. ascites, encephalopathy and coagulopathy which are the signs of liver cell failure).

• Patients with previous lung disease (prior to the diagnosis of HCV).

• Smokers.

• Patients with previous systemic diseases (such as renal failure, congestive heart failure and connective tissue disorders).

• Patients with a current history or past history of anti-viral treatment for hepatitis C.

• Patients with autoimmune liver diseases or any other liver disease.

All of the patients were subjected to the following:

Thorough history taking and clinical examination, serologic assays and PCR for HCV and HBs Ag, liver function tests and alpha fetoprotein (AFP) test for screening of hepato-cellular carcinoma (HCC) were performed. Abdominal ultrasound and triphasic CT abdomen were also performed. To assess the severity of liver disease, liver biopsy was performed according to Ishak et al.'s[8] scoring system.

In addition, all of the patients were subjected to arterial blood gas (ABG) analysis, pulmonary function tests (spirometry) and chest high resolution CT scan (HRCT).

Arterial blood gas analysis:

ABG samples were obtained by a percutaneous radial artery puncture in stable seated patients while breathing room air. Then, the pH, partial pressure of oxygen in arterial blood (PaO2), partial pressure of carbon dioxide in arterial blood (PaCO2) and other parameters were measured with a standard blood gas analyzer (Eschweiler compact PGA, serial no. P2084, GERMANY).

Pulmonary function tests:

Forced expiratory volume in first second (FEV1), forced vital capacity (FVC), forced mid-expiratory flow rate (FEF25-75), and FVC/FEV1% were measured and recorded using a spirometer by a trained, experienced chest physician. Spirometry was performed in accordance with American Thoracic Society criteria. Three technically acceptable measurements were obtained for

each patient, and the highest value was included in the analyses (Spirolab 2, serial no.A23-050.09978MIR, ITALY)

Cryoglobulin testing:

Cryoglobulins were detected by the Winfield method[9]. Twenty milliliters of venous blood was obtained from each patient in a prewarmed (37ºC) syringe. The blood was allowed to clot at 37ºC, and the serum was separated by centrifugation. The supernatant was incubated at 4ºC for 8 d and was examined daily for the cryoprecipitate.

Assessment of tumor necrosis factor- alpha (TNF-α) levels:

The evaluation of serum TNF-α levels was performed using a commercially available enzyme-linked immunosorbent assay, human TNF-α ELISA kit (RayBio, GA, USA), according to the manufacturer's instructions.

Liver biopsy

Liver biopsy specimens were fixed in 10% formalin, and they were stained with hematoxylin and eosin and Masson's trichrome. The specimens with more than six portal areas were used for examination. The severity of chronic liver disease (CLD) was estimated according to Ishak et al.'s[8] scoring system proposed in 1995 (fibrosis 0-6, and liver biopsy was suitable).

Statistical Analysis

The statistical package for the Social Sciences (SPSS) version 16 was used for the statistical analysis. The qualitative data were presented in the form of numbers and percentages. Chi-square test was used as a test of significance for the qualitative data, and Yates correction was used when the expected cell count was less than 5. The quantitative data were expressed as mean and standard deviation. Independent sample t test was used to compare the quantitative data between two groups. Pearson correlation was used to study the relationship between variables. Statistical significance was considered at a p value less than 0.05.

Results

This study showed that the prevalence of cryoglobulinemia

was 61.7% in the studied HCV patients.

Table 1 summarizes the patients' clinical data. Pulmonary symptoms were found in less than half of the patients; however, there was no difference in the pulmonary symptoms between the cryoglobuin positive patients and the cryoglobulin negative patients. Dyspnea and cough were the most common complaints among the symptomatic patients. Some patients had more than one pulmonary symptom. A statistically significant difference was found in the extra pulmonary manifestations, such as vasculitis, rash, renal impairment, arthralgia, lichen planus and diabetes, between the cryoglobulin positive patients and the cryoglobulin negative patients.

Table 2 summarizes the lung function abnormalities (diagnosed by ABG analysis and spirometry) and laboratory findings of the HCV patients with and without cryoglobulinemia. The Spo2, Sao2%, FEV1 and FVC levels were significantly decreased in the cryoglobulin positive compensated HCV patients as compared to the cryoglobulin negative compensated HCV patients; however, the clinical data showed no difference between the groups in terms of age, sex and disease duration. There was a significant increase in the serum albumin level and viremia level in the cryo-positive HCV patients, whereas liver enzymes (ALT and AST) were significantly elevated in the cryo-negative HCV patients.

However, the comparison of chest HRCT findings in both the groups showed no significant difference. The non-septal lines, pulmonary vascular congestion and septal lines were the most common chest HRCT findings, followed by ground glass attenuation. Some patients showed more than one abnormality on chest HRCT.

Table 3 shows that there was a statistically significant correlation between the presence of cryoglobulins and FEV1 level, FVC level, serum albumin level, viremia level, thrombocytopenia and arterial blood gas parameters (PaO2 and O2 saturation). No significant correlation was found between the

presence of cryoglobulins and pulmonary symptoms (such as, cough and wheezing) or extra-pulmonary findings (such as, arthralgia, purpura, lichen planus and lower limb vasculitis). Finally, as shown in Table (3), there were significant negative correlations between cryoglobulin positivity and ALT and AST levels; however, no correlation was found between cryoglobulin positivity and other liver function tests.

Hepatic fibrosis was evaluated using the Ishak score; it showed a significant correlation with pulmonary function parameters, including FEV1/FVC, O2 saturation and PaO2 (P values 0.005, 0.026, respectively), whereas no significant correlation was found between fibrosis score and cryoglobulinemia (Data not shown).

Table (4) demonstrates the TNF-α levels in both the cryonegative patients and the cryopositive patients, which may indicate the underlying pathophysiological role of TNF-alpha in causing pulmonary changes.

The median TNF-alpha level in the cryopositive patients was higher than that in the cryonegative patients; however, this difference was not statistically significant.

Table (1): Clinical data of the studied compensated HCV patients according to the presence or absence of cryoglobulins.

	Cryoglobulin positive patients (n = 52) N (%)	Cryoglobulin negative patients (n = 48) N (%)	Test of significance
Renal involvement	4 (8.3)	-	P = 0.034*
DM	28 (58.3)	8 (15.4)	P < 0.001***
Arthralgia	16 (33.3)	4 (7.7)	P = 0.002**
Elevated Purpura (vasculitis rash)	24 (50)	8 (15.4)	P < 0.001***
Lichen planus	4 (8.3)	-	P = 0.034*
Lower limb pigmentation	20 (41.7)	12 (23.1)	P = 0.046*
Cough	20 (41.7)	16 (30.8)	P = 0.177
Dyspnea	20 (41.7)	20 (38.5)	P = 0.45

Table (2): Clinical data, blood gas analysis, pulmonary function tests and laboratory tests of the studied compensated HCV patients according to the presence or absence of cryoglobulins

		Cryoglobulin negative patients (n = 48)	Cryoglobulin positive patients (n = 52)	Test of significance
Age (Mean ± SD)		45.5± 5.89	47.8±7.98	P=0.115
Disease duration (years)		10.5±3.23	11.3±4.34	P=0.163
Gender male/female		30/18	39/13	P=0.66
ABG	PaO_2 (mmHg)	130.5 ± 29.31	83.5 ± 36.1	P < 0.001***
	O_2 saturation	97.71 ± 1.63	92.7 ± 4.57	P < 0.001***
	pH	7.37 ± 0.148	7.39 ± 0.042	P = 0.73
	$PaCO_2$ (mmHg)	35.66 ± 6.41	32.04 ± 8.08	P = 0.25
Pulmonary function tests	FEV_1/FVC (%)	98.15 ± 12.72	104.81 ± 1.37	P = 0.081
	FEV_1 (%predicted)	89.61 ± 11.45	79.16 ± 16.72	P = 0.013*
	FVC (%predicted)	88.53 ± 12.96	76.41 ± 21.86	P = 0.020*
	FEF25-75	97.84 ± 15.92	73.83 ± 0.83	P = 0.003**
Laboratory tests	Bilirubin (mg/dL)	0.98 ± 0.20	0.96 ± 0.32	P = 0.96
	ALT (IU/dL)	78.58 ± 60.67	59 ± 15.61	P = 0.027*
	AST (IU/dL)	90.16 ± 72.43	65.38 ± 24.29	P = 0.022*
	S.Albumin (g/dL)	3.9 ± 0.64	4.15 ± 0.49	P = 0.035*
	HCV viral load (IU/ml)	222.33 ± 304.51	440.27 ± 337	P < 0.001***

Table (3): Correlation between the presence of cryoglobulins, clinical and laboratory data, pulmonary function tests (PFT) and arterial blood gas parameters (ABG) in the compensated HCV patient group

Clinical data	Presence of cryoglobulins	Laboratory data	Presence of cryoglobulins	PFT & ABG	Presence of cryoglobulins
Diabetes mellitus	– 0.103	**ALT**	– 0.226*	**PaO_2**	– 0.59***
Arthralgia	0.115	**AST**	– 0.242*	**O_2 saturation**	– 0.56***
Elevated Purpura	-0.107	**Serum albumin**	0.20*		
Lichen planus	– 0.189			**FEV_1/FVC**	– 0.22*
Lower limb pigmentation	0.104			**FEV_1**	0.389**
Cough	0.05	**HCV viral load**	0.226*	**FVC**	0.341*
Wheezing	0.189	**Thrombo-cytopenia**	0.390***	**FEF25-75**	0.248

Table (4): Serum TNF-α levels (pg/ml) in the studied compensated HCV patient groups

	Cryoglobulin negative compensated HCV patient group (n = 48)	Cryoglobulin positive compensated HCV patient group (n = 52)	Control (n = 72)	P value
TNF-α	34.39 ± 8.29	39.89 ± 19.11	22.42 ± 6.19	< 0.001

Discussion

This study showed that mixed cryoglobulinemia is prevalent in HCV patients (61.7%). Oxygen saturation (Spo2 and Sao2%), and FEV1 and FVC levels were significantly decreased in the cryoglobulin positive patients than in the cryoglobulin negative patients. A statistically significant correlation was found between the presence of cryoglobulins and FEV1 level, FVC level, serum albumin level, viremia level, thrombocytopenia and arterial blood gas parameters.

This study showed that the prevalence of mixed cryoglobulinemia was 61.7% in the studied HCV patients. This result is within the range of the previous estimates for the prevalence of MC in HCV infection, which varies widely from 10-70%. According to different studies, MC can be found in 19-50% of patients with HCV infection. At the same time, only a small fraction of these HCV infected patients (less than 15%) have symptomatic mixed cryoglobulinemia. However, the asymptomatic mixed cryoglobulinemic patients may develop CG-related symptoms during the course of the disease. Factors that seem to favor the development of MC are female gender, age, alcohol intake (>50g/d), advanced liver fibrosis and steatosis[10,11,12].

HCV is a well-known cause of liver fibrosis, and it could potentially provoke similar abnormalities in the lung, mainly because of its lymphotropism, which can induce chronic immune activation and inflammation[13]. Importantly, in this study, pulmonary symptoms were found in more than half of the patients. Dyspnea, which is the main symptom of pulmonary interstitial affection, was the most common complaint among the symptomatic patients (51.7%), followed by cough in 43.3% and expectoration in 1.7% of patients. Some patients had more than one pulmonary symptom. Our findings

are in agreement with those of previous studies which reported that HCV infection was complicated by a number of extrahepatic manifestations, and it is associated with both the obstructive and restrictive lung diseases[14,15]. However, for some accompanying disorders, such as mixed cryoglobulinemia, the pathogenetic role of the HCV is substantiated by the epidemiologic and experimental data[16,17].

Importantly, a significant increase was found in the serum albumin level (thereby increasing blood viscosity) and HCV viremia levels in the cryo-positive patients, which suggest an increased deposition of cryoglobulins in the pulmonary interstitial tissue and in the airway. These findings are manifested by impaired gas exchange and obstructive ventilatory defect. In addition, these findings are in agreement with those of a previous study, which reported that the most common clinical features of hepatitis C associated with mixed cryoglobulinemia (MC+HCV) are correlated with those of vasculitis affecting various organs and sometimes with those of an increased viscosity of the plasma(18). Many MC+HCV patients experience symptoms, such as fatigue, dyspnea and reduced physical activity. However, in many patients, these symptoms are not proportional to the liver involvement[18].

Some evidence suggests that alveolitis and HCV infection may not be coincidental findings[19,20]. In addition, the prevalence of serum antibodies against HCV (determined using first-generation enzyme-linked immunosorbent assay) has been reported to be higher in patients with idiopathic pulmonary fibrosis (IPF) than in controls, although a subsequent study using similar but improved techniques refuted this finding [21,22]. Further evidence of an association between HCV and interstitial lung involvement was provided by Ferri et al.[23]. Our study reported a significant two fold increase in the HCV viremia levels in the cryo-positive patients compared with the cryo-negative patients.

Mixed cryoglobulinemia is a well-described complication of HCV infection[24]. Fibrotic lung

disease is likely to result from an inciting injurious event within the lung. However, the sequence of events and mechanisms of fibrotic lung disease are not understood. An emerging hypothesis is that occult infections may play a pathogenetic role as cofactors in the development of pulmonary fibrosis, and this hypothesis is based on the assumption that an inflammatory agent (such as a virus) disrupts the physiologic healing response, thereby making the lung highly susceptible to injurious triggers[25]. Chronic HCV infection may contribute to the immune responses that modulate the pathogenic processes underlying pulmonary disorders; therefore, chronic HCV infection may produce a wide spectrum of clinical presentations. The inflammatory cytokines are the potential candidates that play a role in these immune responses [26].

In this study, both cryoglobulinemic patients and non cryoglobulinemic patients showed significantly higher mean TNF-alpha levels than those in controls; however, no significant difference in the mean TNF-alpha levels was demonstrated between cryoglobulinemic and non cryoglobulinemic patients. On the other hand, another study reported significantly higher serum TNF-α levels in the HCV-MC patients than in HCV+ patients or in controls[18]. In addition, other studies reported elevated serum levels of B cell-activating factor (BAFF), a TNF-α family member, which is required for B cell survival in HCV-MC, although the underlying mechanism remains unclear[27-30].

Thus, our study confirmed the finding of a high serum TNF-α level in HCV +ve patients with or without cryoglobulinemia, as was previously demonstrated in other studies of HCV +ve patients[31-33]. The increase in the TNF-α level in MC+HCV patients was unlikely to be due to a more aggressive liver disease; in fact, in our study, no correlation was found between the TNF-α levels and ALT levels, or between the TNF-α levels and the degree of liver inflammation. This result was in accordance with that of a previous study[34]. Other studies have shown an increased production of TNF-α by lymphocytes in MC+HCV patients [35,36], thereby suggesting that the in-

crease in the TNF-α level may be due to the activation of lymphoid cells.

However, the severity of pulmonary involvement may or may not parallel the liver impairment; thus, patient management and prediction of disease outcome can be subject to marked variability. Therefore, early referral of MC+HCV patients who complain of any pulmonary symptoms to a specialized medical team in this field (pulmonologists and hepatologists) is strongly recommended.

Our study has two limitations. First, the study population may not be representative of the general population in Egypt. Therefore, this study may have been affected by the selection bias. Second, genotyping of HCV and cryoglobulin quantitation were not performed because there was not enough financial aid to cover these expenses in the studied HCV cases.

Conclusion

Cryoglobulinemia is a laboratory finding, and it is not a clinical presentation. Cryoglobulinemia may have an impact on both gas exchange and airway parameters. Understanding the respiratory effects of HCV, which is considered to be a major health problem in Egypt, might improve our approach to the treatment of respiratory problems complicating HCV. Future studies should focus on evaluating the pathophysiological mechanisms underlying the relationship between mixed cryglobulinemia and pulmonary affection in chronic HCV patients.

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ASSOCIATION BETWEEN HCV INDUCED MIXED CRYOGLOBULINEMIA AND PULMONARY AFFECTION: THE ROLE OF TNF-ALPHA IN THE PATHOGENESIS OF PULMONARY CHANGES

Abdel Baset M. Saleh MD, Hatem El-Alfy MD, Mona M. Arafa MD, Nashwa Abousamra MD, Adel El-Badrawy MD, Mie A. Mohamed MD, Enaase A. M. E. Barakat MD and Basem S. El Deek MD

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EFFECT OF LISINOPRIL AND VISFATIN ON NON HYPERGLYCEMIC INSULIN RESISTANCE IN ALBINO RATS

Ahmed A. El-Gendy Ph.D

Department of Medical Physiology, Faculty of Medicine Mansoura University. Egypt

Abstract

Background: *Tissue responsiveness to insulin, meaning how successfully the receptor operates to permit glucose clearance, is termed insulin sensitivity. In the case of optimal insulin sensitivity, after a high sugar meal, insulin rises sharply, pushing glucose into the tissues rapidly, then dissipates. In the case of poor insulin sensitivity, however, insulin's elevation is sustained due to an inability to force glucose into muscle tissues. Insulin resistance or compensatory hyperinsulinemia has been associated with dyslipidemia.*

Recent studies have indicated that essential hypertension is an insulin-resistant state. Lisinopril is an oral long-acting angiotensin converting enzyme inhibitor (ACE-I) and it is a potent antihypertensive drug with a low incidence of side effects in humans. However, the effects of lisinopril on insulin sensitivity and glucose metabolism have not been investigated in detail.

One third or even more of all hypertensive individuals have reduced insulin-mediated glucose uptake. Although the association between insulin resistance and hypertension may vary among different populations, it is generally believed that insulin resistance has an important role in the pathogenesis of essential hypertension in a majority of populations.

Visfatin is a novel adipokine or cytokine, also known as pre-B cell colony enhancing factor (PBEF) or nicotinamide phosphoribosyltransferase (NAMPT) which highly expressed in visceral and subcutaneous fat and also secreted by human bone marrow immune cells, liver, and skeletal muscle. Evidence suggests that visfatin is one of the insulin-mimetic adipocytokine that controls insulin resistance.

Aim of the work: *The present study aimed to clarify the effect of both antihypertensive, angitensin converting enzyme inhibitors, (lisino-*

pril) and insulin-mimetic adipocytokine (visfatin) on insulin resistance induced by high fructose diet in albino rats. Other contribution of this work is to find if the tumor necrosis factor (TNF-α) acts as a modulator for these chemicals or not.

Materials & Methods: *In this work, 108 adult male white albino rats were divided into 6 equal groups each group contains 18 animals. Group (1); control group, these animals received standard diet, Group (2); high fructose-fed rats. Rats received a diet in which fructose composed 33.64% of total carbohydrates, Group (3); received standard diet for 9 weeks & rats were injected intra-peritoneally with visfatin once daily (dose = 5 x 10-6 μmol, 0.1 ml) in the last 3 weeks, Group (4); received high fructose diet for 9 weeks and visfatin treatment with the previous dose in last 3 weeks. Group (5); received standard diet for 9 weeks and lisinopril treatment in a dose of 9 mg kg/ day in their drinking water in the last 3 weeks, Group (6); received high fructose diet for 9 weeks and lisinopril in the previous dose in the lat 3 weeks.*

The effects of lisinopril and visfatin on metabolic parameters in rats fed either standard diet or high fructose diet were reflected by analysis of serum fasting glucose, fasting insulin, IV glucose tolerance test and tumor necrosis factor –α (TNF-α) for insulin resistance and β cell function. Also, full analysis for lipid profile including total cholesterol, HDL, LDL and triglycerides were performed.

Results: *Rats received high fructose diet developed a significant insulin resistance as evidenced by impaired response to intraperitoneal insulin dose of 1 u/kg BW. On the other hand, rats fed standard diet showed a significant reduction in plasma glucose in response to intraperitoneal injection of insulin in a dose of 1 u/kg BW. Also, it was shown that rats fed high fructose, there was a significant increase in fasting serum insulin, TNF-α, TG, and LDL. On the other hand, there was a significant decrease in HDL and no change in fasting serum glucose and cholesterol levels as compared with rats fed standard diet. Rats fed high fructose diet showed a significant impairment in response to IV glucose administration compared to rats fed standard diet. Administration of visfatin to rats fed high fructose diet in a dose 5 x 10-6 μmol, 0.1 ml in the last 3 weeks produced a significant decrease in fasting serum glucose, insuln, cholesterol, TG, LDL, and TNF-α but it pro-*

duced a significant increase in HDL, improved insulin sensitivity Moreover, administration of visfatin for 3 weeks to rats fed high fructose diet significantly improved the IV glucose tolerance test. Visfatin administration in rats fed high fructose diet induced a significant increase in HDL and a significant decrease in LDL. Also,visfatin induced a significant decrease in TNF-α in high fructose –fed rats. Administration of lisinopril to rats fed high fructose diet in a dose 9 mg kg/ day in their drinking water in the last 3 weeks induced a significant decrease in fasting LDL,TG, insulin and TNF-α in rats fed high fructose diet but it induced no change in serum cholesterol and glucose levels. On the other hand, lisinopril induced a significant increase in serum HDL. Moreover, administration of lisinopril for 3 weeks to rats fed high fructose diet significantly improved the IV glucose tolerance test. Administration of lisinopril to high fructose fed – rats induced improvement in insulin sensitivity associated with a significant decrease in serum TG, insulin and produced no change in serum cholesterol levels.

Conclusion: *It can be concluded that both lisinopril and visfatin might prove useful in the treatment and/or preventing non hyperglycemic insulin resistance states such as obesity and impaired glucose tolerance as well as in the treatment of established non insulin dependent diabetes mellitus.*

Introduction

In insulin resistance, the body's cells have a diminished ability to respond to the action of the insulin hormone. To compensate for the insulin resistance, the pancreas secretes more insulin. People with this syndrome have insulin resistance and high levels of insulin in the blood as a marker of the disease rather than a cause. Over time people with insulin resistance can develop high sugars or diabetes as the high insulin levels can no longer compensate for elevated sugars[1].

Insulin resistance has been shown in subjects with essential hypertension and has been proposed as a metabolic link between hypertension, noninsulin-dependent diabetes mellitus (NIDDM), obesity, dyslipidaemia and atherosclerotic cardiovascular disease[2]. Therefore, in the treatment of hypertension it is argued that consideration should be given to the

effect of antihypertensive agents on insulin sensitivity.

β-Cell failure coupled with insulin resistance is a key factor in the development of type 2 diabetes. Changes in circulating levels of adipokines, factors released from adipose tissue, form a significant link between excessive adiposity in obesity and both aforementioned factors. Dunmore and Brown[3] consider the role of individual adipokines on the function, proliferation, death and failure of β-cells, focusing on those reported to have the most significant effects (leptin, adiponectin, tumour necrosis factor α, resistin, visfatin, dipeptidyl peptidase IV and apelin). It is apparent that some adipokines have beneficial effects whereas others have detrimental properties; the overall contribution to β-cell failure of changed concentrations of adipokines in the blood of obese pre-diabetic subjects will be highly dependent on the balance between these effects and the interactions between the adipokines, which act on the β-cell via a number of intersecting intracellular signalling pathways[4].

Insulin resistance is a systemic phenomenon associated with several diseases including chronic infection[5], cancer[6], obesity and especially non –insulin dependent diabetes mellitus (NIDDM)[7]. Insulin resistance or hyperinsulinemia has been consistently associated with dyslipidemia in the form of hypertriglyceralehydemia and a decreased serum HDL and increased cholesterol level[8].

Tumor necrosis factor (TNF-α) is a peptide constitutively expressed and secreted by adipose tissue[9]. It has been demonstrated that TNF-α may be a mediator of insulin resistance that is known to occur in obese mice[10]. Tumor necrosis factor interferes with insulin action, probably by inhibiting tyrosine kinase activity of insulin receptors[11]. Phosphorylation of the insulin receptor by this tyrosine kinase is known to be a cardinal step in the post receptor events that follow the binding of insulin to its receptor[12]. Furthermore, it has been shown that, in adipocytes from obese subjects, the expression of TNF-α message and protein falls markedly after weight loss[13].

Visfatin is an adipokine identified in 2005, and thus named for the suggestion that it would be predominantly produced and secreted in visceral fat. It is identical to pre-B cell colony enhancing factor (PBEF), described in 1994 as a cytokine acting on early stage B cell lymphocyte maturation and inflammatory regulation. Visfatin was also recognized as nicotinamide phosphoribosyl transferase (Nampt), the rate limiting enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis[14].

In mammals, Nampt has both intra- and extracellular forms (iNampt and eNampt) respectively. The protein expression of iNampt is highest in brown adipose tissue (BAT), liver, and kidney, intermediate in heart, low in white adipose tissue (WAT), lung, spleen, testis, and skeletal muscle, and undetectable level in pancreas and brain. Extracellular Nampt can be synthesized and released by many different cell types beside adipocytes[15].

Visfatin may have insulin-mimetic action; it binds to and activates the insulin receptor at a site distinct from that of insulin. Visfatin has also been shown to induce the phosphorylation of insulin receptor substrate (IRS)-1, and IRS-2; binding of phosphatidylinositol3-kinase (PI3K) to IRS-1 and IRS-2; and phosphorylation of Akt and MAPK[16].

TNFα suppresses visfatin gene expression in a dose- and time-dependent fashion, which possibly contributes to impaired glucose tolerance seen in. states of increased TNFα levels[17].

Basal visfatin release is enhanced by glucose in adipocytes in vitro and by hyperglycaemia in healthy humans. This effect is counter regulated by insulin and somatostatin in vivo[18].

One third or even more of all hypertensive individuals have reduced insulin-mediated glucose uptake[19]. Although the association between insulin resistance and hypertension may vary among different populations, it is generally believed that insulin resistance has an important role in the pathogenesis of essential hypertension in a majority of populations[20].

Angitensin converting enzyme inhibitors (ACEIs) have well documented effects in reducing blood pressure and improving other cardiovascular parameters in hypertensive individuals. The influence of ACEIs, such as lisinopril, on glucose metabolism has also been studied, several clinical studies have shown that short and long term administration of lisinopril results in a increased insulin stimulated glucose disposal in diabetic or hypertensive individuals[21]. The short term oral administration of lisinopril at dosages that have no effect on blood pressure has been reported to improve peripheral glucose utilization[22]. However, it is currently unclear whether this lisinopril induced improvement in whole body disposal results from an alteration in skeletal muscle insulin signaling pathways, from improved muscle blood flow, or even from the combination of both[23]. It has been suggested that ACEIs may exert their effect on insulin sensitivity not only by blocking the renin angiotensin and kinin system but also by inhibiting production and/or release of endothelin[24].

Rats fed with high dosages of fructose developed insulin resistance therefore, this study was undertaken to determine whether visfatin or lisinopril could improve insulin resistance and the related abnormalities induced by high fructose diet feeding in normal rats.

Materials and Methods

Chemicals used:

1- Lisinopril: 5 mg Pink, oval, biconvex, uncoated tablets debossed “E 54” on one side and bisected on the other side. It stored at 20º to 25ºC and protected from moisture, freezing and excessive heat. Sandoz Inc.

2- Visfatin: Visfatin was supplied as visfatin recombinant from E.coli. Sigma-Aldrich, CH- 9471 Buchs Product of Czech Rep. hh 25 755/081.

Animals used: 108 male albino rats used throughout this study. At the beginning of the experiment, the rats were aged 2 weeks. Food intake was recorded daily & their weight was monitored weekly, they were put under similar housing conditions. They were divided into 6 experimental groups

(n=18 per group).

Group (1): Control group, these animals received standard diet.

Group (2): High fructose-fed rats. Rats received a diet in which fructose composed 33.64% of total carbohydrates[25].

Group (3): Received standard diet & rats were injected intraperitoneal with visfatin once daily (dose = 5 x 10-6 µmol, 0.1 ml) in the last 3 weeks[26].

Group (4): Received high fructose diet for 9 weeks and visfatin treatment with the previous dose in last 3 weeks.

Group (5): Received standard diet for 9 weeks and lisinopril treatment in a dose of 9 mg kg/ day in their drinking water in the last 3 weeks[27].

Group (6): Received high fructose diet for 9 weeks and lisinopril in the previous dose in the lat 3 weeks.

The diets composition are described in (Table 1).

Data are given in grams per 100 gm of dry weight. The salt mixture is expressed in grams per kilogram: $CaHPO_4$, 30 gm ; KCL, 100 gm; NaCL, 100 gm; MgO, 10.5 gm; $MgSO_4$, 50gm; Fe_2O_3, 3 gm; and $FeSO_4$ & H_2O, 5g. Vitamins are expressed per kilogram of the vitamin mixture: retinol, 539 mg; cholecalceferol, 6.25 mg; thiamine, 2.0 mg; riboflavin, 1.5 mg; niacin, 7.0 mg; pyridoxine, 1.0 mg; cyanocobalamine, 5 mg; menadione, 1.0 mg/kg; nicotinic acid, 10.0 mg; o-choline, 136.0 mg; folic acid, 500 mg; p-aminobenzoic acid, 5.0 mg; and biotin, 30 mg/kg.

In animals the following parameter should be evaluated:

Insulin sensitivity assay: In 6 animals of each group, insulin sensitivity was performed according to Surwit et al.[28]. Rats fed either the standard diet or the high fructose diet fasted for 4 hours & injected intraperitoneally with regular insulin at 1 u/kg body weight. Insulin was diluted in sterile saline for a final injected volume of 100 ul. Plasma was collected for glucose quantification

before injection & at 15,30,90 minutes after insulin injection.

Intravenous glucose tolerance test (IVGTT): In 6 animals of each group IVGTT was done according to Rizk et al.[29]. Rats were fed standard diet or high – fructose diet, fasted over night (18 hours) and injected IV glucose in a dose of 1 gm/kg body weight. Plasma was collected for glucose quantification before injection & at 0,15,30, 60 minutes after glucose injection.

At the end of the experiment the remaining 6 rats of each group were decapitated after 14 hours of starvation and blood collected. Blood samples were allowed to clot & were centrifuged. Serum was separated & frozen at -70 0C until time of the assay of the following parameters:

1- Serum glucose: according to the enzymatic glucose oxidase method of Trinder[30].

2- Fasting serum insulin: according to Morgan & Lazarow[31] by radioimmunoassay using iodinated kit, manufactured by diagnostic production corporation.

3- Total serum cholesterol: using kits of biomerieux Co. according to Richmond[32], HDL & LDL: according to Burstein[33] using kits of biomerieux Co. and TG according to Fassati & Prencipe [34] using TG kits of biomerieux.

4- Serum tumor necrosis factor alpha (TNF-α): according to Corti et al(35) using an immunotech (A Cloulter Co.) by enzyme linked immunosorbent assay method.

Regarding food consumption and weight of the rats, all groups had similar food intake throughout the experiment. After 6 weeks, the body weight of the rats was similar in all groups as indicated by measurement of body mass index (BMI= body weight (g)/length2 (cm2) and Lee index (LI= cube root of body weight (g) / nose-to-anus length) and abdominal circumference / thoracic circumference.

Statistical Analysis:

The analysis of data was performed using the SPSS statistical package version 10.0 (SPSS,Chicago. IL.USA). To compare the data , the recorded values were expressed as

means ± standard error of means (mean±SE). A p value of <0.05 was considered statistically significant[36].

Results

Table (1): Diet composition of standard and high fructose diet.

	Standard Diet	High Fructose Diet
Glucose	38	15.96
Fructose	-	33.64
Wheat Starch	20	8.4
Casein	23	23
Cellulose	6	6
Corn oil	5	5
Salt Mixture	7	7
Vitamins	1	1

Table (2): Insulin sensitivity assay in rats fed either standard or high fructose diet for 9 weeks & fasted for 4 hours before intraperitoneal insulin injection in a dose of 1 u/kg body weight (n=6) (M±SE).

Time (Minutes)	**Glucose (mg/dl)**					
	Standard diet	**High fructose diet**	**Standard diet+Vistafin**	**High fructose diet+Visfatin**	**Standard diet+Lisinopril**	**High fructose diet+Lisinopril**
0	88±0.6	90±0.6 P1 NS	86±0.5 P2 NS	89±0.6 P3 NS	87±0.2 P4 NS	91±0.6 P5 NS P6 NS
15	69±0.4	84±0.4 P1 <0.05	71±0.2 P2 <0.05	74±0.5 P3 <0.05	71±0.3 P4 <0.05	70±0.2 P5 <0.05 P6 <0.05
30	61±0.5	74±0.6 P1 <0.05	60±0.4 P2 <0.05	65±0.6 P3 <0.05	60±0.4 P4 <0.05	61±0.6 P5 <0.05 P6 <0.05
90	54±0.5	69±0.6 P1 <0.05	56±0.4 P2 <0.05	57±0.4 P3 <0.05	51±0.3 P4 <0.05	56±0.3 P5 <0.05 P6 <0.05

P1:Test of significance between rats that fed high fructose diet & fed standard diet.

P2:Test of significance between rats that fed standard diet & treated with visfatin versus rats fed standard diet.

P3:Test of significance between visfatin treated rats & fed standard diet versus visfatin treated rats an fed standard diet.

P4:Test of significance between rats received lisinopril & standard diet versus rats fed standard diet.

P5: Test of significance between rats treated with lisinopril & fed high fructose diet versus rats fed high fructose diet.

P6: Test of significance between rats that fed high fructose diet & treated with lisinopril versus that fed high fructose diet + visfatin.

NS: non significant.

SE: standard error

Table (3): Intravenous glucose tolerance test for rats.Rats were fasted over night (18 hours) before IV glucose at dose of 1 gm/kg BW (M±SE).

Time (Minutes)	Glucose (mg/dl)					
	Standard diet	High fructose diet	Standard diet+Vistafin	High fructose diet+Visfatin	Standard diet+Lisinopril	High fructose diet+Lisinopril
0	82.2±0.3	86.5±0.2 P1 NS	84.3±0.4 P2 NS	83.8±0.1 P3 NS	75.7±0.2 P4 NS	82.2±0.2 P5 NS P6 NS
15	99.6±0.2	143.2±0.3 P1 <0.05	117.6±0.8 P2 <0.05	130.1±0.4 P3 <0.05	114.5±0.8 P4 NS	110.1±0.8 P5 <0.05 P6 <0.05
30	134.4±0.3	175.4±0.6 P1 <0.05	133.3±0.7 P2 <0.05	165±0.2 P3 <0.05	130.2±0.4 P4 NS	154.3±0.6 P5 <0.05 P6 <0.05
60	95.4±0.5	135.2±0.4 P1 <0.05	100.9±0.6 P2 <0.05	113.4±0.6 P3 <0.05	94.3±0.4 P4 <0.05	101.6±0.5 P5 <0.05 P6 <0.05

P1: Test of significance between rats that fed high fructose diet & fed standard diet.

P2: Test of significance between rats that fed standard diet & treated with visfatin versus rats fed standard diet.

P3: Test of significance between visfatin treated rats & fed standard diet versus visfatin treated rats and fed standard diet.

P4: Test of significance between rats received lisinopril & standard diet versus rats fed standard diet.

P5: Test of significance between rats treated with lisinopril & fed high fructose diet versus rats fed high fructose diet.

P6: Test of significance between rats that fed high fructose diet & treated with lisinopril versus that fed high fructose diet + visfatin.

NS: non significant

SE: standard error.

Table (4): Effect of either visfatin or lisinopril on fasting serum glucose, insulin, cholesterol, HDL,LDL,TG and TNF-**α** in rats fed either standard diet or high fructose diet (M±SE).

Animal group(n=6)	Fasting serum glucose(mg/dl)	Fasting serum insulin(nu/ml)	Cholesterol(mg/dl)	HDL(mg/dl)	LDL(mg/dl)	TG(mg/dl)	TNF-α(pg/ml)
Standard diet(9weeks)	74.3±0.4	13.5±2	83.6±0.7	50.3±03	38.9±0.7	93.5±06	0.89±0.2
High fructose diet(9weeks)	77.4±0.2 P1 NS	23.4±0.8 P1 <0.05	80.3±0.8 P1 NS	24.4±0.3 P1 <0.05	51.6±0.7 P1 <0.05	137.3±1 P1 <0.001	3,76±0.27 P1 <0.001
Standard diet+visfatin treatment for 3 weeks	62.4±0.5 P2 <0.05	13.0±0.7 P2 NS	82.2±0.5 P2 NS	50.0±0.4 P2 NS	37.6±0.4 P2 NS	87.3±0.7 P2 <0.05	0.89±0.22 P2 NS
High fructose diet+visfatin treatment for 3 weeks	65.7±0.3 P3 <0.05	19.6±0.8 P3 <0.05	76.6±0.8 P3 NS	47.8±0.7 P3 NS	16.6±0.7 P3 <0.05	94.6±0.4 P3 NS	0.93±0.2 P3<0.001
Standard diet+lisinopril treatment for 3 weeks	72.7±0.6 P4 NS	12.9±0.4 P4 NS	80.3±0.7 P4 NS	49.3±0.2 P4 NS	36.8±0.5 P4 NS	92.7±0.4 P4 NS	0.94±0.3 P4 NS
High fructose diet+lisinopril treatment for 3 weeks	74.2±0.3 P5 NS P6 NS	11.6±0.4 P5 <0.05 P6 <0.05	81.6±0.7 P5 NS P6 NS	50.1±0.4 P5 <0.05 P6 <0.05	37.2±0.1 P5 <0.05 P6 NS	95.3±0.4 P5 <0.05 P6 NS	0.94±0.23 P5 <0.001 P6 NS

P1: Test of significance between rats that fed high fructose diet & fed standard diet.

P2: Test of significance between rats that fed standard diet & treated with visfatin versus rats fed standard diet.

P3: Test of significance between visfatin treated rats & fed standard diet versus visfatin treated rats and fed standard diet.

P4: Test of significance between rats received lisinopril & standard diet versus rats fed standard diet.

P5: Test of significance between rats treated with lisinopril & fed high fructose diet versus rats fed high fructose diet.

P6: Test of significance between rats that fed high fructose diet & treated with lisinopril versus that fed high fructose diet + visfatin.

NS: non significant

SE: standard error.

Discussion

In the present study, the effect of either visfatin or lisinopril on high fructose diet induced insulin resistance was studied. Insulin sensitivity is based on intravenous glucose tolerance and insulin sensitivity test[37]. Furthermore fasting serum insulin has been a an index of insulin sensitivity in several epidemiological studies, assuming that hyperinsulinemia is a proxy of insulin resistance[38].

Table (2) showed that rats received high fructose diet developed a significant insulin resistance as evidenced by impaired response to intraperitoneal insulin dose of 1 u/kg BW. On the other hand, rats fed standard diet showed a significant reduction in plasma glucose in response to intraperitoneal injection of insulin in a dose of 1 u/ kg BW.

Also, it was shown that rats fed high fructose, there was a significant increase in fasting serum insulin, TNF-α, TG, and LDL. On the other hand, there was a significant decrease in HDL and no change in fasting serum glucose and cholesterol levels as compared with rats fed standard diet.

Table (3) demonstrated that rats fed high fructose diet showed a significant impairment in response to IV glucose administration compared to rats fed standard diet.

Insulin resistance by high fructose diet has been proposed to: (i) elevated serum TG through increasing the production of very low density lipoprotein (VLDL) and reducing the catabolism of VLDL due to low lipoprotein lipase activity and to (ii) lower serum HDL through decreasing the synthesis of HDL from LDL-triglycerides due to low lipoprotein lipase activity elevating fractional catabolic rate of apolipoprotein A-1(the major apolipoprotein for HDL) elevating hepatic lipase concentration and increasing cholesteryl ester transfer protein activity. The results are in agreement with previous study [39] which concluded that high-fructose diet produced insulin resistance in rats within 6 weeks due to decrease insulin receptor binding and post-receptor defects which are the most underlying explanation[40].

Moreover, high- fructose fed rats showed a significant increase in TNF-α which suggests insulin resistance[10]. TNF-α induces an effect antagonistic to insulin, through the inhibition of insulin receptor tyrosine kinase[12]. In addition, TNF-α may cause insulin resistance in vivo by raising free fatty acid concentration, which in turn impairs muscle glucose metabolism[41].

As shown in table (4), administration of visfatin to rats fed high fructose diet in a dose 5 x 10-6 μmol, 0.1 ml in the last 3 weeks produced a significant decrease in fasting serum glucose, insuln, cholesterol, TG, LDL and TNF-α but it produced a significant increase in HDL. Moreover, administration of visfatin for 3 weeks to rats fed high fructose diet significantly improved the IV glucose tolerance test indicating improvement of insulin sensitivity.

Whether visfatin influences glucose and lipid homeostasis is unknown, so that this study was designed to investigate the role of visfatin in metabolic homeostasis in non hyperglycemic high fructose induced insulin sensitivity.

These findings were in accord with Tobey et al.[42] and Faure et al.[25] who reported that the improvement of insulin sensitivity produced by visfain may be due to its potential antioxidant effect. Furthermore, visfatin improved glucose uptake by the peripheral tissues which could result from increased insulin binding to its membrane receptors, from the activation of post-receptor metabolic pathways as well as from a beneficial effect on lipid metabolism[43].

One of the mechanism involved in this effect suppose that visfatin plays a central role in maintaining the activity of NAD dependent enzymes and is implicated in the regulation of cellular metabolism. On the other side, visfatin can exert a wide range of actions in a paracrine or endocrine manner[15].

Visfatin administration in rats fed high fructose diet induced a significant increase in HDL and a significant decrease in LDL. These findings were in agreement with Jin et al.[44].

This could be explained on the basis that visfatin improved insulin resistance. In addition, visfatin induced a significant decrease in TNF-α in high fructose –fed rats which may explain the improving of insulin sensitivity. The finding of Dan Don et al.[10] supports this result as they reported that a fall in TNF-α concentration contributes to the restoration of insulin sensitivity.

In this study, there was a significant decrease in glucose level after injection of visfatin in normal animals. This result was in agreement with the insulin mimetic effect of visfatin showed by Fukuhara et al,[14] who observed the insulin mimetic activity during various experiments with cultured cells, whereas visfatin lowered plasma glucose level in mice.

Revollo et al,[15] who came with another theory of the role of visfatin. They proved that not the insulin mimetic activity, but synthesis of NAD is more important in glucose homeostasis in vivo. After chemical inhibition of Nampt by specific inhibitor, the defects in NAD synthesis and in glucose stimulated insulin secretion were observed. In view of that, visfatin has both central mechanism on insulin secretion from β cells and peripheral mechanism on insulin receptors and glucose utilization. But, which is the predominant during normal or pathological conditions still a point of controversy [45] Otherwise, several investigators demonstrated a significantly positive correlation between level of circulating visfatin and type – 2 diabetes mellitus(T2DM)[46].

The similar results showed by El-mesallamy et al[47] in T2DM, obese and non-obese patients. By comparing these patients to healthy controls the increased plasma visfatin levels were observed. The possible explanations for this relation are still unclear but there is a realistic belief of direct pathophysiological linkage.

As regard the lipid profile, the physiological role of circulating visfatin seems to be elusive. Some papers found positive associations of plasma visfatin concentrations with HDL and negative associations with triglycerides in nondiabetic persons[48].

The relationship with lipid metabolism seems to be not dependent on visceral obesity and insulin resistance and is probably linked to intracellular enzymatic function in NAD synthesis. These authors concluded that circulating visfatin levels are an indicator of beneficial lipid profile in non-diabetic persons[49]. Similar findings were also recorded by the present study, as there were a significant decrease in plasma levels of both triglycerides and total cholesterol in non hyperglycemic animals. Whereas there was a significant increase in the serum levels of HDL.

Chen et al.[46] revealed the effect of visfatin in diabetic animals. There were significant increases in serum levels of HDLcholesterol after injection of visfatin in diabetic animals. Besides, there were a significant decrease in serum levels of total cholesterol, triglycerides and LDL-cholesterol after injection of visfatin in diabetic animals. This may situate a point of view that visfatin affects glucose metabolism by a different mechanism by which it affects lipid metabolism, especially in diabetic conditions.

As shown in table (4), administration of lisinopril to rats fed high fructose diet in a dose 9 mg kg/ day in their drinking water in the last 3 weeks induced a significant decrease in fasting LDL,TG, insulin and TNF-α in rats fed high fructose diet but it induced no change in serum cholesterol and glucose levels. On the other hand, lisinopril induced a significant increase in serum HDL. Moreover, administration of lisinopril for 3 weeks to rats fed high fructose diet significantly improved the IV glucose tolerance test.

Adminstration of lisinopril to high fructose fed – rats induced improvement in insulin sensitivity associated with a significant decrease in serum TG, insulin and produced no change in serum cholesterol levels. These findings are in accordance with previous studies[21]. They reported that in human & animal models of insulin resistance. ACE inhibitors increase sensitivity to insulin. Most previous investigations have attributed the influence of ACE inhibitors on glucose disposal to improved capillary flow & an accompanying increased delivery

of insulin & glucose to muscle[50]. Other investigators indicated that the improvement of insulin sensitivity following treatment with ACE inhibitors was due to increase in insulin-induced insulin receptor substrate-1 phosphorylation(IRS-1) as well as IRS-1 phosphatidylinositol (PI3) kinase association. Furtheremore in the liver and muscle[51], it has been suggested that lisinopril induced improvement of insulin sensitivity via endothelin-1 inhibition[52]. Endothelin-1 has potent glycogenolytic effect on hepatocytes & may cause insulin resistance in rat adipocyte.

Moreover, lisinopril induced a significant decrease in TNF-α in high fructose fed rats as compared to rats fed standard diet treated with lisinopril. As TNF-α induced an effect an antagonistic to insulin through the inhibition of insulin receptor tyrosine kinase[11], so the normalization of TNF-α levels can explain the improvement of insulin sensitivity with lisinopril treatment.

It can be concluded that this study provided additional evidence that feeding rats with high dosage of fructose leads to insulin resistance. On the light of this study, it could be concluded that lisinopril and visfatin treatment has a beneficial effect in the reversal of insulin resistance and its accompanying metabolic changes. TNF-α may be an important circulating cytokine which may provide a potentially reversible mechanism for mediating insulin resistance. Furthermore both lisinopril and visfatin might prove useful in the treatment and/or preventing non hyperglycemic insulin resistance states such as obesity and impaired glucose tolerance as well as in the treatment of established non insulin dependent diabetes mellitus.

Ethical approval: This present study was conducted in the Medical Physiology Department, Faculty of Medicine, Mansoura University and was performed in accordance with the ethical standards of the "local medical committee" of faculty of medicine in Mansoura University, Egypt

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EFFECT OF LISINOPRIL AND VISFATIN ON NON HYPERGLYCEMIC INSULIN RESISTANCE IN ALBINO RATS

Ahmed A. El-Gendy Ph.D

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CHANGES IN BLOOD GLUCOSE LEVELS IN CHRONIC HEPATITIS C EGYPTIAN PATIENTS TREATED WITH ANTIVIRAL THERAPY AND IT'S IMPACT ON VIROLOGICAL RESPONSE

Mostafa Soliman El Kady MD, Ibrahim Ahmed Awadin MD, Maha Zein Elabedin Omar MD and Wael Ahmad Shahin MD

Abstract

There is a complex relationship between Diabetes mellitus (DM) and chronic Hepatitis C (CHC). DM is common in CHC patients and at the same time it reduces the therapeutic effectiveness of pegylated interferon (Pegyl.IFN) and ribavirin therapy. The aim of the study was to examine the influence of DM on the Sustained Virological Response (SVR) after Pegyl IFN, ribavirin therapy in Egyptian CHC patients and to study the incidence of glucose abnormalities developed in non diabetic CHC patients with treatment and its relation to the SVR. ***Methods:*** *256 Egyptian CHC patients were divided into 2 groups: Group (1): 116 diabetic patients, and Group (2): 140 Non diabetic patients. All patients received Pegyl IFN and ribavirin for 48 weeks and monitored for SVR and glucose abnormalities development during 12 months after end of treatment.* ***Results:*** *SVR of the studied groups was 50.8%, diabetic patients had less SVR than non diabetic (40.50% vs. 59.30%, p=0.003), and on multivariate analysis, diabetic patients had significantly older age [O.R 1.06, 95% C.I (1.016-1.114) p 0.008], higher BMI [1.204 (1.020-1.422) p 0.03] and more steatosis [1.565 (1.21-2.20) p 0.001]. Higher SVR was associated with lower fasting and post prandial blood sugar (p <0.05) for both, less steatosis (p=0.04), lower stage of fibrosis (p=0.039) and lower viral load (p=0.001). Glucose abnormalities developed in 25 patients (18%) who were normoglycemic with antiviral therapy and their SVR was (36% vs. 64%, p=0.009). Multivariate analysis reveled that those patients had older age (p=0.001), higher BMI (p=0.001) and stage 3 fibrosis (p=0.001). Out of those 25 patients; 18 patients (13%) devel-*

oped impaired glucose tolerance and their SVR was 50% and 7 patients (5%) developed DM and their SVR was Zero %. ***Conclusion:*** *DM has a major effect on the SVR in Egyptian CHC patients; SVR of the studied groups was 50.8% and in diabetic patients it was 40.5%. In non diabetic patients, 18% developed glucose abnormalities with SVR of 36% and 5% developed DM and their SVR was Zero%.*

Introduction

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide. It is estimated that approximately 130–210 million individuals are chronically infected with HCV[1]. The prevalence varies markedly from one geographic area to another; the highest reported prevalence is in Egypt (15% - 22%), approximately 90% of them have HCV genotype[2,3,4] Chronic Hepatitis C leads to the development of cirrhosis, the rate of progression towards the cirrhosis varies widely, it seems to depend on host-related cofactors, such as age, sex, overweight, immune status and type 2 diabetes (T2D) even at the pre diabetic stage (impaired fasting glucose)[4,5] High prevalence of glucose abnormalities and type 2 DM has been reported in chronic hepatitis C patients. a mutual positive relationship was found between type 2 DM and chronic hepatitis C; type 2 DM was seen three folds more frequent in chronic hepatitis C compared to HCV negative patients and in some studies, HCV was found to be up to seven folds more prevalent in type 2 diabetic patients compared to controls[6,7,8].

DM has been reported to reduces the therapeutic effectiveness of interferon (IFN) and ribavirin therapy in patients with chronic hepatitis C[9]. Also, IFN therapy is often implicated in the development of diabetes in HCV patients. However, this association is rare, and the few cases of DM developed during IFN therapy had T2DM, in line with other autoimmune manifestations induced by IFN[10,11].

The aim of the study was to study the influence of DM on the outcome of pegylated IFN alpha plus ribavirin therapy in Egyptian

chronic HCV patients, and also to study the incidence of glucose abnormalities (diabetes mellitus and impaired fasting glucose) happened during treatment and its relation to SVR.

Materials and Methods

This prospective study was carried out at the Department of Hepatology, Gastroenterology and Infectious diseases, Benha University Hospital, Benha, Egypt and Agouza Police Hospital, Cairo, Egypt, from May 2010 till April 2012. The study was done on 256 adult chronic hepatitis C (CHC) patients who received pegylated IFN and Ribavirin for 48 weeks. Out of the 256 patients; 116 patients were diabetic (group 1) and 140 patients were normo-glycemic (group 2). Informed written consent to participate in the study was obtained from each patient. All patients were selected according to the inclusion and exclusion criteria of the Egyptian ministry of health (Inclusion criteria include: age from 18 to 60 years, Positive HCV- RNA and compatible liver biopsy, S. albumin ≥ 3.5 gm/dl, negative HBs Ag, WBCs> 3.500/ μL, Neutrophil count >2.000/μL, Platelets > 75.000/μL, Serum creatinine <1.2 mg/dl and Hb >13 gm/dl in males & 12 g/dl in females. Exclusion criteria include: liver cirrhosis, other causes of CLD, other chronic diseases (as ischemic heart disease, uncontrolled psychiatric disease or hemolytic anemia) and Pregnancy or breast feeding[12].

The patients were treated with pegylated interferon (PEG-IFN) plus ribavirin for 48 weeks. PEG-IFN alfa was given subcutaneous once weekly and ribavirin was given orally daily (1000-1200 mg/dl). Patients will not continue treatment if they failed to have 2 log drop or more of HCV PCR at 12 weeks or clear the virus at 24 weeks, otherwise treatment continued for 48 weeks. All patients were subjected to the following: CBC, Fasting and 2h post prandial blood glucose level, Liver and kidney function tests and coagulation profile, viral markers (HCV Ab, HCV- RNA, HBsAg), ANA titer, TSH level, α feto protein, Pregnancy test for females in child bearing period, Liver biopsy and Abdominal Ultrasound. Glucose abnormalities were diagnosed

according to American Diabetes Association 2011[13] when: 1. Fasting plasma glucose (FPG) ≥126 mg/dl (Fasting is defined as no caloric intake for at least 8 hours), 2. Two hours plasma glucose ≥200 mg/dl during an oral glucose tolerance test (OGTT) or 3. A random plasma glucose ≥200 mg/dl in a patient with classic symptoms of hyperglycemia. The definition of Impaired fasting glucose (IFG): when FBG =100 – 125 mg/dl or Impaired glucose tolerance (IGT) = 2h OGTT=140 - 200 mg/dl.

Liver biopsy was done and histological evaluation of the stage of fibrosis were evaluated according to the Metavir scoring system. (F) fibrosis (F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with rare septa, F3 = numerous septa without cirrhosis, and F4= cirrhosis).[14] A Sustained Virological Response (SVR) was considered when undetectable HCV RNA in serum was maintained 6 month after cessation of antiviral therapy.

Patients follow up: patients were seen in the Hepatology outpatient clinic weekly during the first month and monthly thereafter along the course of therapy, during follow up visits, signs and symptoms of possible adverse effects were evaluated and laboratory studies were performed and 6 months after discontinuation of treatment patients were assessed for SVR. Patients were monitored for fasting blood sugar and 2h (OGTT) at 0, 12, 24, 36, 48 weeks of treatment and during follow up 12 months after the end of treatment.

Statistical analysis: results were expressed as means ± standard deviation for continuous variables or number and percentage (%) for categorical variables. Comparison between the two groups was performed using unpaired t test and comparison between categorical data was performed using Chi square test. Pearson chi-square and likelihood-ratio were used. Fisher's exact test and Yates' corrected chi-square were computed for 2*2 tables. Stepwise logistic regression was used for multivariate analysis and Cox-regression for multivariate analy-

sis of variables associated with the appearance of impaired fasting glucose in the course of the follow up. SPSS Statistics 16 program was used for data analysis. Significance was considered at P value 0.05 or less.

Results

This prospective study was conducted on 256 Egyptian chronic hepatitis C patients, out of them; 140 patients were non diabetic and 116 patients were diabetic. All patients were treated with peg interferon alfa plus ribavirin for 48 weeks.

Table (1) showed the basic characteristics of all patients including the demographic, laboratory and histopathological data, males were more than females (57.4%), the mean age of patients was 46.9±7.58 years, the mean BMI was 27.71 ± 1.54Kg/m2, F3 fibrosis was found in 28.9% while steatosis was found in 31.6% of patients. SVR was achieved in 50.8% of patients.

Table (2) compared diabetic and non diabetic chronic hepatitis patients prior to antiviral treatment, using univariate analysis, diabetes mellitus was significantly associated with advanced age (49.84 ± 5.82 vs. 44.46 ± 8.02 yrs, p value <0.001) , high BMI (28.32±1.36 vs. 27.2 ± 1.5 kg/m2, p value <0.001) and high ALT level (27.55 ± 9.27 vs. 22.72 ± 9.45 U/L, p value <0.001), F3 fibrosis (40.5% vs. 19.3%, p value 0.001), steatosis (41.4% vs. 23.6% p value 0.002) and failure of SVR (40.7% vs. 59.5%, p value 0.003). On multivariate analysis, age (p=.008), BMI (p=0.03) and steatosis (p=.001) were significantly higher in diabetic patients while ALT and Fibrosis were not significantly different.

Table (3) compared sustained virological responders and non responders, age, fasting blood glucose, 2h OGTT, viral load, advanced fibrosis and steatosis were all significantly higher in non sustained virological responders in both univariate and multivariate analysis while BMI was significantly different in univariate but not multivariate analysis. Patients with SVR were younger

(44.51±7.75 vs. 49.37±6.58), had lower BMI (27.05±1.39 vs. 28.39±1.39), lower viral load (662.415±641.912 vs. 1.053.442±880.771), lower 2h.P.P (166.67±66.97 vs. 196.30±82.90) and lower fasting blood sugar (113.47±46.63 vs. 128.45±51.57). Multivariate analysis revealed that, the independent variables related to SVR were viral load (P=0.001), fasting blood sugar (P=0.000), 2HPP (P=0.02), fibrosis grade 3 (P=0.039) and steatosis (p=0.04).

According to table 4, factors associated with development of glucose abnormalities in normoglycemic group after antiviral treatment were elderly group (51.2±4.7 vs. 43.00±7.85), higher BMI (28.47±1.14 vs. 26.92±1.43), presence of steatosis (40% vs. 20%), advanced fibrosis F3 (48% vs. 13%) and lower SVR (36% vs. 64%). On multivariate analysis, age (p 0.001), BMI (p 0.001), advanced Fibrosis, F3 (p 0.001) were significantly related to the development of glucose abnormalities after antiviral treatment. It is important to mention that SVR is lower in patient who developed glucose abnormalities in both univariate and multivariate analysis.

In table (5), out of 140 non diabetic patients, 25 patients (18%) developed glucose abnormalities after antiviral treatment. None of the patients who developed DM during treatment had SVR (zero% SVR), 18 patients had impaired glucose tolerance (IGT), and the SVR is 50%.

Table 1: Baseline characteristics of the studied patients

parameter	Mean ± SD
Male (no, %)	147 (57.4%)
Age(y)	46.90 ± 7.58
Height(m)	1.71± 0.06
Weight(kg)	81.21 ± 6.26
BMI	27.71± 1.54
Fasting blood sugar(mg/dl)	120 ± 49
2hOGTT(mg/dl)	181 ± 76
Creatinine(mg/dl)	1.04 ± 0.08
Albumin(gm/dl)	4.22 ± 0.39
Alk.phosphatase (U/L)	108 ± 16
AST(U/L)	22 ± 9
ALT(U/L)	24.91 ± 9.66
Bilirubin(mg/dl)	0.98 ± 0.07
WBC(c/ μL)	5,253 ± 2,542
ANC(c/ μL)	2,816 ± 797
HB(gm/dl)	13.50 ± 0.87
Platelet (c^3/ μL)	205 ± 45
INR	1.00 ± 0.00
Alpha FP(ng/dl)	11.50 ± 8.27
TSH(mIU/L)	3.13 ± 0.38
Viral load(IU/ ml)	854,874
POSITIVE SVR	130 (50.78%)
Fibrosis F1	89 pt (34.8%)
Fibrosis F2	93pt (36.3%)
Fibrosis F3	74 pt (28.9%)
steatosis	81 pt (31.6%)

Table 2: Comparison of diabetic and non diabetic chronic hepatitis C patients using univariate and multivariate analysis.

Significant parameters	Univariate analysis			Multivariate analysis	
	Group 1 Diabetic	Group ll Non Diabetic	P value	OR(95%CI)	P value
Age(yr)	49.8 ± 5.8	44.5 ± 8.0	<0.001	1.064(1.016-1.114)	0.008
BMI (kg/m^2)	28.32 ± 1.36	27.20 ± 1.50	<0.001	1.204(1.020-1.422)	0.03
ALT(U/L)	27.55 ± 9.27	22.72 ± 9.45	<0.001	0.98(0.608-1.537)	0.18
F 3 fibrosis	47(40.50%)	27(19.30%)	0.001**	1.167(0.83-1.64)	0.37
steatosis	48(41.40%)	33(23.60%)	0.002**	1.565(1.21-2.20)	0.001

Table 3: Comparison of sustained virological responders and non responders' chronic hepatitis C patients using univariate and multivariate analysis.

Significant parameters	Univariate analysis			Multivariate analysis	
	Positive SVR (n= 130)	Negative SVR (n=126)	P-value	OR(95%CI)	P-value
Age(yr)	44.51 ± 7.75	49.37 ± 6.58	<0.001	3.568(.899-14.161)	.071
BMI	27.05 ± 1.39	28.39 ± 1.39	<0.001	0992(0.977-1.009)	.355
Fasting blood sugar (mg/dl)	113.47 ± 46.63	135.45 ± 51.57	0.001	1.863(1.445-2.401)	.000
2h OGTT (mg/dl)	166.67 ± 66.97	196.30 ± 82.90	0.000	0.57(0.35-0.92)	.02
Viral load (IU/ ml)	662,415 ± 641,912	1,053,442 ±880,771	<0.001	1.000(1.000-1.000)	0.001
FIBROSIS (F 3)	29(22.30%)	45(35.70%)	0.009	1.491(1.020-2.178)	0.039
STEATOSIS	31(23.80%)	50(39.70%)	0.006	0.48(0.24-0.98)	0.04

Table 4: Comparison of normo-glycemic chronic hepatitis C patients who developed glucose abnormalities after antiviral treatment and those who remained normo-glycemic using univariate and multivariate analysis.

Significant parameters	Univariate analysis			Multivariate analysis	
	Glucose abnormalities (n= 25,18%)	Normo-glycemic (n=115, 82%)	P-value	OR(95%CI)	P-value
Age(y)	51.20 ± 4.70	43.00 ± 7.85	<0.001	1.837(0.952-1.931)	0.001
BMI	28.47 ± 1.14	26.92 ± 1.43	0<0.001	1.565(1.211-2.202)	0.001
steatosis	10 (40.00%)	23 (20.00%)	0.03	1.035(0.289-3.706)	0.958
Fibrosis F3	12(48.00%)	15 (13.00%)	<0.001	4.43(2.49-8.30)	0.001
SVR	9(36.00%)	74 (64.30%)	0.009	0.472(0.295-0.756)	0.002

Table 5: The relation between SVR and glucose abnormalities developed in non diabetic chronic hepatitis C patients after pegylated IFN and ribavirin treatment.

parameter		Non SVR	SVR	total
Glucose abnormalities	DM	7	0	7 (5%)
	IGT	9	9	18 (13%)
No glucose abnormalities		46	69	115 (82%)
Total		62	78	140 (100%)

Discussion

Diabetes mellitus is more likely to develop in patients infected with HCV genotype 4, which is the predominant genotype in Egypt and the Middle Eastern countries [15] In Egypt the prevalence of DM was 25.4% among HCV patients, CHC patients are three times more likely to develop DM than HCV sero-negative patients[16]. Diabetes mellitus (DM) has been reported to reduce the therapeutic effectiveness of interferon (IFN), ribavirin therapy in patients with chronic hepatitis C[9].

This prospective study was conducted on 256 Egyptian CHC patients, 140 patients were non diabetic and 116 patients were diabetic. All patients were treated with peg interferon and ribavirin.

In this study, male patients constituted 57.4%, and the average age was 46.90 ± 7.58yrs. Diabetic CHC patients were older in age CI = 1.020- 1.422; p=0.03) and higher number of cases having CI= 1.21- 2.20; p=.001). In a recent study using US population data. The National Health and Nutrition Examination Surveys (NHANES) collected 19,741 participants between 1999 and 2010,

173 individuals (0.88%) had Chronic Hepatitis C who were predominantly, men (66.6% vs. 46.1%, P = 0.0001), between 45 and 55 years of age (41.9% vs. 20.4%, P = 0.0001), and had higher rate of insulin resistance (44.1% vs. 31.1%, P = 0.0301). In multivariate analysis, CHC was independently associated with the presence of insulin resistance [OR (95% CI) = 2.06 (1.19–3.57)] and DM [OR = 2.31 (1.18–4.54)].[17], In a study from Mexico on CHC patients treated with Pegyl IFN, ribavirin, 18% of patients had DM, None of the responders had T2DM, but 25 % of the non-responders had diabetes. T2DM patients were older than those without diabetes (57.7 vs. 44.5 years, p < 0.001), and after multivariate analysis, only age was significantly associated with diagnosis of T2DM[18], also, Romero-Gomez et al., 2008[19] reported that DM was detected more often in older patients (OR: 1.08; CI=1.05- 1.19; p= <0.001). EL-Hawary et al., 2011[20] reported that DM was detected more often in chronic hepatitis c patients with advanced age. Le cube et al., 2004 and Zein et al., 2005[6,21] reported that insulin resistance was shown to increase in older patients, and to correlate with steatosis severity and with body mass index. Similar results were also obtained by Chen et al ., 2003[22] who reported that old age, high BMI and family history of diabetes were all independent factors for the development of type 2 DM in patients with chronic hepatitis C. Also, Mehta et al., 2003[23], studied 1084 patients, a total of 548 subjects developed T2DM over 9 years of follow up. Among those at high risk for DM, persons with HCV infection were more than 11 times as likely as those without HCV infection to develop T2DM.

In the current study, SVR in diabetic patients was 40.7% while in normo-glycemic patients; it was 59.5% (18.8% difference). This result was in agreement with Romero-Gomez et al., 2008[19] who reported that in patients with abnormal glucose values the rate of SVR lower than normo-glycemic patients. Similar results were obtained by (Elgouhari et al., 2009)[24] who reported that patients with T2DM were less likely

to achieve sustained virological response. Also,[25,26,27] reported that DM and insulin resistance in HCV infection was associated with reduced rates of initial virological response as well as SVR in patients treated with a combination of pegylated IFN-a and ribavirin. This negative association had been reported not only in patients infected with the HCV genotype 1, but also in those with the so-called "easy-to-treat" genotypes2 and 3. On the other hand, (Cammá et al., 2006)[28] reported that there was no significant reduction in the response to combination therapy (pegylated IFN-α and ribavirin) in CHC patients and Insulin Resistance and DM, this could be explained by the character of patients included in this study who had genotype 1, high baseline viral load and advanced hepatic fibrosis.

In this study, the independent variables related to SVR were CI=0.35- 0.92; p=0.02). Romero-Gomez et al., 2008[19] reported that sustained virological response depends on several factors as age, CI=0.35- 0.92; p=0.02). Also, (Miyanari et al., 2007)[29] reported that HCV patients having steatosis are thought to have increased lipid droplets in hepatocytes which increase HCV replications that result in poor responses to antiviral treatment. Similarly, (EL-Zayadi and Anis., 2012)[30] reported that hepatitis C virus infection and hyperglycemia are considered significant risk factors for development of insulin resistance (IR). IR is associated with poor response to antiviral treatment both for initial virological response and SVR. On the other hand, (Guidi et al., 2007)[31] reported that hepatic steatosis in chronic hepatitis C, irrespective of its grade, is not a negative prognostic factor of response to combined anti-viral therapy. But, this study was limited by the relatively small sample size (89 cases) and included easy to treat genotypes[2,3].

In this study, non diabetic patients who maintain normoglycemia during follow up period have higher SVR compared to those who develop CI=0.295-0.756; p = 0.002). Similar result was obtained by[19,32] they observed that glucose abnormalities

were significantly lower in CI=0.24- 0.98; p=0.04).[33,34,35] reported that in patients who achieved SVR, glucose homeostasis may be improved and hence the risk of T2DM may be potentially reduced. On the other hand, Giordanino et al., 2008[36] reported that there was no significant difference between responders and non responders after period of follow up (8years) regarding glucose abnormalities (DM and IFG). But, this study was limited by the relatively small sample size.

In the present study, after antiviral treatment, glucose abnormalities developed more in patients with advanced age (OR: CI=0.295-0.756; p = 0.002). Romero-Gomez et al., 2008 (19) CI=.1.06- 2.01; p = 0.02) were found to be independently associated with a higher risk for impaired fasting glucose or diabetes development following treatment for viral infection. Similarly, Simo et al., 2006 [32], reported that patients who developed glucose abnormalities had more diabetic risk factors such as older age, higher BMI, negative SVR and high triglyceride level compared with patients who remained without glucose abnormalities.

In this study, patients with sustained virological response did not develop diabetes during follow up, whereas diabetes was detected in 7 patients with non sustained virological response (5%). Similar result was obtained by Simo et al., 2006[32], who studied 234 patients, 96 of them with SVR and 138 with no SVR. During follow up, patients with SVR did not develop diabetes, whereas 9 cases (6.5%) of diabetes were detected in non sustained responders. Also, Romero-Gomez et al., 2008[19], reported that normo-glycemic patients with SVR (432/734) during follow up did not develop diabetes, whereas 7/152 cases (4.6%) of diabetes were detected in non sustained responders. On contrast to all these works, Giordanino et al., 2008[36], reported that there is no significant difference between responders and non responders as regard to developing DM after antiviral therapy.

The SVR rate was lower in diabetic patients than normo-glycemic patients [47/116

(40.50%) vs. 83/140(59.30%); p=0.003]. Also, the independent variables related to SVR were steatosis (p=0.04), grade 3 fibrosis (p=0.039) and viral load (p=0.001). During follow up of normoglycemic patients, glucose abnormalities developed more in negative SVR than positive SVR patients [16/25(64.00%) vs.9/25 (36.00%); p=0.002] , patients with positive SVR did not develop diabetes, whereas 7 cases of diabetes were detected in negative SVR .Also, it was found that advanced age (p=0.001), high BMI (p=0.001), and higher fibrosis score (p=0.001) were the independent variables associated with the development of glucose abnormalities.

Conclusion

Abnormal glucose values was found to be associated with a lower rate of SVR. The achievement of SVR induces a decrease in the incidence of glucose abnormalities and T2DM and this supports the concepts that HCV infection could be a cause of type2 diabetes in predisposed individuals like those who were older, had higher BMI and had steatosis. Sustained virological response rate , in the current study was lower in patients with higher viral load , higher fibrosis score, steatosis and in

Recommendations: large scale studies are needed to support the impact of antiviral treatment on blood glucose levels in Egyptian CHC patients. Correcting the modifiable factors as BMI and steatosis will help to increase SVR.

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CHANGES IN BLOOD GLUCOSE LEVELS IN CHRONIC HEPATITIS C EGYPTIAN PATIENTS TREATED WITH ANTIVIRAL THERAPY AND IT'S IMPACT ON VIROLOGICAL RESPONSE

Mostafa Soliman El Kady MD, Ibrahim Ahmed Awadin MD, Maha Zein Elabedin Omar MD and Wael Ahmad Shahin MD

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FREQUENCY OF EXTRAHEPATIC CHOLESTASIS IN TROPICAL MEDICINE DEPARTMENT IN ZAGAZIG UNIVERSITY HOSPITALS

Ghada A. Salem MD, Samy Eissa MD, M.I. Radwan MD and Ramy Ibrahim MD

Tropical Medicine Department, Faculty of Medicine, Zagazig University

Abstract

Background and study aim: *Production of bile is a complex process comprising hepatic uptake of bile salts, bilirubin, cholesterol and other solutes with conjugation or metabolic modification of selected solutes and transport or diffusion of the compounds across the hepatocyte to the bile canliculs. Disruption of one or more of the steps in the bile formation can cause cholestasis or jaundice. Cholestatic jaundice is often accompanied by a broad spectrum of laboratory, clinical and histological abnormalities. The various causes of enterohepaticcholestasis may not be easily detected in many cases admitted to our department which may pass undiagnosed. Our aim was to find if the extrahepatic cholestasis may a big problem in cases admitted to our department and try to determine the possible underlying causes of this health problem aiming to resolve that problem.*

Patients and methods: *in this work, we studied 506 cases admitted to our department who met the inclusion criteria of high serum alkaline phosphatase level 1.5 times the upper limit of normal, high gamma glytamyl transferase more than 3 times the upper limit of normal, ultrasonogrpahic features of extrahepaticholestasis (dilated extra-or intrahepatic ducts) whatever the cause. Then 61 selected patients underwent the following tests: Full history taking and thorough clinical examination. Complete blood picture, liver and kidney function tests, total cholesterol were done, abdominal CT and ERCP were done.*

Results: *Frequency of extrahepatic cholestasis in 61 cases out of 506 cases was 12.1%, the female was 54.1% while the male was 45.9% with mean ± SD was 51.1 ± 11.7. The CBD stone was 42.4% in female while 39.3% in male which was the most common cause of extrahepaticcholestasis in our study. There was positive correlation between alka-*

line phosphatse and gamma glutanyl transferase with P= 0.0001 there is increase in the percentage of females with BMI 25 – 30 and > 30 in cases of GB stones more than the males but it is non-significant P= >70.05 and there is no statistical difference between BMI and age in cases of GB stones.

The predictive value of US compared to ERCP in detection of CBD stone was 100% positive and 85.7% negative, while the predictive value of CT compared to ERCP in detection of CBC stone is 100% positive an 92.3% negative, the predictive value of US compared to ERCP in detection of cancer head of pancreas is 100% positive and 94.5% negative, while the predictive value of CT compared to ERCP in detection of cancer head of pancreas is 100% positive.

We can concluded, that, extrahepatic cholestasis has a frequency of 12.1% is in our department, choledocholithiasis is the commonest benign aetiology, while cancer head of pancreas is the commonest malignancy ERCP is considered a gold standard modalities in diagnosis and treatment of extrahepaticcholestasis.

Introduction

Production of bile is a complex process comprising hepatic uptake of bile salts, bilirubin, cholesterol and other solutes, production of bile is the most distinctive and specific liver functions. Conjugation or metabolic modification of selected solutes; transport or diffusion of the compounds across the hepatocyte to the bile canclicululus, simultaneous regulated de novo synthesis of bile salts, phospholipids and cholesterol; secretion of bile salts, cholesterol, phospholipids and conjugated organic solutes across the cancliculus membrane; formation of bile is the bile ducts; and flow of bile to the gallbladder and duodenum.

Following its synthesis by hepatocytes, bile is secreted into bile ducts before entering the gall bladder, where it is concentrated and stored. After meals the gall bladder contracts and bile flow through the cystic and common bile duct into the intestine, where it mixes with food and helps to solubilize and absorb fats. Bile salts are then actively reabsorbed in the distal small bowel and taken up by liver from the portal blood as a part of the enterohepatic circulation[1].

Disruption of one or more of these steps in bile formation can cause cholestasis or jaundice[2]. Bile facilitates digestion and absorption of cholesterol and also acts as the major vehicle for elimination of cholesterol from the body[3].

Cholestatic jaundice is often accompanied by a broad spectrum of laboratory, clinical and histological abnormalities[4]. The causes of extrahepatic cholestasis may not be easily detected in many cases admitted to our department which may pass undiagnosed.

The primary aim of our study was to find if the extrahepatic cholestasis may consider a big problem in cases admitted to our department and try to determine the possible underlying causes of this health problem, aiming to resolve this problem.

Patients and Methods

The present study was conducted in Tropical Medicine Department Zagazig university hospital, informed consent obtained from all patients, this study was approved by Ethical committee of faculty of medicine zagazig university. There were no conflicts of interests and no founding during study.

The study was conducted on 506 cases admitted to our department and they were subjected to:

• Serum alkaline phosphatase level (with or without hyperbilirubinemia).

• Pelvi abdominal ultrasound.

The inclusion criteria were high serum alkaline phosphate level 1.5 times the upper limit of normal, high gamma glutanyl transferase more than 3 times the upper limit of normal, ultrasonography features of extrahepatic cholestasis (dilated extra or intrahepatic ducts) whatever the cause. All patients underwent the following tests:

• Full history taking and through clinical examination.

• Complete blood picture using sympx K x 21 cell counter (Roche diagnostics Manheim, Germany).

• Liver, kidney function tests.

• Total cholesterol, triglyceride were done by selectra XI autoanalyzer system (vital scientific, Daren, Holland).

• Abdominal C.T. especially when mass lesion is suspected by

ultrasonography.

• ECRP to determine the etiology of obstruction.

Statistically analysis:

The data were statistically analyzed using microstate soft were program and the following statistically tests were applied:

• Studied "t" test for comparison of means two independent group.

• Paired "t" test: $\frac{\text{mean difference}}{\text{SD of difference}}$

• The standard deviation (SD) as measure of dispersion of the results wound the mean.

• Chi-square test: used to find the association between row and column variables.

• Validity of screening tests.

• Sensitivity= $\frac{\text{true positivie}}{\text{true positive + false negative}}$

• Specificity= $\frac{\text{true negative}}{\text{true negative + false positive}}$

• Negative predictive value=

$$\frac{\text{true negative}}{\text{true negative + false negative}}$$

• Accuracy=

$$\frac{\text{true positivie + true negative}}{\text{true positive + ture negative + false positive + false negative}}$$

Level of significance:

• P value of >0.05 → non significant.

• P value of <0.05 → significant

• P value of <0.001→highly significant .

Results

Table (1): Demographic data of cases with extrahepatic cholestasis.

	Sex		Age	
	Male	Female	Male	Female
No	28	33	25-72	51.1 ± 11.7
%	45.9	54.1		
Total No = 61				

Table (2): Frequency of extrhepatic cholestasis:

Studied cases	Cases with extrahepathic cholestasis	%
506	61	12.1%

Table (3): Agreement between alkaline phosphate and gamma glutamyl transferase in extrahepatic cholestasis.

		ALP				Total		Kappa	P
		<1.5		<1.5		No	%		
		No	%	No	%				
GGT	<3 folds	5	%	Zero	%	5	8.2%	0.68	0.0001
	>3 folds	4	6.5%	52	85.2%	56	91.8%		
Total		9	14.7%	52	85.2	61	100%		

- As shown in the table there is significant agreement between ALP and GGT, P value is < 0.05.

Table (4): Relation between BMI and sex in cases with GB stones.

		BMI											
		> 25				25-30				> 30			
Sex		Male		Female		Male		Female		Male		Female	
		No	%	No	%	No	%	No	%	No	%	No	%
GB stone	ve	1	100%	-	0%	20	87%	14	63.6	3	60%	4	44.4%
	+ve	-	0%	1	100%	3	13%	8	36.4%	2	40%	5	55.6%
X2		-				3.311				0.311			
P		-				0.069				0.577			

- As shown in the table there is increase in the percentage of females with BMI more than 30 in cases of GB stones, it is non significant as p value is > 0.05.
- Regarding the group with BMI < 25, there are only 2 patients (this is not sufficient for statistically study).

Table (5): Relation between BMI and age in case with GB stones.

		BMI													
		<25		25-30						> 30					
age		>60		20-40		41-60		>60		20-40		41-60		>60	
		No	%	No	%	No	%	No	%	No	%	No	%	No	%
GB stones	-ve	1	50%	6	75%	17	77%	11	74%	-	0%	6	55.5	1	50%
	+ve	1	50%	2	25%	5	23%	4	27%	1	100%	5	45.5%	1	50%
X2	-	0.077								1.091					
P	-	0.962								0.580					

- As shown in the table there is no statistical difference between BMI and age in cases with GB stones, p value > 0.05.
- Regarding patients of age > 60 years in the group of BMI < 25, there is only 2 patients (number is not sufficient for statistical study).
- As regards patients <60 years in the same group (BMI <25), there were no patients at all.

Table (6): Ultrasound compared to ERCP in detection of CBD stones.

		ERCP		Total
		Positive	Negative	
U/S	Positive	19	0	19
	Negative	6	36	42
Total		25	36	64

- As shown in the table sensitivity of U/S is 76% while specificity is 100% the positive predictive value is 100% and the negative predictive value is 85.7%.

Table (7): CT compared to ERCP in detection of CBD stones.

		ERCP		Total
		Positive	**Negative**	
U/S	Positive	22	0	22
	Negative	3	36	39
Total		25	36	61

Table (8): U/S compared to ERCP in detection of cancer head of pancreas.

		ERCP		Total
		Positive	**Negative**	
U/S	Positive	6	0	6
	Negative	3	52	55
Total		9	52	61

- As shown in the table sensitivity of U/S is 66.7% while specificity is 100%, the positive predictive value is 100% and the negative predictive value is 94.5%.

Table (9): CT compared to ERCP in detection of cancer head of pancreas.

		ERCP		Total
		Positive	**Negative**	
CT	Positive	9	0	9
	Negative	9	52	52
	Total	9	52	61

- As shown in the table sensitivity and specificity of both CT and ERCP is 100% in detection of cancer of pancreas.

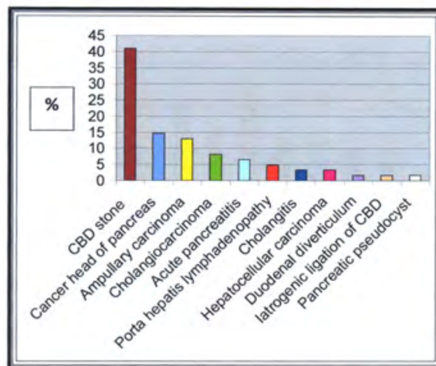

Fig. (1): Frequency of causes of extrahepatic cholestasis.

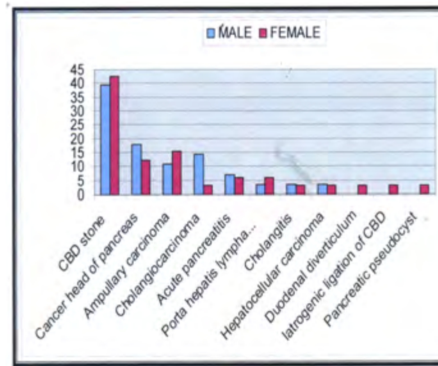

Fig. (2): Relation between etiology of extraheaptic cholestasis and sex.

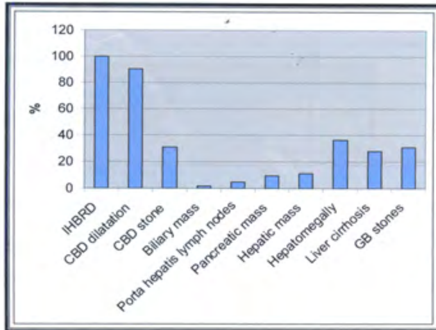

Fig. (3): Frequency of ultrasonographic features of extrahepatic cholestasis.

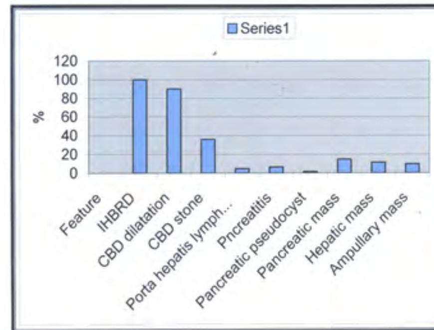

Fig. (4): Frequency of CT features of extra-hepatic cholestasis.

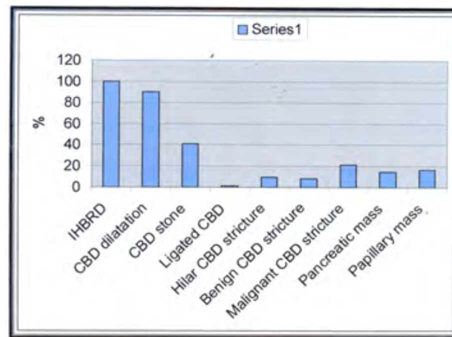

Fig. (5): ERCP features of cases with extrahepatic cholestasis.

Discussion

Among the many functions of the liver, production of bile is the most distinctive and liver specific. Adults humans produce approximately 500ml of bile per day which is an aqueous solution containing bile salts, cholesterol proteins, bilrubin conjugates and others[5].

Extrahepatic cholestasis is caused by mechanical obstruction of large bile ducts or masses present the portahepatics[6].

This study was conducted to assess the frequency of extrahepatic cholestasis as a health problem in our department with its possible underlying causes, it was found to be 61 cases out of 506 cases admitted to our tropical department with a percentage of (12.1%).

The frequency of extraheaptic cholestasis in our study may be due to conduction of this study on a defined population who were admitted to our department, with high prevalence of chronic liver disease and cirrhosis which increase the risk of gall bladder and common bile ducts stones as noted by[7] who studied the prevalence of gall bladder stones diseases in Egyptian patients with chronic liver disease and found that chronic hepatitis C virus (HCV) infection is considered an important risk factor for the development gallstone disease in those patients.

The lower incidence of obstructive jaundice in other studies as reported by[8] who found the frequency was 5/100 cases have obstructive jaundice may be due to assessment of this incidence by a large scale study conducted on general population.

In our study, the frequency of extra-hepatic cholestasis was common among middle aged patients with a mean age of 51 years and a range of 25-72 years. This is in agreement with[9] who found similar results.

The majority of patient is this study had benign obstructive jaundice (57.4%) while malignant causes were (42.6%) which is in agreement with many authors [10,11,12] who found the percentage of benign to malignant causes 60% : 40%.

In our patients choledocholithiasis was the most common cause among various causes of extrahepatic cholestasis with a percentage of (40.9%)[13] also found the percentage of choledocholithiasis to be (35%) being the most common cause of obstructive jaundice in their study.

In our work extrahepatic cholestasis was more common in females (54.1%) which is usually benign in nature (60.6%) and common bile duct stone (CBD) was the most common cause in females (42.2%). This may be due to the high prevalence of cholesthiasis in females.

Women are at greater risk of developing gall bladder cholesterol

stone, this may be attributed to estrogen hormone level. Many studies[14] have shown that, female steroid hormones significantly alter hepatobiliary physiology. As the gall bladder volume increase during pregnancy and the emptying of the gall bladder is showed by pregnancy and by using contraceptive pills, the cholesterol content of bile is increased and the bile acid metabolism is altered by pregnancy and contraceptive pills.

The percentage of malignant extrahepatic cholestasis was higher in males (50%) than female (36.4%), being mainly cancer head of pancreas and cholangiocarcinoma with a percentage of (17.9%) and (14.3%) respectively[15] also found malignant obstructive jaundice to be more in males than females.

Cancer head of pancreas has higher incidence with more mortality in males than females. This may be due to increased frequency of tobacco use in males which is considered a risk factor of malignancy[16].

According to clinical presentation, 58 cases presented with jaundice with a percentage of 95.1%. The three cases that were not presented with jaundice one of them presented with vomiting and weight loss and it was diagnosed as ampullary carcinoma[17] postulated that intermittent jaundice in ampullary carcinoma may be due to necrosis that occur in the tumor allowing passage of bile with transient relief of symptoms.

The other two cases were presented with itching and they were diagnosed as CBD stone[18] mentioned that CBD stone may lead to intermittent jaundice due to the bell and valve action of stone at the lower end of CBD leading to partial and intermittent obstruction.

In selected cases in our study with extrahepatic choletasis, the frequency of gall bladder (GB) stones was higher among the cirrhotic patients with a significant relation between them. The percentage of GB stone in non cirrhotic was 22.7 while it was 52.9 in cirrhotic patient. The pathophysiologic mechanism responsible for GB stone in cirrhotic patients

may be related to altered pigment secretion, increased estrogen levels and / or abnormal gallbladder motility in cirrhosis[19]. The abnormal bile secretion in patients with liver cirrhosis may be due to diminished liver reserve and damaged bile ductules. Increased gall bladder wall thickness caused by hyperemia and edema and decreased contractility and impaired GB emptying contribute to gallstone formation[20].

In our study, patients with BMI > 30 showed increased percentage of GB stones (5.56%) while those with BMI (25-30), the percentage was (36.4%). Obesity is associated with increased bile stastis and cholesterol saturation, and increased risk of gallstone development, high dietary cholesterol increase biliary cholesterol secretion and decreases bile acid synthesis and pool in cholesterol gallstone [21].

In our study, there was positive correlation between both GGT and ALP in 52 cases of extrahepatic cholestasis with a percentage of 85.2%. this indicates that both markers together can be used for confirmation of cholestasis. This was previously mentioned by[22].

According to imaging modalities ultrasound (U/S) was done to all cases with extrheaptic cholestais. It picked dilated intrahepatic channels 100%, dilated CBD in 90.2%, while CBD stones are detected in 31.1%, mass was detected in only 27.8% and most of the times, it was in the head of pancreas. The diagnostic accuracy of ultrasound was conducted by[23] and showed it to be 85% accuracy.

Abdominal computed tomography (CT) was done to all cases to confirm the diagnosis of (U/S) or to detect the cause of obstruction when U/S couldn't detect it. It picked dilated intrahepatic channels in 100% dilated CBD in 90.2%, while CBD stones were detected in 36.1%. Mass was detected in 41%. It was superior to ultrasound in detection and localization of masses. The efficacy of CT in diagnosis and staining of tumours causing obstructive jaudince has also reported by[24].

Endoscopic retrograde cholangi-pancreatography (ERCP) was

done to the cases which were not diagnosed by US or CT or as a therapeutic tool.

The sensitivity of US, CT and ERCP is detection of the various causes of extrohepatic obstruction was 76%, 88% and 100% respectively. These findings are broadly in agreement with studies done at various other centers for CT[25] and ERCP[26].

We can concluded that, the extrahepatic cholestasis has a frequency of 12.1% in our department. Ultrasonography is considered the best primary diagnostic modality for extrahepatic cholestasis. While CT scans has more accuracy in detection of biliary and pancreatic masses, the ERCP is considered a gold standard modality in diagnosis and treatment of extrhepatic cholestasis with high accuracy in detection of the cause.

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FREQUENCY OF EXTRAHEPATIC CHOLESTASIS IN TROPICAL MEDICINE DEPARTMENT IN ZAGAZIG UNIVERSITY HOSPITALS

Ghada A. Salem MD, Samy Eissa MD, M.I. Radwan MD and Ramy Ibrahim MD

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IMMUNE PROFILE IN SPLENECTOMIZED AND NON-SPLENECTOMIZED CHILDREN WITH MAJOR BETA-THALASSEMIA

Maha A. Nouh MD, Anwar A.A. El-Sayed MD, Wafaa F. El-Saeed MD and Mona Afify MD*

*Department of Pediatrics, Factulty of Medicine, Zagazig University and Department of Biology, Science College for Girls, King AbdulAziz University**

Abstract

Beta-Thalassemia major patients suffer from too many problems rather than severe anemia, including increased susceptibility to bacterial infections which plays, constitutes the second most common cause of death after cardiac complications. This investigation was conducted to evaluate the impact of splenectomy in Beta (β)- thalassemia major children on the function of different components of the immune system. Study population included 40 β-thalassemia major children (20 splenectomized and 20 non-splenectomized) and 20 age- and sex- matched healthy control children. Parental consents were obtained before inclusion to the study. All children were subjected to history-taking, clinical examination and laboratory investigations including measurement of serum levels of immunoglobulins (Igs) (G, M and A), T-lymphocyte subsets, serum interleukin-6 (IL-6) and phagocytic activity of neutrophils. Obtained results revealed that splenectomized patients had significantly higher levels of serum IgA compared to that in non-splenectomized patients and control. Patients, especially, splenectomized ones had significantly lower levels of serum IL-6 and phagocytic activity compared to control. There were nonsignificant differences between patients and control as regards levels of IgG, IgM and CD4/CD8 ratio. There was significant positive correlation between IgG levels and frequency of transfusions, and between IgM levels and both age and mean ferritin levels, and between serum IL-6 levels and age of start transfusion. In conclusion various components of the immune system were impaired in β-thalassemia major patients, especially splenectomized ones.

Keywords: *Immune function - infection - thalassemia.*

Introduction

Thalassemias, a group of genetic disorders characterized by a disturbance of globin chain production, are among the most common genetic disorders in the world. Predisposing factors for infection in thalassemic patients include severe anemia, iron overload, spenectomy, and a range of immune abnormalities (Vento et al., 2006).

Numerous immune abnormalities have been described in thalassemic patients. As far as T-lymphocytes are concerned, greater numbers and activity of CD8 supperssor cells, decreased CD4/CD8 ratio, and reduced proliferation have been reported (Khalifa et al., 1988; Ezer et al., 2002). B-lymphocytes have been found to be increased in number, activated, and with impaired differentiation. Increased levels of Igs have also been described (Dua et al., 1993). Neutrophils and macrophages appear to have defects in chemotaxis and phagocytosis (Matzener et al., 1993). Finally, reduced levels of the complement components C3 and C4, and defective natural killer cell function have been reported (Ezer et al., 2002).

Splenectomy is justified in patients with thalassemia when the spleen becomes hyperactive, leading to excessive destruction of red blood cells (RBCs) and thus increasing the need for frequent blood transfusions, which in turn results in more iron accumulation. As a result, spenectomy is frequently done, and has been correlated with changes in immune responses (Koren et al., 1984; Ahluwalia et al., 2000). So, this study aimed to evaluate the impact of splenectomy in children with β-thalassemia major on the functions of the different components of the immune system including humoral, cellular, nonspecific immunity, and cytokines.

Subjects and Methods

This study was carried out in the Pediatric Hematology Unit of Zagazig University Hospitals, during the years 2011 and 2012, on 40 children with β-thalassemia major (20 non-splenectomized and 20 splenectomized), of ages ranging from 8-16 years (mean ± standard deviation: X ± SD = 12.8 ± 2.49 years), 20 were males and 20

were females. In addition, 20 age- and sex-matched healthy children served as a control group.

Inclusion criteria:

1) Patients kept on hyper-transfusion schemes to maintain hemoglobin concentration at or greater than 10 gm/dl (Lukens, 2004).

2) Patients receiving chelation therapy recommended with evidence of chronic iron overload (i.e, transfusion of about 100 ml/Kg packed RBCs and serum ferritin levels of > 1000μg/L) (Deborash and Eliezer, 2000).

3) Patients receiving vitamin E and folic acid supplementation (Yaish, 2010).

4) Criteria for splenctomy included a blood consumption greater than 50% above the mean requirement of the splenectomized population, i.e., more than 200-250 ml/Kg year of pure RBCs, to maintain a pre-transfusion hemoglobin around 9 gm/dl (Cohen et al., 1989).

All study populations were subjected to:

1. Immunoglobulins assay.

Immunoglobulin levels were measured, using quantitative turbiditmetric test for measurement of serum IgA, IgG and IgM (Young, 1997).

2. IL-6 serum levels were measured, using Avibion human IL-6 ELISA kit (Allen, 1997).

3. The percentage of T-Lymphocyte subsets were analyzed on a flow cytometer (BD FASC Calibur), using anti-human CD4/FITC, anti-human CD8/PE (Dako, Multimix TM).

4. Phagocytic function of macrophages was evaluated by following the uptake of candida albicans at 37°C for 20-25 minutes followed by counting the number of ingested candida albicans associated with each cell in gram smear. The percentage of phagocytosis was calculated by the number of phagocytes that have ingested 20 or more candida out of 200 phagocytes examined (Wood and white, 1978).

Statistical analysis

Data were checked, entered

and analyzed using SPSS version 11 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD for quantitative variables, number and percentage for qualitative one. Paired t-test, ANOVA, Chi square (χ^2) and correlation coefficient were used when appropriate. A P-value of < 0.05 was considered to be statistically significant.

Results

Their were nonsignificant differences between splenectomized and non-splenectomized patients as regards age of start transfusion, frequency of transfusion, age of start chelation and serum ferritin (Table1).

Table 2 presents immunological parameters of studied groups. Splenectomized patients had significantly higher levels of Ig, and significantly lower levels of each of IL-6 and phagocytic activity, compared to control children. Meanwhile, there were nonsignificant differences between studied groups as regards CD4/ CD8 ratio and serum levels of IgM and IgG.

Correlation between immunological profile and other parameters showed significant positive correlation between serum IgG and frequency of transfusions; serum IgM and each of age and serum ferritin; IL-6 and age of starting transfusion. On other hand, other components of immunological profile showed nonsignificant correlation with other parameters (Table 3).

Table (1): Transfusion data, chelation data and serum ferritin of 20 splenectomized thalassemic patients versus 20 nonsplenectomized thalassemic patients, presented as mean ± standard deviation (X ± SD).

	Non splenectomized (n = 20)	Splenectomize (n = 20	t- value	P- value
Age of start transfusion (months)	12.1 ± 4.4	9.6 ± 4.2	0.85	0.39(NS)
Frequency of transfusion (weeks)	3.2 ± 0.76	3.5 ± 1.8	1.68	0.49(NS)
Age of start chelation (years)	4.1±2.3	4.35±3.56	0.32	0.74(NS)
Serum ferritin (µg/dl)	2366.1±1626	2678.9±1164.7	0.67	0.5(NS)

n: number NS: nonsignificant µg:microprogam dl: deciliter

Table 2: Immunoligical parameters of studied groups, presented as mean ± standard deviation (X±SD) and percentage (%).

Parameter	Thalassemic patients		Control	F-value	p-value
	Non-splenectomized n = 20	Splenectomized n = 20			
IgA (mg/dl)	121.9±49.7	178.9±53.6*	117.4±48.7	9.1	0.001(S)
IgG (mg/dl)	759.9±232	850±234.9	779±156.8	1.008	0.37(NS)
IgM(mg/dl)	100.59±47.8	99.3±60.5	99.9±37	0.003	0.99(NS)
CD4/CD8 ratio (%)	1.47±0.76	1.43±1.02	1.44±49	0.01	0.98(NS)
IL-6 (pg/ml)	8.6±4.5	7±4.4*	12.3±6.4	5.48	0.006(S)
Phagocytic activity (%)	72.6±8.8	62.6±10.5*	97.0±1.86	98.02	0.001(S)

n: number * significant S: significant mg: milligram NS: nonsignificant
dl: deciliter Ig: immunoglobulin pg: picogram IL-6: interleukin-6

Table 3: Correlation between Immunoligical profile and other parameters.

Immunological parameter	Age		Age of start transfusion (months)		Frequency of transfusion		Age of start chelation (years)		Ferritin level (Mg/dl)	
	r	p	r	p	r	p	r	p	r	p
IgA	-0.01	> 0.05	-0.17	> 0.05	0.19	>0.05	0.11	>0.05	0.01	>0.05
IgG	0.03	> 0.05	0.21	>0.05	0.32	<0.05*	-0.19	>0.05	-0.01	>0.05
IgM	0.3	<0.05*	-0.1	>0.05	-0.08	>0.05	-0.04	>0.05	0.57	<0.001*
CD4/CD8 ratio	-0.11	>0.05	0.17	>0.05	-0.18	>0.05	0.2	>0.05	-0.11	>0.05
IL-6	0.18	>0.05	0.35	<0.01*	0.001	>0.05	0.08	>0.05	-0.03	>0.05
Phagocytic activity (%)	-0.21	>0.05	0.26	>0.05	0.22	>0.05	0.17	>0.05	-0.19	>0.05

n:number * significant µg: microgram dl:deciliter Ig: immunoglobulin %:percent IL-6: interleukin-6

Discussion

The substantial improvement in survival rates and quality of life achieved in β-thalassemia major over the last several decades has directed the attention of investigators to other collateral abnormalities that were previously neglected or overlooked. Abnormalities of the immune system represent one of the neglected areas in β-thalassemia major (Amin et al., 2005). A wide spectrum of immune abnormalities has been described in β-thalassemia major patients with multiple transfusions (Ezer et al., 2002). Besides causing iron overload multiple transfusions lead to continuous allo-antigenic stimulation (Weiss, 2002).

In this study, we found that splenectomized β-thalassemia major patients had significantly higher levels of serum IgA compared to

that in non-splenectomized patients and control children, with nonsignificant differences of serum IgG and IgM. Nearly similar results were reported by other studies (Dwyer et al., 1987; Amin and Daneshi, 2011). On the contrary, other studies reported higher levels of IgA, IgG, IgM and IgE in β-thalassemia patients, particularly splenectomized ones. This result was explained by repeated exposure to antigens due to repeated transfusions and infections which stimulates IgG, IgM, and IgE production (Amin et al., 2005; Farmakis et al., 2003).

In this study, there was nonsignificant differences of CD4/CD8 ratio, between patients and control. However, Ahluwalia et al. (2000). reported that β-thalassemia patients, particularly spelenectomized ones, had significantly lower CD4/CD8 ratios, compared to control children. On the contrary, other studies reported increased CD4/CD8 ratio in β-thalassemia patients (Hodge et al., 1999; Pattanapanyasat et al., 2000; Gharagozloo et al., 2009).

In this study, β-thalassemia patients, particularly splenectomized ones had significantly lower serum levels of IL-6 compared to control children. This result is consistent with that reported by Lombardi et al (1994) who explained this by reduced activity of $CD4^+$ lymphocyte subsets which indirectly induce the production of IL-6 via IL-2.

This study showed that β-thalassemia patients, especially splenectomized ones had significantly lower phagocytic activity compared to controls. Nearly similar results were obtained by other studies. The impaired phagocytic function was most marked for salmonella typhi in splenectomized patients (Khalifa et al., 1983; Cantinieaux et al., 1987). Impaired phagocytic function might be due to development of anti-ferritin antibodies, However, the most important mechanisms is its direct inhibitory effect on the activity of interferon-gamma, leading to the loss of ability of iron-loaded macrophages to kill intracellular pathogens (Walker and Walker, 2000).

In this study, there were signifi-

cant positive correlation between serum IL-6 levels and age of starting transfusion. This means that patients transfused at younger ages had lower IL-6 levels than those transfused at older ages. This can be explained by the possible reduced activity of $CD4^{+}$ helper cells in polytransfused patients with the resultant decrease in IL-6 levels.

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EFFECT OF HEAT SHOCK PROTEIN-90 INHIBITION ON PERIPHERAL NEUROPATHY IN RATS WITH STREPTOZOTOCIN-INDUCED DIABETES

Ahmed A. El-Gendy Ph.D and Amr M. Abbas Ph.D

Medical Physiology Department, Faculty of Medicine, Mansoura University, Egypt

Abstract

Background & aim of work: *Increasing the expression of heat-shock protein 70 (Hsp70) can inhibit sensory neuron degeneration after axotomy. Since diabetic peripheral neuropathy (DPN) is associated with the gradual decline of sensory neuron function, we evaluated whether increasing Hsp70 was sufficient to improve several indices of neuronal function. Hsp90 is the master regulator of the heat-shock response and its inhibition can up-regulate Hsp70. KU-32 (N-{7- [(2R,3R,4S,5R)-3,4-dihydroxy-5-methoxy-6,6-dimethyl-tet- rahydro-2H-pyran-2-yloxy]-8-methyl-2-oxo-2H-chromen-3-yl}acetamide) was developed as a, novobiocin-based, C-terminal inhibitor of Hsp90 whose ability to increase Hsp70 expression is linked to the presence of an acetamide substitution of the prenylated benzamide moiety of novobiocin.*

Methods: *Glucose-induced death of embryonic DRG neurons cultured for 3 days in vitro and neuregulin 1-induced degeneration of myelinated Schwann cell DRG neuron co-cultures, prepared from Sprague-Dawley rats, were examined. In addition, forty Sprague-Dawley rats were divided into four groups; control, control received KU-32, diabetic (70 mg\kg streptozotocin, intra-peritoneal injection) and diabetic treated with KU-32 (20 mg/kg i.p. once per week for 6 weeks to rats that had been rendered diabetic with streptozotocin for 12 weeks). Blood samples were taken for measuring glucose, insulin and HBA1c. In addition, motor nerve conduction velocity (MNCV), hot plate test, formalin test and open field test were performed. Also, histological examination of rat sciatic nerve was done.*

Results: *KU-32 protected against glucose-induced death of embryonic DRG neurons cultured for 3 days in vitro. Similarly, KU-32 significantly decreased neuregulin 1-induced degeneration of myelinated*

Schwann cell DRG neuron co-cultures prepared from Sprague-Dawley rats. On the other hand, after 12 weeks of diabetes, rats developed deficits in MNCV, formalin test, open field test and hot plate test along with development of a sensory hypoalgesia. Although KU-32 did not improve glucose levels, HbA1c or insulin levels, it reversed the MNCV and sensory deficits. These results were confirmed by the histopathological studies

Conclusion: *Inhibition of Hsp90 can protect against neuron death and demyelination and reverse the neuron deficits associated with DPN.*

Key words: *diabetic neuropathy, dorsal root ganglia neuron, heat-shock protein 70, molecular chaperone, nerve conduction velocity, neurodegeneration.*

Introduction

Diabetic peripheral neuropathy (DPN) is a neurodegenerative complication of diabetes that has proved difficult to manage pharmacologically since it does not result from a single biochemical etiology that is uniformly manifested for the disease's duration (10–30 years)[1]. Hyperglycemia in diabetic patients as the main factor of diabetic neuropathy induces oxidative stress through various cellular pathways such as increasing aldose reductase activity[2], increasing glycation end-products [3] and altering protein kinas C activity[4]. Long- standing hyperglycemia through producing a large amount of Reactive Oxygen Species (ROS) can damage mitochondrial DNA in dorsal root ganglia leading to peripheral nerves dysfunction[5]. Several studies have proposed that oxidative stress is one of the major factors impairing sensory nerves and dorsal root ganglia[6]. Many studies have focused on the beneficial effects of various antioxidants such as melatonin on diabetic neuropathy[7]. Indeed, small molecule inhibitors against targets that are relatively diabetes-specific, i.e. aldose reductase and advanced glycation end products, have not effectively halted the progressive degeneration of sensory fibers in human DPN[1]. On the other hand, targeting pathways that contribute to disease progression, but that are not necessarily diabetes- specific, has met with some success. For example, oxidative

stress contributes to neuron and glial degeneration in DPN[8] and some small molecule antioxidants have shown efficacy in reversing clinical and electrophysiological deficits associated with the disease[9]. A common theme in the above approaches has been the pharmacological targeting of one specific biochemical pathology associated with DPN. An alternative paradigm for treating DPN is to up-regulate a broad cytoprotective response.

Heat-shock protein 90 (Hsp90) is the master regulator of the heat-shock response (HSR) since it binds heat-shock factor 1 (HSF1). Disruption of the Hsp90–HSF1 complex by cellular stress induces the transcriptional up-regulation of antioxidant genes and molecular chaperones, such as Hsp70, that characterize the cytoprotective HSR[10]. The induction of molecular chaperones can minimize the accumulation of damaged proteins by enhancing their refolding and interfering with pro-apoptotic pathways[11]. Since small-molecule N-terminal Hsp90 inhibitors can mimic cell stress and promote the release of HSF1 from Hsp90[12], their ability to decrease protein aggregation has been proposed for treating neurodegenerative diseases whose etiology is linked to the accumulation of specific misfolded or aggregated proteins[13]. Although the pathogenesis of DPN is unlikely to result from accumulation of any one specific misfolded or aggregated protein, hyperglycaemia can increase oxidative modification of proteins[14]. This can damage protein structure, impair protein folding, decrease refolding of damaged protein or induce protein aggregation[14]. Thus chaperone induction by Hsp90 inhibitors may help to minimize hyperglycemic stress; however, their use is potentially complicated by the dual role of Hsp90 in regulating protein folding.

In the presence of co-chaperones, the folding of nascent and damaged polypeptides into their biologically active structures is dependent on Hsp90 and Hsp70. 'Client proteins' form a stabilized complex with an Hsp90 homodimer, and ATP hydrolysis by the chaperone's intrinsic N-terminal ATPase provides the energy necessary for conformational

maturation of the client. Inhibiting the chaperone activity of Hsp90 blocks protein folding and leads to client protein degradation[15]. Since many oncoproteins are Hsp90 clients, N-terminal Hsp90 inhibitors promote oncoprotein degradation and cytotoxicity[16]. Thus Hsp90 inhibitors can be useful as both chemotherapeutic and neuroprotective agents, and their primary biological outcome is dictated by the therapeutic window that dissociates client protein degradation and cytotoxicity from the cytoprotective induction of HSR [10]. Although many N-terminal Hsp90 inhibitors have been designed[17], we used novel Hsp90 analogues that effectively promote neuroprotection in the absence of cytotoxicity.

Novobiocin is an inhibitor of bacterial DNA gyrase that also exhibits weak affinity for the C-terminal ATP-binding domain of Hsp90. Since novobiocin promotes client protein degradation at concentrations that also induce HSR [18], a systematic modification of novobiocin (Figure 1) was undertaken to identify structure–activity relationships that yielded high-affinity C-terminal analogues that diverged client protein degradation from Hsp70 induction (Donnelly et al., 2008). KU-32 (N-{7-[(2R,3R,4S,5R)- 3,4-dihydroxy- 5-methoxy- 6,6-dimethyl-tetrahydro-2H-pyran-2-yloxy]- 8-methyl-2-oxo-2H-chromen-3-yl} acetamide; (Figure 1) emerged from this library as a potent inducer of Hsp70, but a poor inducer of client protein degradation. Importantly, KU-32 was found to be minimally cytotoxic to primary cortical neurons and protected against neuronal death induced by amyloid b-peptide[19].

Few studies investigate the relationship between heat shock protein (HSP) and diabetic neuropathy. Tidwell et al.,[20]; has suggested that Hsp70 can improve the survival of motor and sensory neurons after axotomy of neonatal sciatic nerves. However, it is unknown whether pharmacologically manipulating molecular chaperones in adult animals is sufficient to improve clinically relevant indices of unmyelinated and myelinated fiber function. Since KU-32 inhibits Hsp90 and increases Hsp70 levels, we examined whether it decreased neurodegeneration

of non-myelinated or myelinated sensory neurons in vitro and attenuated the pathophysiological progression of DPN in Sprague-Dawley rats.

Materials and Methods

Chemicals: STZ (streptozotocin) was obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). KU-32 (Figure 1) was synthesized and structural purity was verified as described previously[21]. The antibodies used and their sources were: SMI-94R (Covance, Princeton, NJ, U.S.A.); compact myelin protein zero (P0), ubiquitin C-terminal hydrolase (PGP 9.5; Chemicon, Temecula, CA, U.S.A.); monoclonal Hsp70 C92F3A-5 (Stressgen, Ann Arbor, MI, U.S.A.); Akt (also called protein kinase B), β-actin and horseradish- peroxidase- conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); Alexa FluorH 488 rabbit anti-mouse and Alexa FluorH 568 goat anti-rabbit antibodies (Molecular Probes, Eugene, OR, U.S.A.). MCF7 cells were maintained in DMEM (Dulbecco's modified Eagle's medium)-F12 medium containing 10% (v/v) FCS (fetal calf serum) and 100 units/ml penicillin and 100 mg/ml streptomycin.

In vitro experiments:

Preparation of non-myelinated and myelinated dorsal root ganglion (DRG) neurons

DRG neurons were dissected from embryonic day 15–18 rat pups[22] and ganglia were collected into L15 medium and sedimented at 1000 g for 5 min. After dissociation, the cells were resuspended in serum-free neurobasal medium containing 2 mM glutamate, B27 supplement, 100 units/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamicin and 50 ng/ml NGF (nerve growth factor; Harlan Biosciences, Indianapolis, IN, U.S.A.) and seeded at a density of (2–3) 6104 cells per well. Mitotic cells were partially depleted by treating the neurons with 10 mM each of fluorodeoxyuridine and cytosine b-D-arabinoside for 2 days. The cells were switched to neurobasal medium containing 50 ng/ml NGF and were pretreated for 6 h with the indicated concentration of KU-32. Hyperglycemia was induced by the addition of 20 mM excess glucose (final glucose concentration 45

mM), and cell viability was assessed after 24 h using calcein AM (acetoxymethyl ester) and propidium iodide as previously described [23].

Schwann cells were isolated from postnatal day 3 rat pups, and myelinated rat SC-DRGs (Schwann cell DRGs) neuron cocultures were prepared as described previously[24]. At 3 weeks after initiating myelination, the cultures were treated with vehicle or 0.1–1 mM KU-32 for 6 h, followed by 100 ng/ml of NRG1 (human recombinant neuregulin-1-b1 epidermal growth factor domain; amino acids 176–246; R&D Systems, Minneapolis, MN, U.S.A.). After 48 h, the cultures were fixed and stained for myelin basic protein (MBP). Degenerated myelin segments were quantified as previously described[24].

Myelinated rat neuron cultures were prepared using DRGs isolated from 1-day-old rat pups by collecting the ganglia into L15 medium and dissociating the tissue with 0.25% trypsin at 37°C for 30 min. The cells were resuspended in DMEM containing 25 mM glucose and 10% FCS (Atlas Biologicals, Fort Collins, CO, U.S.A.), triturated with a fire-polished glass pipette and plated in maintenance medium (DMEM containing 25 mM glucose, 10% FCS, antibiotics as above and 50 ng/ml NGF) in the centre of collagen-coated glass coverslips. Proliferating cells were removed by treating the neurons with the antimitotics for 3 days. After 1 week in culture, myelination was induced by the addition of 50 mg/ml ascorbic acid in maintenance medium. The cells were maintained for 15–18 days with medium replenishment every 2 to 3 days. Demyelination was induced by the addition of 100–200 ng/ml NRG1 for 2–4 days. Some cultures were treated overnight with vehicle or the indicated concentration of KU-32 prior to the addition of NRG1. The cultures were co-stained for MBP and PGP9.5 and nuclei were visualized with DAPI (49,6-diamidino-2-phenylindole). Degeneration of the myelin segments was quantified with the aid of the open source imaging software, Cell Profiler. Individual myelin internodes were identified using Otsu's method for thresholding and segmentation[25].

Segmentation was visually inspected for errors or regions where segments were closely apposed and manually edited where necessary. The length was computed for each identified myelin internode. In cases where segments intersected and a minimum minor axis width was exceeded, lengths were not included in the average of the population of segments surveyed. However, total area of coverage for myelin segments did include the intersecting regions. In some experiments, cell lysates were prepared and immunoblot analyses were performed as previously described[24,26].

In vivo experiments:

Animals: Forty male Sprague Dawley rats, weighing 220–240 g, were used in the present study. They were purchased from Vaccine and Immunization Authority (Helwan, Cairo, Egypt) and housed (Animal House, Medical Physiology department, Faculty of Medicine, Mansoura University, Egypt) under controlled conditions (temperature 23±1°C, and a 12:12 light/dark cycle). The animals were allowed free access to food and tap water. All animal procedures were performed in accordance with protocols approved by the Medical Research Ethics Committee of Mansoura University, Egypt and in compliance with standards and regulations for the care and use of laboratory rats set by the National Institutes of Health.

Grouping: These rats were divided into four groups: First group consisted of untreated control (normal) animals. The second group consisted of normal animals given KU-32 treatment (20 mg/kg i.p. once per week for 6 weeks). The third group served as untreated diabetic group. The fourth group was the diabetic group treated with KU-32. Diabetes was induced by single intra-peritoneal (i.p.) injection of 70 mg\kg streptozotocin[27]. In order to confirm diabetes, three days after streptozotocin injection, blood glucose was measured using glucometer instrument (Accu-check-active, ROCHE, Germany) and animals with blood glucose over 200 mg\dL were considered as diabetics[28]. After 12 weeks of diabetes, animals were given a once per week intraperitoneal injection

of 5% Captisol (CyDex Pharmaceuticals, Lenexa, KS, U.S.A) (third group) or 20 mg/kg KU-32[29] in 5% Captisol (forth group) for 6 weeks. At the end of the study, pain rating was carried out using the hot plate and formalin tests. In addition, open field test and MNCV were performed. Also, fasting blood glucose (FBG) and HbA1c levels were measured via tail clip sampling. Briefly the tip of the tail (approximately 2mm) was clipped off using a sterile blade and 1-2 drops of blood from the cut surface were used for measurement of blood glucose concentration and HbA1c levels. FBG (One-Touch Ultra glucometer) and HbA1c levels were determined prior to killing the animals. Plasma insulin levels were determined by ELISA using a commercial kit from Mercodia AB (Uppsala, Sweden).

Behavioral assessment

Hot plate & open field tests: At the last week of study, hot plate test for assessing sensory nerve function and open field test for detecting motor impairment were done. In hot plate test, rats were placed on a 52±0.2°C heated plate (socrel hot-plate model DS37, Ugo Basile, Italy) and time spent until the first episode of heat sensitivity including jumping, forepaw or hind paw licking was measured. Response latencies were measured at 15 minutes intervals and the average of the results was taken[30]. Similarly, in open field test animals were placed into an area (diameter 1.4 m) and their locomotion within the area were tracked over a 10 minuets period. Its data was recorded using a high resolution monochrome camera and analyzed with Ethovision software (v.8) and total distance moved was calculated[31].

Formalin Test: The rats were acclimatized to the experimental arena for 15 minutes and anaesthetized with 5% halothane[32]. Formalin (50μL, 0.25- 5%) was injected sub-cutaneously (sc) into the large lateral foot pad on the plantar surface of the left hind paw. The rats were placed in a transparent rectangular plastic box with the top opened for an unobstructed view of the response to formalin injection which was measured using the weighted scores method[33].

Electrophysiological evaluation: Animals were anesthetized by injection of Ketamin/Xylozine solution (50/20 mg kg, i.p). After shaving the animals' back, a small incision was made in right sciatic notch and ankle. Then, by bi-polar electrodes, proximal part of sciatic notch and distal part of ankle were stimulated and after stimulation, Motor Nerve Conductivity Velocity (MNCV) of sciatic-tibial was recorded (powerlab/ML856). Immediately after each stimulation, action potential of first interosseous muscle of the hind paw was recorded by unipolar electrodes. The obtained records are biphasic responses with a primary M-wave produced due to the stimulation of motor fibers[34]. MNCV was calculated by dividing the distance between the two stimulated sites (mm) by the difference between proximal and distal latencies (ms). After measuring NCV, the animals were killed, and the sciatic nerves were dissected and flash frozen.

Histological & Morphological study of sciatic nerve: For morphometric evaluation, sciatic nerve was isolated and divided into 2mm segments. They were fixed immediately with glutaraldehyde solution 2.0% in 0.1 M Phosphate Buffer Solution (PBS) for 24h. The specimens were washed with PBS for 3 times and post fixed for 1.5 h in 1% tetroxide osmium and dehydrated in graded concentration of ethanol and finally embedded in Epoxy resin. Semithin sections (350 nm) were stained with 1% toluidin blue and examined by light microscopy. Ten fields of transverse sections were morphometrically analyzed by computerized image analysis system (Motic Images China e-kup Co., Ltd). Myelinated Fiber Diameter (MFD), axon diameter (AD) and myelin sheath diameter (MSD) were measured for each section.

In vivo pharmacokinetics

KU-32 (2 mg/ml) was administered intraperitoneally to 60 Sprague-Dawley rats and blood was collected from six rats at the indicated time via cardiac puncture while the rats were under isoflurane anaesthesia. The animal was perfused with saline to remove any residual blood from the organs and the brain tissue was harvested.

Plasma (0.05 ml) or 0.25 ml brain homogenate was spiked with trideutero KU-32 as the internal standard, the protein was precipitated with acetonitrile, KU-32 was extracted from the supernatant with t-butyl methyl ether and the solvent was evaporated. Samples were reconstituted with 0.1 ml of CH3CN/water (20:80) and 0.01 ml was used for LC-MS (liquid chromatography MS) analysis. Chromatographic separation was performed using a 5 mm Agilent Zorbax SB 2.1 mm650 mm column and a linear gradient of CH3CN/water/formic acid (5:95: 0.2) to CH3CN/water/formic acid (95:5.0:0.1) at a flow rate of 0.20 ml/min. The effluent was introduced to a Sciex API3200 Linear Ion Trap detector using turbo ion spray in the positive mode. Assay response was linear (r2.0.997) and validated over the range of 1-1000 ng/ml for plasma samples and 1-500 ng/ml for rat brain samples. Recoveries ranged from 65 to 75%, and PK Solutions software (Summit Research Services, Montrose, CO, U.S.A.) were used for data analysis.

Statistical analysis: Data are presented as mean±SEM. One-way ANOVA was used in order to determine statistical difference and in the case of significant difference, Tukey test was used to determine the difference among the groups. LSD test was used in morphological study and $p<0.05$ was considered as statistical significant level.

Results:

Effect of KU-32 on cell death and demyelination in culture models of unmyelinating and myelinating sensory neurons

KU-32 is a novobiocin-based Hsp90 inhibitor containing an acetamide substitution on the coumarin ring (Figure 1). KU-32 significantly increased the expression of Hsp70 in MCF7 cells at 10 nM, which is consistent with the induction of an HSR. Although inhibiting Hsp90 can destabilize its association with client proteins such as Akt and lead to their degradation, 5 mM KU-32 only induced a 35% decrease in the Akt client (Figures 2A and 2B). Thus substitution of the prenylated benzamide of novobiocin with the simplified acetamide is responsible for differentiating client protein degradation

from Hsp70 induction.

Since DPN affects both non-myelinated and myelinated sensory fibers, we assessed whether KU-32 could protect cultured sensory neurons against death and demyelination. We first determined whether KU-32 could protect unmyelinated embryonic primary sensory neurons from glucose-induced cell death[35]. Rat embryonic DRG neurons were cultured for 3 days and hyperglycaemia was induced by the addition of 20 mM excess glucose (45 mM final glucose concentration) to the medium. Cell death was assessed by the uptake of propidium iodide. Hyperglycemia increased neuronal death by 1.5-fold, but a 6 h pretreatment with either 0.1 or 1 mM KU-32 prevented glucose-induced cell death (Figure 3A). Regardless of any arguments surrounding the physiological relevance of the glucose concentration, these in vitro data support that targeting molecular chaperones can protect unmyelinated sensory neurons from acute glucotoxicity.

To ascertain whether KU-32 could protect myelinated axons against degeneration, we assessed its ability to prevent NRG1-induced demyelination. Neuregulin-1 is a family of EGF (epidermal growth factor)-like ligands that bind ErbB receptors and can induce demyelination of myelinated rat SC-DRG neurons[36]. Myelin degeneration is readily visualized and quantified by staining the SC-DRG co-cultures for MBP and assessing the number of damaged myelin segments[22]. At 2 days after treating rat SC-DRG co-cultures with 100 ng/ml NRG1, the percentage of damaged myelin segments was approx. 34% (Figure 3B). Overnight pretreatment with 1 mM KU-32 was sufficient to prevent this damage. Similarly, KU-32 also prevented the NRG1-induced decrease in the expression of the compact myelin protein zero, P0 (Figure 3C). Importantly, any alterations in client protein levels by KU-32 were not sufficient to affect viability since 1 mM KU-32 neither promoted the death of the unmyelinated sensory neurons (Figure 3A) nor increased the number of damaged myelin segments in the SC-DRG co-cultures (Figure 3B).

Metabolic characteristics: Plasma glucose and HbA1c levels significantly increased while insulin decreased in diabetic group as compared to the control group ($p<0.05$). Moreover, control or diabetic rats receiving KU-32 showed no changes in FBG, HbA1c or plasma insulin levels compared with the control or diabetic rats receiving vehicle, respectively (Table 1).

Effect of KU-32 on the pathophysiological progression of DPN

The onset of DPN in rats (3–12 weeks) is characterized by early metabolic alterations in the conduction velocity of peripheral nerves and the development of altered sensory thresholds to noxious thermal (unmyelinated fibres) or mechanical (myelinated fibres) stimuli presented to the foot. Since our in vitro readouts support the notion that KU-32 protects unmyelinated and myelinated nerves from death and degeneration, we rationalized that it may be similarly protective in a rat model of DPN.

KU-32 is highly bioavailable and has a systemic clearance of 71.4 ml/min per kg. The drug was detectable to similar levels in both brain tissue and plasma after intraperitoneal administration in rat and had a mean half-life (tK) of 105.6 and 106.9 min in plasma and brain respectively (Figure 4A). Although the accumulation of KU-32 in peripheral nerve has not been assessed, the level of Hsp70 was modestly increased in sciatic nerve at 1 week after drug administration (Figure 4B). Preliminary studies indicated that more frequent dosing at these concentrations did not increase the expression of Hsp70 (results not shown).

A powerful indicator of the ability of KU-32 to improve neurodegeneration is whether it could reverse pre-existing deficits in MNCV, hot plate latency, formalin score and explorative behavior in open field test in rats with DPN. Therefore diabetes was induced in 8-week-old Sprague-Dawley rats and allowed to progress for 12 weeks prior to drug treatment. Rats then received vehicle or 20 mg/kg KU-32 once a week for 6 weeks. MNCV showed significant decrease in diabetic groups compared to the control group ($p<0.05$). Diabetic rats treated

with KU-32 for 6 weeks showed a significant increase in MNCV as compared with untreated diabetic rats ($p<0.05$) while no significant difference relative to the control group ($p>0.05$) was reported (Figure 5).

Consistent with the development of a sensory hypoalgesia, diabetic rats showed increased hot plate latency in comparison with control non diabetic rats ($P<0.05$) (Table 1). KU-32 treatment of diabetic rats significantly improved these deficits to near control levels after 6 weeks of treatment (Table 1). The mean formalin scores in diabetic rats were significantly lower ($P<0.05$) than those in control, non – diabetic rats (Table 1). The results are consistent with decreased pain sensitivity in the diabetic state. When these same diabetic rats were tested after KU-32 administration, the formalin scores were similar to those of control rats (Table 1).

In open field test, performed to find the effect of diabetic neuropathy and KU-32 treatment on rats' explorative behavior, the frequency of rearing (Figure 6a) and grooming (Figure 6b), the total distance moved (TDM) (Figure 6c) and mobility duration (Figure 6d) in diabetic rats showed significant decrease compared to the control group ($p<0.05$). Ku-32 administered to diabetic rats significantly improved these deficits to near the control levels after 6 weeks of treatment (Figure 6).

Histological & Morphological study of sciatic nerve: Light microscopy study of sciatic nerves sections showed that normal structure and morphology of myelinated fibers in control group (Figure 7a). In diabetic group, some abnormalities including increased numbers of mast cells, edema and myelin sheath splitting were seen (Figure 7b). In addition, increased number of abnormal myelinated fibers was observed in diabetic rats (Figure 7b). Treatment of diabetic rats with KU-32 decreased all of these abnormalities (Figure 7c). In addition, myelinated fiber diameter (MFD) and axon diameter (AD) of diabetic group were decreased as compared to control group. Pretreatment with KU-32 for 6 weeks significantly reversed each diameter reduction in diabetic rats (Table 1).

Table (1): Metabolic characteristics, behavioral assessment and histomorphometric parameters of rat sciatic nerve in normal and diabetic rats with and without KU-32 treatment.

	Control rats (vehicle) (n=10)	**Control rats (vehicle+KU-32) (n=10)**	**Diabetic rats (STZ+vehicle) (n=10)**	**Diabetic rats (STZ+KU-32) (n=10)**
Metabolic characteristics				
Fasting blood glucose (mg/dl)	135±5	123±6	380±25[ab]	418±21[ab]
Insulin (nM)	0.26±0.03	0.33±0.05	0.11±0.005[ab]	0.12+0.004[ab]
HbA1C (%)	4.9±0.1	4.8±0.1	12.5±0.5[ab]	12.7±0.3[ab]
Behavioral assessment				
Hot plate latency (sec)	20.0±1.5	19.0±1.6	45.0± 2.8[ab]	22.0±2.7[c]
Formalin score	2.5±0.08	2.6 ± 0.09	1.8 ± 0.06[ab]	2.4 ± 0.09[c]
Histomorphometric parameters of rat sciatic nerve				
MFD (μ m)	9.75± 0.45	9.78± 0.43	8.14±0.18[ab]	8.68±0.3
AD (μ m)	5.11±0.34	5.15±0.31	4.12±0.15[ab]	4.21±0.4
MSD (μ m)	4.64±0.35	4.69±0.34	4.06±0.23	4.66±0.30

Data are presented as Mean±SEM. MFD: Myelinated Fiber Diameter, AD: Axon Diameter, MSD: Myelin Sheath Diameter.

a: significant ($p<0.05$) compared with control rats (vehicle).
b: significant ($p<0.05$) compared with control rats (vehicle+KU-32).

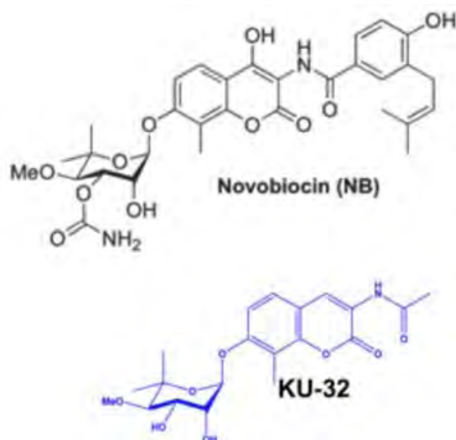

Fig. (1): Novobiocin (NB) and KU-32.

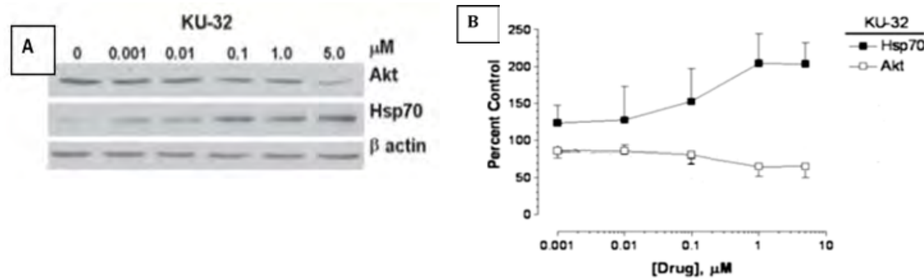

Fig. (2): KU-32 diverge client protein degradation from induction of Hsp70. **(A)** KU-32 induces Hsp70 expression but shows limited degradation of the Hsp90 client protein Akt. **(B)** Band intensities were normalized to β-actin and expressed as a percentage of the control.

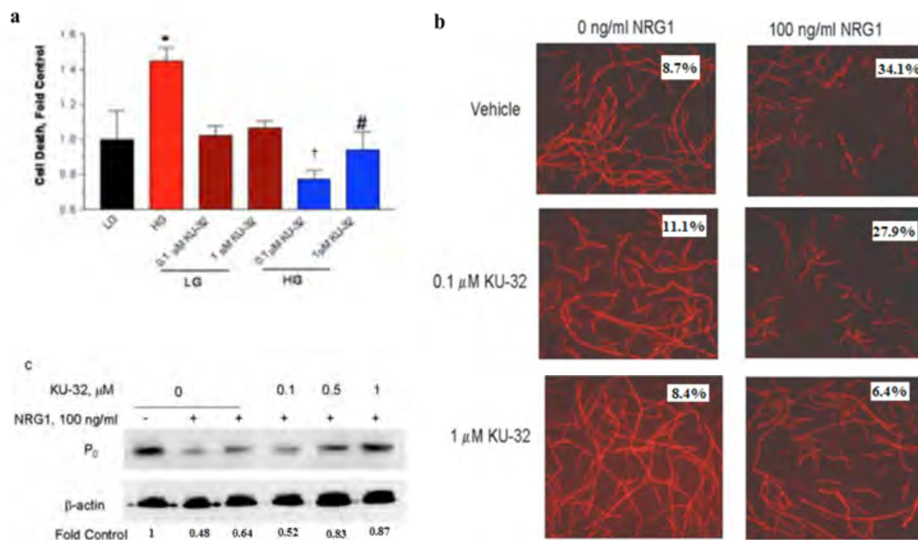

Fig. (3): KU-32 protects rat sensory neurons against glucose-induced death and neuregulin-induced demyelination.

(a) Embryonic sensory neurons were treated for 6 h with 1% DMSO or 0.1–1 mM KU-32 in a medium containing 25 mM glucose (LG). The glucose concentration was raised to 45 mM to induce hyperglycaemia (HG) and the cells were incubated for an additional 24 h. Cell death was assessed using calcein AM and propidium iodide. *$P<0.05$ versus LG control; †$P<0.003$ versus HG; #$P<0.02$ versus HG. **(b)** Myelinated rat SC-DRG neuron co-cultures were treated overnight with 1% DMSO, 0.1 or 1 mM KU-32 and the cultures treated with PBS or 100 ng/ml NRG1 for 48 h. The myelin segments were visualized by staining for MBP. Numbers show percentage degenerated segments in each culture. **(c)** Myelinated rat SC-DRG neuron co-cultures were treated as above and immunoblot analysis for the P0 compact myelin protein was performed. Band intensities were normalized to β-actin and expressed as a percentage of the control.

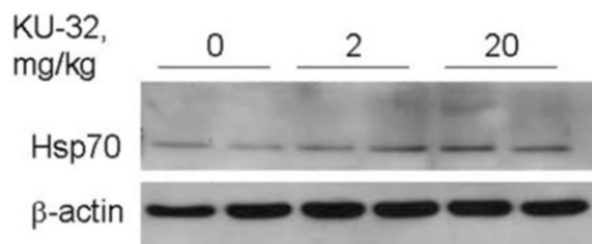

Fig. (4): Pharmacokinetic profile of KU-32 uptake in plasma and brain after intra-peritoneal administration.

(a) KU-32 (2 mg/ml) was injected intraperitoneally in 5% Captisol, and plasma and brain samples were taken at the indicated time. KU-32 levels were quantified by LC-MS. Plasma AUC0-∞, 27.4 mg/min per ml. Results are the means±S.E.M. for six mice per time point. **(b)** Effect of KU-32 on expression of Hsp70 in sciatic nerve. Rats were injected with KU-32 and sciatic nerve was harvested after 1 week. Hsp70 and β-actin levels were determined by immunoblotting. The level of Hsp70 was normalized to β-actin, and KU-32 increased Hsp70 expression by 1.2- and 1.35-fold at 2 and 20 mg/kg respectively

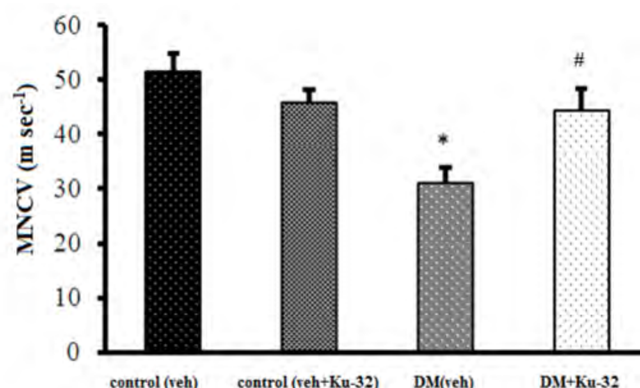

Fig. (5): KU-32 reversed pre-existing indices of diabetic sensory neuropathy in rat. Rats were rendered diabetic for 12 weeks and then treated with weekly doses of vehicle (veh) or 20 mg/kg KU-32 for 6 weeks. After 18 weeks of diabetes, MNCV significantly decreased in untreated rats, but KU-32 treatment for 6 weeks improved the deficits in MNCV. *: $p<0.05$ compared with the control group; #: $p<0.05$, compared with the DM (veh) group.

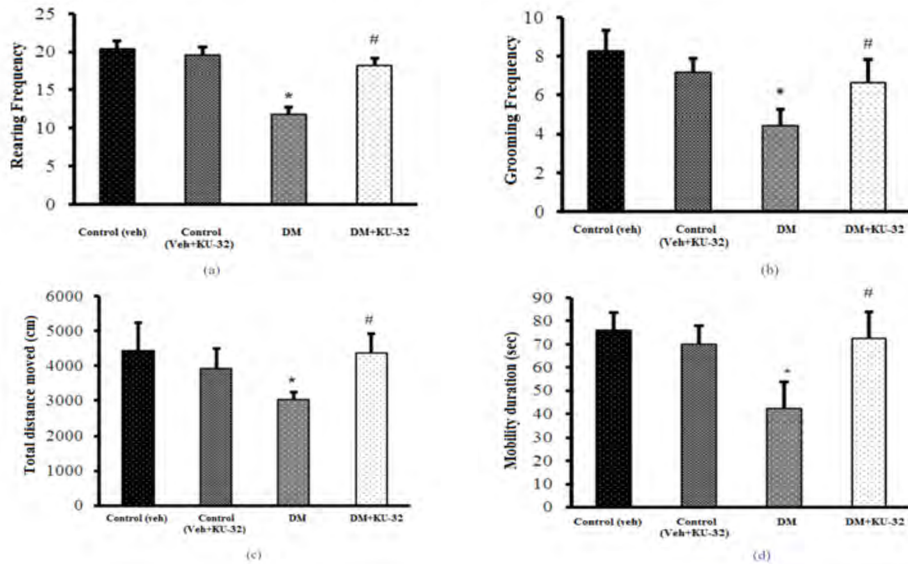

Fig. (6): Effect of KU-32 on explorative behavior of rats in open field test. Rearing frequency (a), Grooming frequency (b) ,total distance moved (c) and Mobility duration (d), *:p<0.05 compared with untreated control groups #: p<0.05 compared with the untreated diabetic group. Values are expressed as mean ± SEM. (n=10 rats in each group).

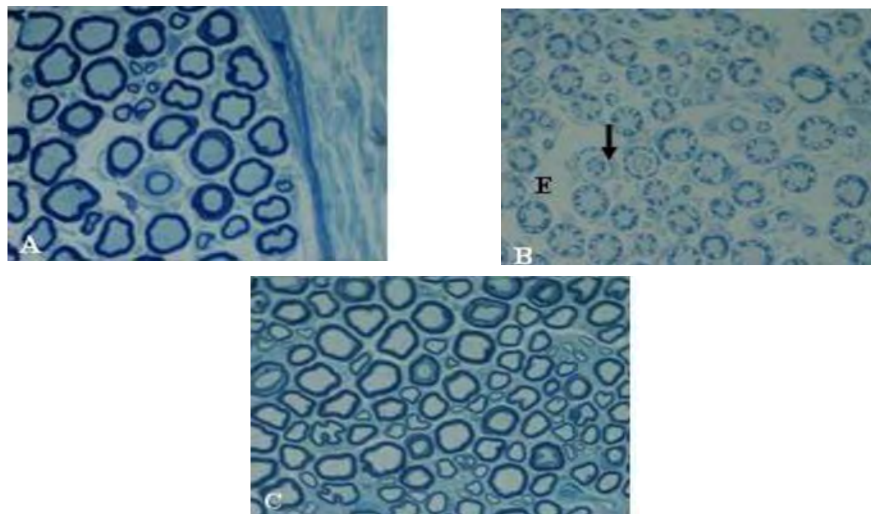

Fig. (7): Light micrograph of transverse semi-thin sections of rat sciatic nerves. A: Control group, myelinated nerve fibers are in normal structure and morphology. B: Sham group, nerves fibers show some abnormalitities such as myelin splitting (black arrow), and Edema (E). C: The KU-32 treated group, the proportion of nerve fibers with abnormalities was reduced. *1000. Toluidin blue staining.

Discussion

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes and it is associated with several problems such as cardiovascular defects, retinopathy and muscular pain or weakness[37]. Although hyperglycemia is the definitive cause of DPN[38], the vascular, glial, and neuronal damage that underlies the progressive axonopathy in DPN has a complex biochemical etiology involving oxidative stress[39], protein glycation, protein kinase C activation[40], polyol synthesis [41], and the hexosamine pathway [42]. Altered neurotrophic support also contributes to sensory neuron dysfunction in DPN[43]. In the present study, our finding shows that STZ administration caused a significant increase in plasma glucose, insulin and HBA1C (Table 1) in rats. In addition, STZ administration induced a marked peripheral sensorimotor neuropathy with behavioral, electrophysiological and histological alterations in rats. A significant increase in hot plate latency (Table 1) as an indicator of sensory neuropathy and a decrease in total distance moved, frequency of rearing and grooming and mobility duration as indicators of abnormal locomotion activity (Figure 6) demonstrated neuropathy potentially induced by diabetes. The detected decrease in MNCV in sciatic nerve (Figure 5) confirmed this nerve injury in diabetic rats. Some studies have reported that MNCV decrease in diabetes is due to alterations of Na-K ATPase pump activity and sodium gradient[44]. Alteration of Na-K pump activity can be attributed to the tissue injuries and metabolism abnormality following hyperglycemia in diabetic patients. Decrease of nerve blood flow in diabetic neuropathy leads to nerve metabolic abnormality and consequently defect of ATP-sensitive ion exchanger pumps like Na-K pump. Defect of Na-k pump is finally led to the membrane disability in preserving resting potential and consequently disturbs nerve conductivity[44].

Also, the mean formalin scores in diabetic rats were significantly lower than those in control rats (Table 1) which is consistent with decreased pain sensitivity in the diabetic state. Our results were further confirmed by the light microscopic study of sciatic nerves

which showed some abnormalities including increased numbers of mast cells, edema, myelin sheath splitting and abnormal myelinated fibers in diabetic rats (Figure 7). Also, myelinated fiber diameter (MFD) and axon diameter (AD) of diabetic group were decreased as compared to control group (Table 1).

Since DPN affect the quality and quantity of life, its treatment or prevention of its accompanying symptoms has been considered as a major goal. In addition, there are no effective treatment options specific to the neuropathy[45]. Targeting molecular chaperones has shown promising results in treating both neurodegenerative and diabetes-associated phenotypes. For example, N-terminal Hsp90 inhibitors decreased motor neuron degeneration in a murine model of spinal and bulbar muscular atrophy[46] and reduced aggregated or hyperphosphorylated forms of tau protein in JNPL3 mice[47] and humanized tau protein transgenic mice (48). Similarly, induction of Hsp70 with HSF1 activators (bimoclomol and related hydroxylamine derivatives) improved insulin resistance[49], diabetic wound healing[50] and was sufficient to prevent the slowing of NCV in diabetic rats[51]. In addition, KU-32, a C- terminal Hsp90 inhibitor, is a small molecule designed to inhibit Hsp90, thereby increasing Hsp70 levels. Both Hsp70 and Hsp90 are molecular chaperones that are critical for the proper folding of proteins[45]. In the present study, we provide an evidence that KU-32 that increases Hsp70 expression is similarly effective in preventing death of unmyelinated neurons, demyelination of myelinated sensory neurons and was able to reverse preexisting sensory deficits associated with the development of DPN in rats.

According to our results, KU-32 has been shown to have dramatic effects in protecting neurons from stress. KU-32 decreased cell death and demyelination in culture models of unmyelinating and myelinating sensory neurons (Figure 2). KU-32 protects the embryonic primary sensory neurons from glucose-induced cell death and myelinated axons against degeneration as evidenced by its ability to prevent NRG1-induced demyelination

(Figure 2). Also the results of the current work demonstrated that KU-32 reverses the clinical signs of DPN in rat model. Treatment of diabetic rats with KU-32 decreased the hot plate latencies (Table 1) and increased the MNCV in sciatic nerve (Figure 5), total distance moved, frequency of rearing and grooming, mobility duration (Figure 6) and mean formalin scores (Table 1) in comparison with untreated diabetic rats. The results of the present work confirm those of previous studies [29,52] which demonstrated that treatment of diabetic rodents with KU-32 reversed multiple clinical indicators of diabetic peripheral neuropathy including thermal hypoalgesia[29] and mechanical sensitivity[52]. In rodent nerves, KU-32 increased Hsp70, and this protein was necessary for the neuroprotective efficacy of the drug in reversing indices of diabetic peripheral neuropathy[29]. The improvement of clinical signs of diabetic neuropathy by KU-32, demonstrated in our results, was confirmed by light microscopic study of sciatic nerves which showed that treatment of diabetic rats with KU-32 decreased all abnormalities of sciatic nerve revealed in diabetic rats (Figure 7). Also, treatment with KU-32 reversed each diameter reduction, myelinated fiber diameter (MFD) and axon diameter (AD), in diabetic rats (Table 1). No significant changes in plasma glucose, insulin and HBA1C were reported in diabetic rats with KU-32 administration (Table 1). Thus the improvements in nerve function with Ku-32 are not related to decreased hyperglycemia or an outright increase in insulin levels. However, since STZ does not completely ablate insulin production[49], we cannot rule out the possibility that KU-32 may improve insulin action.

Although Hsp70 is a critical mechanistic component necessary for the efficacy of KU-32 in improving a major neurodegenerative complication of diabetes, the molecular basis by which Hsp70 intersects with biochemical mediators that contribute to the pathophysiology of DPN is unclear [29]. It is well recognized that induction of the HSR can upregulate numerous genes that may have a beneficial effect in

ameliorating DPN, i.e. Mn superoxide dismutase and haem oxygenase 1[29]. The reliance on Hsp70 expression for neuroprotection suggests that if KU-32 promotes a broader HSR via the release of HSF-1, this is not sufficient for drug efficacy[29]. Previous work has shown that up-regulation of Hsp70 can improve insulin resistance and that this is associated with a decrease in phosphorylation of JNK (c-Jun N-terminal kinase)[49]. Although increasing the expression of Hsp70 inhibits JNK-dependent apoptosis[53], neuronal apoptosis is not a substantial contributing feature to the pathophysiology of DPN[54]. Indeed, adult neurons are relatively insensitive to JNK-induced apoptosis [55]. However, JNK activation does mediate death of embryonic neurons[53,55], and the ability of Hsp70 to block this activity may protect against glucose-induced death of the immature, embryonic DRG neurons. Interestingly, although increased expression of the c-jun transcription factor contributes to NRG1-induced demyelination, its phosphorylation by JNK is not essential to mediate Schwann cell degeneration[56]. These results suggest that any inhibitory effect of Hsp70 on JNK activity would not be critical to the ability of KU-32 to attenuate demyelination. However, an interaction of Hsp70 with JNK is possible in the context of DPN, since JNK activity increases in diabetic nerves[57] and contributes to features of neuropathic pain[58]. Thus up- regulating Hsp70 may be an effective treatment for both insensate and painful diabetic neuropathy.

The ability of Hsp70 to refold aggregated or oxidatively damaged proteins may also contribute to improving nerve function. It is generally accepted that enhanced production of reactive oxygen species and subsequent mitochondrial dysfunction[59] and fission [60] contribute to DPN. Oxidative modification of the mitochondrial fission protein Drp1 (dynamin-related protein 1) by S-nitrosylation favors the formation of Drp1 tetramers and larger aggregates that display enhanced GTPase activity, which promotes mitochondrial fission[61]. It will be important to determine whether an interaction of Hsp70 with Drp1

may aid its refolding or prevent aggregation to minimize increased GTPase activity and organelle fission. However, Hsp70 mutants that are folding-incompetent, but are able to bind and maintain the solubility of denatured proteins, were as effective as Hsp70 in decreasing oxidative stress and the rate of proton leak across the inner mitochondrial membrane in glucose-deprived astrocytes(62). Thus the folding competence of Hsp70 may not be a critical feature of neuroprotection, at least in transient models of oxidative stress that are not associated with protein aggregation. On the other hand, Hsp70 may also help maintain mitochondrial protein integrity via its interaction with TOM70 (translocase of outer membrane 70), which is important for mitochondrial protein import(63).

It is notable that the diabetic Hsp70 KO mice developed a similarly severe neuropathy as in the wild type (WT) mice. This outcome indicates that inducible forms of Hsp70 are not necessary for the pathogenesis and progression of neuropathy(29). However, since constitutive forms of Hsp70 (Hsc70) may substitute for Hsp70.1 and 70.3, the possibility that diabetes-induced changes in chaperone activity may contribute to decreased neuronal function cannot be ruled out(29). Indeed, the effect of diabetes on Hsp70 protein expression and its role in the development of diabetic complications are not clear. In short-term diabetic rats (1 month), Hsp70 levels did not change in various skeletal muscles, heart, liver or kidney(64). However, diabetes may affect Hsp70 in a cell-selective manner since immunoreactivity increased in the kidney outer medulla, but not the glomeruli, of rats rendered diabetic for 4–24 weeks(65). Unfortunately, whether this contributed to the nephropathy that developed in these animals was not determined. Similarly, some reports indicate that a 15 min heat stress enhanced the expression of Hsp70 in diabetic heart relative to levels observed in heat-stressed non-diabetic rats(64). In contrast, induction of Hsp70 has been reported to be similar in hearts from heat stressed control and diabetic rats and that Hsp70 induction in diabetic heart was not cardiopro-

tective against an ischaemia/ reperfusion injury[66]. These results raise the issue of whether Hsp70 induction may be broadly protective in reversing diabetic complications associated with other organs. However, to the best of our knowledge, the potential efficacy of Hsp90 inhibitors and Hsp70 induction against other diabetic complications has not been adequately examined.

In summary, our results provide an evidence that targeting molecular chaperones can be sufficiently cytoprotective to reverse pre-existing symptoms associated with DPN in experimental animals. Inhibition of Hsp90 can protect against sensory neuron death and demyelination and reverse the sensory deficits associated with DPN. Moreover, Hsp70 expression is critical to this neuroprotection. These results establish proof-of-principle that pharmacological modulation of molecular chaperones may be useful toward decreasing neurodegeneration associated with the onset of DPN.

The potential of this approach as a monotherapy or in conjunction with pharmacological agents that antagonize a specific biochemical pathology that contributes to DPN may broaden our translational options toward developing effective therapies for the medical management of DPN.

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EFFECT OF HEAT SHOCK PROTEIN-90 INHIBITION ON PERIPHERAL NEUROPATHY IN RATS WITH STREPTOZOTOCIN-INDUCED DIABETES

Ahmed A. El-Gendy Ph.D and Amr M. Abbas Ph.D

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PATHOPHYSIOLOGY OF BREATH-HOLDING SPELLS

**Ahmed G. Siam MD, Magdy A. Aidaros MD*,
El-Sayed M. Farag MD**, Amal F. Gharib MD***
and Abdel Nasser A. Mourad MD******

Departments of Pediatrics, Neurology, Cardiology** and Biochemistry****
Zagazig & Benha Universities (Egypt)

Abstract

Objectives: *The mechanism of breath-holding spells (BHS) is not fully understood and most probably multifactorial; So, this study was designed to clarify the pathophysiology of BHS through assessing laboratory parameters and electrocardiographic (ECG) changes which might be contributed to the occurrence of the attacks. Another aim of the study is to evaluate the differences in the pathophysiology between pallid and cyanotic types of BHS.*

Methods: *Seventy-six children aged 15-48 months were subjected to the study: 32 child with cyanotic BHS, 14 child with pallid BHS, and 30 healthy children as a control group. All children were subjected to: full history taking, clinical examination, laboratory work up in the form of CBC, serum iron, ferritin and zinc. Twenty-four hours ambulatory ECG (Holter) recording was also performed for all.*

Results: *No significant statistical difference was found between cyanotic and pallid groups regarding family history of BHS, severity and precipitating factors of the attacks. Frequent runs of respiratory sinus arrhythmia (RSA) during 24 hours ECG were significantly higher in children with BHS; the frequency of RSA was significantly correlated with the frequency (severity) of the attacks. Low serum ferritin was significantly associated with BHS groups but not correlated with the severity of the attacks.*

Conclusion: *Autonomic dysregulation evidenced by frequent RSA is the main cause of BHS in children and is correlated with the frequency of the attacks. Low serum ferritin is additional factor in the*

pathophysiology. Both pallid and cyanotic BHS are types of the same disease sharing the same pathophysiology.

Key word: *Breath-holding, reflex anoxic seizures, pallid, cyanotic, BHS.*

Introduction

Breath holding spells (BHS) is a common problem in children, and particularly so in infants and is a frightening experience for the parents[1,2]. BHS is apparently due to acute cerebral hypoxia, and the child recovers spontaneously after a period of unconsciousness and sometimes opisthotonic posturing[3]. The diagnosis is based on stereotyped sequence of clinical events which begin with crying, trauma or emotional upset leading to noiseless state of expiration associated with color changes and ultimately loss of consciousness[4,5]. The spells begin most commonly during the first 12 months of life and almost always by 2 years of age. Most children outgrow those spells approximately at the age of 6 years[6]. Rarely BHS continue and replaced by vasovagal attacks[7].

Two types of BHS are present based on the color of the child during the apneic episode following the end of prolonged expiration either pale (pallid attacks) or blue (cyanotic attacks); rarely both types may occur in the same child (mixed type)[1,4,8].

The mechanism of BHS is not fully understood and most probably multifactorial[7,9,10]. Iron deficiency anemia is claimed to be associated with the occurrence of BHS supported by the response of some cases to iron therapy; associated zinc deficiency may be additional factor[10,11]. Autonomic dysregulation that leads to alteration in cardiac function and simultaneous decrease in cerebral blood flow is important risk factor[12,13], so, electrocardiogram (ECG) should be strongly considered in any patient with BHS[14].

Objectives:

(1) To clarify the pathophysiology of breath-holding spells by assessing factors claimed to be associated with the attacks including laboratory parameters and continuous

ECG monitoring.

(2) To identify differences in the pathophysiology between pallid and cyanotic BHS.

Methods :

This cross-sectional study was conducted in Pediatrics, Neurology and Cardiology Departments of Zagazig University Hospitals (Egypt).

Seventy-six children aged 15-48 months were included in the study comprising 3 groups:

- Group I: 32 patients with cyanotic BHS.
- Group II: 14 patients with pallid BHS.
- Group III: 30 healthy children as a control group. Children with primary cardiac or central nervous system disease were not included as well as any child with uncertain history of the type of BHS or suspected mixed type. Any child with possibility of seizure was subjected to EEG assessment and children with abnormal EEG were also excluded. Initial basic ECG rhythm strip for 30 seconds was done to exclude prolonged QT syndrome.

*** All children were subjected to:**

- Full history taking including age, gender, parental consanguinity & thorough clinical examination.
- Detailed history of the attacks with stress on family history, age of onset, triggering factors and severity; Severity of the attacks was assessed by determining the average frequency of occurrence:
- Mild → < one attack/week
- Moderate → 1-3 attacks/week
- Severe → >3 attacks/week
- Laboratory investigations including RBC's count and hemoglobin level (to assess anemia), serum iron (by colorimeteric chromazuol B)[15], serum ferritin (by electrochemluminescence using elecsys and cobas (2010) immunoassay analyzers)[16] and serum zinc (by atomic absorption spectrophotometer [GBC GF 3000])[17].

- Laboratory results were described in the form of normal, low or high according to their reference range for each age.
- Electrocardiographic (ECG)

study: 24 hours ambulatory ECG recording was done for all children using (VX3 series E) recorders analyzed by H7000 Holter software for detection of changes in ECG during and in-between the attacks of BHS.

**** Ethical considerations:** A permission was taken from the ethical committee of Zagazig University and the head of Pediatrics, Neurology and Cardiology Departments before the beginning of the study. An informed written consent was taken from the care givers of all children included in the study.

**** Statistical analysis:**

Data management was done using SPSS version 14.

Results

Table (1): Characteristics of the studied population.

		Group I (n=32) Cyanotic BHS		Group II (n=14) Pallid BHS		Group III (n=30) Control group		Test of significance		Sig.
								F	P	
Age (months)		31.4±8.7 15-48		32.3±11.5 15-48		31.7±8.5 15-48		0.05	0.94	NS
		No	%	No	%	No	%	X^2	P	
Gender	Male	18	56.3	9	64.3	17	56.7	0.29	0.86	NS
	Female	14	43.7	5	35.7	13	43.3			
Consanguineous marriage	+ ve	9	28.1	3	21.4	8	26.7	0.23	0.89	NS
	- ve	23	71.9	11	78.6	22	73.3			

Table (2): Features of BHS in cyanotic and pallid types.

		Group I (n=32)		Group II (n=14)		Test of significance		Sig.
						t	P	
Onset of the attacks (months)		9.8±1.4		9.7±1.7		0.78	0.26	NS
		No	%	No	%	X^2	P	
Positive family history		10	31.3	5	35.7	0.001	0.96	NS
Severity of the attacks	Mild	8	25	4	28.6	0.11	0.94	NS
	Moderate	13	40.6	5	35.7			
	Severe	11	34.4	5	35.7			
Precipitating factors:	Head injury	6	18.8	6	42.9	1.82	0.17	NS
	Pain	11	34.4	6	42.9	0.3	0.58	
	Cry	24	75	7	50	1.75	0.18	
	Fear	6	18.8	4	28.6	0.13	0.72	

Table (3): Laboratory findings.

	Group I (n=32)		Group II (n=14)		Group III (n=30)		X^2	P	Sig.
	No	%	No	%	No	%			
Anemia	19	59.4	7	50	15	50	0.66	0.72	NS
Low serum iron	11	34.4	5	35.7	9	30	0.2	0.9	NS
Low serum ferritin	17	53.1	8	57.1	7	23.3	7.23	0.026**	Sig.
Low serum zinc	8	25	6	42.9	6	20	2.62	0.26	NS

Table (4): Frequency of respiratory sinus arrhythmia (RSA) in 24 hours ECG monitoring among different groups**.

RSA	Group I (n=32)		Group II (n=14)		Group III (n=30)		X^2	P	Sig.
	No	%	No	%	No	%			
Few (<5/24 hrs)	5	15.6	2	14.3	21	70	30.72	0.001*	Sig.
Moderate (5-10/24 hrs)	19	59.4	4	28.6	7	23.3			
Frequent (>10/24 hrs)	8	25	8	57.1	2	6.7			

** Significant run of RSA were found in group I and group II when compared with group III.

Table (5): ECG changes during the attack.*

ECG findings	Group I (n = 7)	Group II (n = 3)	Total (n = 10)
RSA	3	1	4 (40%)
Sinus bradycardia	1	-	1 (10%)
Siuns tachycardia	3	1	4 (40%)
Asystole	-	1	1 (10%)
Prolonged Q-T	-	-	-
Normal	-	-	-

* Only 10 cases experienced a breath-holding attack during 24 hours ECG recording.

Table (6): Factors affecting severity of BHS:
6 (a): Relation between severity of the attacks and frequency of RSA in 24 hours ECG recording:

	RSA in 24 hours ECG monitory					
	Few (<5)		Moderate (5-10)		Frequent (>10)	
	No	%	No	%	No	%
Severity of the attacks:						
- Mild	5	35.7	1	5.6	1	7.2
- Moderate	9	64.3	9	50.0	5	35.7
- Severe	0	0.00	8	44.4	8	57.19

$X^2 = 14.08$ P = 0.007* Significant

6(b): Relation between severity of the attacks and low serum ferritin:

	Low serum ferritin			
	Absent		Present	
	No	%	No	%
Severity of the attacks:				
- Mild	7	33.3	7	28
- Moderate	10	47.6	8	32
- Severe	4	19.1	10	40

$X^2 = 2.46$ P = 0.29 NS

6(c): Correlation between severity of the attacks and other parameters.

	r	P	Sig.
- 24 hours ECG recording	0.49	<0.001*	HS
- Low serum ferritin	0.17	>0.05	NS

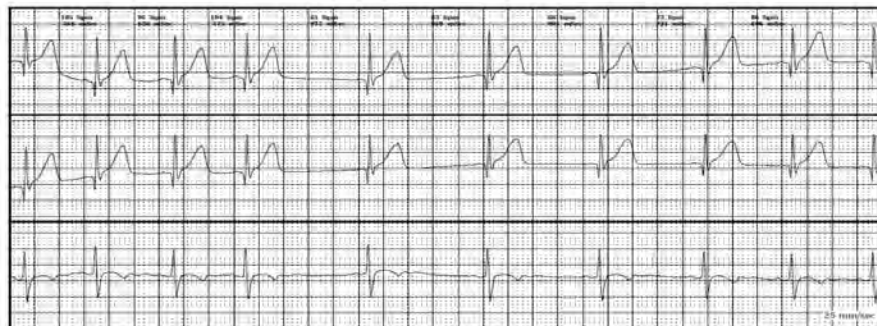

Figure 1: Respiratory Sinus Arrhythmia

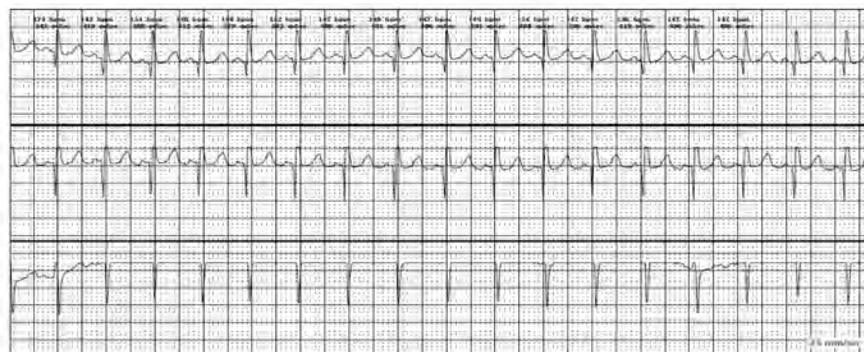

Figure 2: Sinus tachycardia

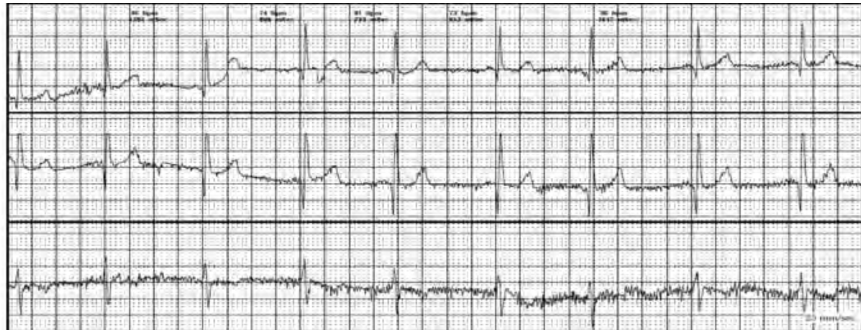
Figure 3: Sinus bradycardia

Discussion

The pathophysiologic mechanism of BHS remain controversial and no study had identified the exact etiology of the attacks(6,9). The present study included 46 children with BHS and 30 healthy children as a control group. The control group were age and gender matched to children with BHS with no statistical difference regarding consanguineous parental marriage (Table 1). The average age of onset of BHS was 9.8 and 9.7 months for cyanotic and pallid groups respectively (Table 2) which is not far from other studies which stated that most cases of BHS are manifested before the first birthday(5,18,19). Similar attacks of BHS in first degree relative (positive family history) were present in about one-third of cases of BHS (31.3-35.7%) (Table 3), which is consistent with multiple studies(4,5,6). DiMario, 1997 explained this considerable positive family history by the autosomal dominant inheritance with incomplete penetrance of BHS(20); However, as no specific gene had been identified for inheritance of BHS, the presence of family history may be related to the inheritance of the cause of BHS rather than BHS themselves.

Multiple precipitating factors were associated with the attacks (Table 2), of which, cry was the commonest event preceding cyanotic (75%) and pallid (50%) BHS; However, no single factor showed statistical significant association with either type of BHS.

Children with BHS showed non significant statistical difference

regarding anemia and low serum iron when compared with the control group (Table 3). Multiple studies had reported a high incidence of iron deficiency anemia in children suffering from BHS[6,9,11,12,21]. No other types of anemia were claimed to be associated with BHS in any study, so, it is obvious that the problem is related to "iron" and not to "anemia"; as body iron is better assessed by serum ferritin rather than serum iron which is affected by many factors[25], our results still prove the role of iron deficiency in BHS pathogenesis alike other studies in the literature.

Low serum zinc was not associated with increased risk of BHS (Table 3). A single study performed by Gencgonul, et al in Turkey (2002) had suggested a role of zinc deficiency in the pathogenesis of BHS[11]; however, Gencgonul study only suggested a role of zinc deficiency in association with iron deficiency-and not zinc alone-in the pathogenesis of BHS; as zinc deficiency is a common association in iron deficiency anemia our results regarding this issue seem logic.

24 hours ECG monitoring was done for all children subjected to our study. Respiratory sinus arrhythmia (RSA) was the only significant finding in 24 hours ECG monitoring in cases with BHS; most cases of either cyanotic or pallid BHS showed higher runs of respiratory sinus arrhythmia compared with the control group (Table 4). Respiratory sinus arrhythmia was identified if PP intervals were present that exceeded the previous PP by more than 10%[26].

A study of DiMario, et al. 1998 had suggested the same association with pallid and not with cyanotic BHS[22]; the difference between the results of Di-Miario, et al and ours may be attributed to the fewer number of cases in their study (46 versus 76); also, the 24 hours ECG recording performed to our studied children is probably more accurate than Di-Mario, et al., study who performed a 5-min, ordinary ECG. Infact our study may be the only study that performed a Holter's monitoring for children with BHS and -to our knowledge- all published reviews of ECG interpretation in BHS de-

pend on ordinary short ECG strips.

Only 10 cases experienced an attack of BHS during 24 hours ECG recording and revealed RSA and sinus tachycardia to be the most common findings (Table 5), although statistical analysis could not be performed due to small sample size. Among the ten patients who experienced an attack during Holter ECG monitoring, no data of prolonged QT interval was obtained. Cases with persistent prolonged Q-T were not included in our study according to our exclusion criteria as prolonged Q-T syndrome associated syncope is a serious condition which may need interference up to pacemaker implantation[4,7,10,19,23]. On the other side, Akalin et al. (2004) had linked the BHS with increased QT dispersion in ECG[12] but their study didn't exclude patients of prolonged QT syndrome which may extend their study outside the scope of BHS.

Our study revealed a significant correlation between the frequency of RSA in Holter monitoring and the frequency of BHS obtained from parental history (Table 6), which is an important evidence indicating the basic role of cardiac rhythm abnormality in the pathophysiology of BHS and strengthen the results of other studies which concluded that autonomic dysregulation is the primary abnormality in children with BHS that leads to defective cerebral blood flow followed by the sequence of events observed[12,13,22]. On the other side, low serum ferritin despite being significantly associated with BHS but was not correlated with the frequency of the attacks (Table 6) which may indicate that iron deficiency assessed by low serum ferritin is not the primary pathophysiology of BHS but an additional factor contributed to the etiology. The association of iron deficiency and BHS may be related to:

1) Iron deficiency is usually associated with irritability and excess crying(5,6) which is the commonest triggering factor for the attacks (Table 2).

2) Iron deficiency may lead to catecolamine disruption and subsequent autonomic dysregula-

tion[6,24] and this was proved by Orri et al.(2002) who reported an improvement of autonomic dysregulation after iron supply in three children with BHS[24].

Lastly, among all clinical, laboratory and ECG data obtained from the present study no significant difference was found between cyanotic and pallid BHS which indicates that both types of BHS share the same pathophysiologic mechanism i.e. pallid and cyanotic BHS are "one" and not "two" diseases. This conclusion is not in agreement with some studies which postulated that the pathophysiology of pallid and cyanotic spells is not the same[7,14,22]. Again, the use of 24 hours ECG recording in our study which is more accurate than ordinary ECG may add more confidence to our results. Moreover, the presence of a considerable percentage of children who experienced both types of BHS i.e mixed type, may indicate that the pathogenesis of both types may be similar.

Differences between cyanotic and pallid BHS regarding sequence of events and color changes might be explained by the dominant autonomic dysregulatory component which may be sympathetic overactivity in cyanotic and parasympathetic in pallid BHS(4).

Conclusion

Both pallid and cyanotic BHS are types of the same disease sharing the same pathophysiologic mechanisms. The main etiology of BHS is autonomic dysregulation manifested by frequent respiratory sinus arrhythmia in ECG recording; this heart rate disturbance was positively correlated with the frequency of breath-holding spells. Defective body iron assessed by low serum ferritin is an additional factor which may contribute to the etiology of BHS to lesser extent as low serum ferritin was not correlated with the frequency of the attacks. Positive family history was present in one-third of cases but it is not known whether this family history is an indicator of inheritance of BHS or inheritance of autonomic dysregulation responsible for the attacks.

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PATHOPHYSIOLOGY OF BREATH-HOLDING SPELLS

Ahmed G. Siam MD, Magdy A. Aidaros MD, El-Sayed M. Farag MD, Amal F. Gharib MD and Abdel Nasser A. Mourad MD

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OCULAR AND CERVICAL VESTIBULAR EVOKED MYOGENIC POTENTIALS IN PATIENTS WITH VESTIBULAR MIGRAINE

Elsaeid M. Thabet, MD

Audiology Unit, ENT-HNS Deptartment, Mansoura University, Mansoura, Egypt

Abstract

Objective: *Vestibular migraine (VM) is one of the most common vestibular disorders. However, the pathogenesis of VM is not totally understood. The aim of the present study was to increase the understanding of VM pathophysiology by assessing the otolith organ function and its related brainstem pathways through recording both cervical and ocular vestibular evoked myogenic potentials (cVEMP and oVEMP) in VM patients. Study Design: Both cVEMP and oVEMP were recorded in both ears of 19 patients having definite VM and of 13 age and sex matched control subjects. For cVEMP, analyzed parameters were latency and interaural latency difference of p13 and n23, peak amplitude difference between the p13 and n23, and the p13-n23 amplitude asymmetry ratio (AR). For oVEMPs, parameters were latency of the first positive peak n10 and interaural latency difference of n10, peak n10 amplitude and the n10 AR. Results: None of the control subjects or VM patients had absent cVEMPs or oVEMPs responses and there were no statistically significant differences between right and left ears in either group as regards VEMPs parameters in both VEMPs responses. Compared to the control subjects, VM patients had significantly longer mean peak latencies of both VEMPs responses in both ears and longer both cVEMPs p13 and oVEMPs n10 interaural latency difference. In addition, the mean peak amplitudes of cVEMPs p13-n23 and oVEMPs n10 were significantly lower in VM patients with statistically significant difference in the amplitude AR. While 89% of ears of the VM patients had abnormal oVEMPs n10 peak latency and 100% abnormal amplitude, 74% of ears had only abnormal cVEMPs p13 peak latency and 29% ears had abnormal cVEMPs amplitude. Conclusion: VEMP abnormalities are prevalent in VM patients suggesting involvement of both otoliths and related brainstem pathways in the pathogenesis of VM.*

Key words: *Vertigo, VEMP, vestibular migraine, Brainstem.*

Introduction

Migraine as a cause of recurrent vertigo is supported by epidemiologic studies[1,2]. Vestibular migraine (VM) is a vestibular syndrome caused by migraine and presents with attacks of spontaneous or positional vertigo, lasting seconds to days, in conjunction with migrainous symptoms during the attack[3]. It is a clinically heterogeneous disorder that can present with any combination of recurrent spontaneous vertigo, positional vertigo, or head motion intolerance[4]. At present the International Headache Society (IHS) classification does not include a specific category for VM. Therefore, this most suffering segment of headache patients still does not have their own diagnostic criteria. Vertigo is included only in the framework of basilar migraine. However, less than 10% of VM patients fulfill the criteria for basilar migraine which makes basilar migraine an inappropriate category for these patients[5]. To resolve this situation, Neuhauser and her colleagues proposed operational clinical criteria modeled on IHS headaches classification and defined two diagnostic categories for definite and probable VM[6]. In specialized audio-vestibular and migraine clinics, definite and probable VM accounted for 7% and 9% of the patients in Neuhauser et al. studies respectively[4]. Unfortunately, the pathophysiology of VM is still a matter of speculation. In any paroxysmal disorder, the physical examination during the acute episode is indispensable for an understanding of the underlying pathophysiology. However, reports on the clinical findings in patients with VM during the acute episode are scarce[7]. It appears that between attacks, the functional state of the vestibular system may be normal or near normal, but at the time of the attack, there are major changes in vestibular and cochlear function which are responsible for the clinical symptoms.

Vestibular evoked myogenic potentials (VEMPs) are short latency electromyographic (EMG) responses that can be recorded from various muscles during the contraction phase in response to acoustic stimulus[8-10]. VEMPs recorded from ipsilateral sternocleidomastoid (SCM) muscle known as cervi-

cal VEMPs (cVEMPs) are a clinical demonstration of vestibulocollic reflex[8,9,11]. cVEMPs pathway is believed to originate in the saccular macula and continues through the vestibular nerve and nucleus, the medial vestibulospinal tracts, spinal motor nucleus and SCM muscles[8,11]. cVEMPs are characterized by biphasic waves with initial positivity (p13) followed by a negative wave (n23). Recently, a short latency negative potential (n10) probably reflecting activation of inferior oblique muscle in response to acoustic stimuli has been reported to be a manifestation of crossed otolith-ocular response and named ocular VEMPs (oVEMPs). It predominantly reflects contralateral utricular function[12].

Utricular afferents have strong projections to the oculomotor system whereas saccular projections to the oculomotor system are weak and polysynaptic. Saccular neurons project strongly to the cervical SCM muscle whereas utriculo cervical projections to the ipsilateral cervical SCM muscle is not as strong. On the basis of this differential pattern of oculomotor and cervical projections, it was suggested that measuring oVEMPs predominantly indexes utricular function and measuring cVEMPs predominantly indexes saccular function. There is no afferent specificity but the differential motor projections of the utricular and saccular maculae allow differential evaluation of the functional status of the utricular and saccular maculae[12].

VEMPs are thought to provide useful information about brainstem functions, as the neural pathway of both cervical and ocular VEMPs pass through the brainstem[13,14]. The aim of the present study was to increase the understanding of VM pathophysiology by assessing the otolithic (utricle and saccule) organ function in VM using vestibular evoked myogenic potentials testing (cervical and ocular VEMPs).

Material and Methods

The study group included a total number of 19 patients diagnosed as having definite VM based on the criteria proposed by Neuhauser et al.[4]: recurrent episodic vestibular symptoms in the form of rotational vertigo of at least

moderate severity to be present in a patient with current or a previous history of migraine diagnosed according to IHS criteria. Migraine symptoms (headache, photophobia, and phonophobia, visual or other auras) occur in at least two of vertiginous attacks. The diagnosis of definite VM was established after diagnostic imaging studies were used to rule out other diseases (magnetic resonance imaging of the brain with particular interest in the posterior cranial fossa using paramagnetic contrast enhancement) in all patients. The patients were tested between episodes of migraine. The control group consisted of 13 subjects with age and sex matched to the study group. None of the control subjects reported any auditory, vestibular or neurological problems. All procedures were approved by Mansoura University ethical research committee and all subjects and patients gave informed consent. Audio-vestibular test battery included a detailed vertigo history, hearing evaluation and neuro-otologic examination (cranial nerves and bedside vestibular examination). Laboratory investigation in the form of videonystagmography using a two channel Visula Eyes recording system from Micromedical Technologies, Chatham, Illinois, USA.

cVEMPs and oVEMPs testing were done using a two channel evoked potential recording system Intelligent Hearing System (IHS) with Smart EP software, Miami, Florida, USA. The patients were tested in a sitting position with the following parameters:

a- Electrode montage: For the cVEMPs test, EMG activity was ipsilaterally recorded from the upper half of the SCM muscle using surface (active) electrode, with a reference electrode on the upper edge of the sternum and a ground electrode on the forehead. Care was taken to place the bilateral electrodes symmetrically. During each recording session, the subject was instructed to rotate the head towards the contralateral side of the tested ear to keep the SCM muscle under tension. Subjects were instructed to tense the SCM muscle during acoustic stimulation and relax in between recording sessions. For oVEMPs test, the head pitched slightly nose down, and surface EMG activity was recorded

from active electrodes placed on the face 1cm below the lower eye lid on the infra orbital ridge with the reference electrodes placed 1-2 cm below the first electrode. The electrodes were aligned with the center of the pupil as the subject looked up at a distant target exactly in the midline. The ground electrode was placed on the forehead. To optimize the oVEMP responses, the subjects were instructed to look upward with a visual angle of approximately 30º during recording(15,16).

b- Stimulus and recording parameters:

Tone bursts of 500 Hz with a two cycle rise /fall time and plateau were used. They were presented at a rate of 5 cycles per second (TDH 39 headphones) at 100 dBnHL (125 dBSPL). The EMG signal was amplified (10000 times), bandpass filtered (30 to 1500 Hz) and was averaged after 100-200 sweeps. The analysis window was 49.92 ms wide. Each ear was stimulated separately and the first ear to be tested was randomly allocated. For cVEMPs, The EMG activity was rectified online then averaged. To decrease the effect of tonic activity of the SCM muscle on the recorded VEMPs and ensure equal muscle contraction between both sides, the Smart EP software accepted data acquisition only when the rectified rms EMG activity was between 50 and 100 µv. Data acquisition was rejected when rectified rms EMG activity was below 50 µv or above 100 µv. EMG activation was maintained at 60-80 µV throughout recording using a visual biofeedback technique. For the oVEMPs, online automatic artifact rejection was used to reject the blink artifacts and prevent them from contaminating oVEMPs response.

c- Response analysis:

Measurements made on cVEMPs responses were latency of the first positive peak (p13), latency of the next negative peak (n23), interaural p13 latency difference, interaual n23 latency difference, and peak amplitude difference between the p13 and n23. Amplitude was measured as the peak-to-peak difference between the p13 and n23 components of the response. Raw amplitudes were corrected for underlying EMG activity by dividing the non rectified amplitude (A

RAW) by the integral rectified EMG (A EMG) i.e. normalised amplitude ratio = A RAW/ A EMG. The p13-n23 amplitude asymmetry ratio (AR) was evaluated using the equation [(larger response – smaller response)/(larger response + smaller response)] x 100[8]. Measurements made on oVEMPs responses were n10 latency, and n10 baseline to peak amplitude, interaural n10 latency difference. The n10 amplitude asymmetry ratio was evaluated using the same equation. Statistical analysis was done using Microsoft Excel release 2000. Student t-test was used for comparison of both the study and the control groups. Differences with P values less than 0.05 were considered to be statistically significant.

Results

A total of 32 subjects were investigated (19 VM patients and 13 control subjects). The VM group consisted of 13 (68%) females and 6 (32%) males (age range was 18-49 years; mean age was 33±8.1 years) with duration of illness 42.21±44.23 months. The control group consisted of 8 (62%) females and 5 (38%) males (age range was 19-48 years; mean age was 34±7.4 years). There were no statistically significant differences between the VM and control group as regards their demographic data. Both groups were of bilateral normal hearing sensitivity at frequency range between 250 and 8000 Hz. In the study group, complaints of false sensation of self motion or spinning of the surround or positional vertigo precipitated by head movement or motion (11 patients), visual stimulated vertigo by a moving visual target (3 patients), visual auras characterized by bright lights or zigzag lines restricted to one hemifield (2 patients only), transient auditory symptoms as aural fullness or tinnitus associated with migraine attacks in addition to photophobia and phonophobia (3 patients). Clinical bedside vestibular examination revealed positive head thrust test (catch up saccades) to the same side of headache or pain (10 patients) and positive Fukuda stepping test (19 patients). Videonystagmography revealed positional nystagmus on supine position mimicking benign paroxysmal positional vertigo in 53% of the study group (10/19) but differed

in being more persistent and not aligned with a single semicircular canal. Caloric stimulation revealed unilateral hypo excitable response on the same side of headache in 10 patients (53%), and within normal response in 9 (47%) of patients.

Both ocular and cervical VEMPs responses were detectable from both sides in all the investigated study and control groups. Individual and average values of each VEMPs parameter for the VM and control groups are shown in tables 1 through 4. There were no significant differences between right and left ears in either group as regards the different VEMP parameters.

cVEMPs and oVEMPs mean peak latencies were significantly prolonged in right and left ears of the VM group in comparison to the control group, and cVEMPs p13-n23 and oVEMPs n10 amplitudes were significantly reduced (tables 5, 6). In addition, there was statistical significant difference in the amplitude AR of both VEMPs responses between VM patients and the control subjects (t value = 3.78; p value <0.5). There was no significant differences in cVEMPs mean n23 latency between the VM and control subjects. Mean interaural latency difference for cVEMPs p13 and oVEMPs n10 latencies were significantly prolonged in VM group with no significant difference in interaural latency difference for cVEMPs n23 (table 7). The upper limit of normal values in each VEMP parameter (control group mean + 2.5 SD) was determined in order to detect the number of abnormal cervical and ocular VEMPs responses. For the oVEMPs response, 89% of ears of the VM patients had abnormal n10 peak latency and 100% abnormal n10 amplitude, for the cVEMPs, 74% of ears had only abnormal p13 peak latency and 29% ears had abnormal p13 - n23 amplitude (table 8).

Table (1): cVEMPs data in VM patients

Patient no.	right ear			left ear			AR	Rp13-Lp13	Rn23-Ln23
	p13 latency (msec)	n23 latency (msec)	p13n23 amp	p13 latency (msec)	n23 latency (msec)	p13n23 amp			
1	13.4	19	17.90	14	20.6	19.87	5.22	-0.6	-1.6
2	13.8	20.8	20.78	13.4	19.6	18.70	5.27	0.4	1.2
3	17.4	23.6	21.73	18	23.4	19.40	5.66	-0.6	0.2
4	15.8	22.4	17.87	17.4	24.2	16.43	4.47	-1.6	-1.8
5	13.4	20.4	8.98	13.4	20.4	6.96	12.67	0	0
6	13.4	21	29.10	14	20.8	23.40	10.86	-0.6	0.2
7	14.8	20.6	20.25	18.8	24.2	16.3	10.81	-4	-3.6
8	13.4	22.6	22.26	14	20.4	23.59	2.90	-0.6	2.2
9	17	23	13.11	14.6	21.6	14.10	3.64	2.4	1.4
10	14.3	21	11.58	13.9	20.2	10.62	4.32	0.4	0.8
11	15.6	22.6	15.78	15.8	23.3	17.52	5.23	-0.2	-0.7
12	13.3	20.2	23.04	13.4	23.5	22.55	1.07	-0.1	-3.3
13	13	20	19.88	14.8	23.8	21.64	4.24	-1.8	-3.8
14	16	23.3	14.33	15.3	23	17.26	9.28	0.7	0.3
15	15.2	22.3	21.73	14.3	20	19.40	5.66	0.9	2.3
16	15.8	22	7.85	15.2	21	8.41	3.44	0.6	1
17	14.6	23.8	21.76	13.9	23.6	20.16	3.82	0.7	0.2
18	15.2	23	22.23	15.7	23.4	20.54	3.95	-0.5	-0.4
19	13.8	23.44	13.53	14	20.8	15.46	6.66	-0.2	2.64
X	14.69	21.84	18.09	14.94	21.99	17.49	5.75	0.89	1.45
SD	1.32	1.42	5.43	1.58	1.64	4.74	3.04	0.97	1.22

AR = amplitude asymmetry ratio; X= Mean; SD= standard deviation; Rp13-Lp13 = inter-aural latency difference between right and left p13; Rn23-Ln23 = inter-aural latency difference between right and left n23.

Table (2): cVEMPs data in control subjects

subject no.	right ear			left ear			AR	Rp13-Lp13	Rn23-Ln23
	p13 latency (msec)	n23 latency (msec)	p13n23 amp	p13 latency (msec)	n23 latency (msec)	p13n23 amp			
1	11.89	20.23	21.35	11.89	20.23	24.64	7.15	0	0
2	12.12	20.26	31.37	12.12	20.26	23.09	15.20	0	0
3	13.2	21.2	25.42	12.95	22.09	27.56	4.04	0.25	0.89
4	11.88	21.67	17.83	11.88	21.67	22.37	11.29	0	0
5	11.76	21.55	35.03	11.76	21`.55	29.61	8.38	0	0
6	11.85	22.24	27.92	11.85	22.24	20.71	14.83	0	0
7	12.21	22.82	32.73	12.21	21.29	26.13	11.21	0	1.53
8	10.77	20.21	22.63	10.77	20.21	35.26	21.82	0	0
9	12.23	22.98	19.91	11.88	22.98	23.44	8.14	0.35	0
10	12.75	23.24	26.76	12.75	23.24	34.31	12.36	0	0
11	12.66	23.28	18.33	12.66	23.28	23.96	13.31	0	9
12	11.94	20.23	27.65	11.94	22.28	31.65	6.75	0	2.05
13	11.99	20.22	24.93	11.99	23	37.71	20.40	0	2.78
X	12.10	21.55	25.53	12.05	21.87	27.73	11.92	0.05	0.56
SD	0.58	1.25	5.45	0.54	1.13	5.50	5.25	0.11	0.96

AR = amplitude asymmetry ratio; X= Mean; SD= standard deviation; Rp13-Lp13 = inter-aural latency difference between right and left p13; Rn23-Ln23 = inter-aural latency difference between right and left n23.

Table (3): oVEMPs data in VM patients

Patient no.	right ear		left ear		AR	Rn10 - Ln10
	n10 latency (msec)	n10 amp (µv)	n10 latency (msec)	n10 amp (µv)		
1	10.78	0.91	11.03	0.93	0.01	-0.25
2	10.56	0.80	11.78	0.75	0.03	-1.22
3	11.52	0.91	12.86	0.85	0.03	-1.34
4	11.23	0.84	11.53	0.76	0.05	-0.30
5	11.33	0.92	11.65	0.84	0.05	-0.32
6	10.89	0.89	10.75	0.81	0.05	0.14
7	10.67	0.94	10.91	0.85	0.05	-0.24
8	11.22	0.80	10.95	0.80	0.00	0.27
9	11.42	0.84	11.96	0.81	0.02	-0.54
10	11.00	0.87	11.28	0.79	0.05	-0.28
11	11.22	0.84	11.42	0.85	0.01	-0.20
12	11.33	0.86	11.65	0.77	0.06	-0.32
13	10.34	0.81	11.54	0.84	0.02	-1.20
14	11.33	0.95	11.65	0.85	0.06	-0.32
15	11.00	0.74	10.75	0.65	0.06	0.25
16	11.57	0.72	11.65	0.85	0.08	-0.08
17	11.52	0.90	12.86	0.87	0.02	-1.34
18	12.23	0.95	10.75	0.85	0.06	1.48
19	11.42	0.79	11.96	0.88	0.05	-0.54
X	11.19	0.86	11.52	0.82	0.04	-0.33
SD	0.43	0.07	0.62	0.06	0.02	0.65

AR = amplitude asymmetry ratio; X= Mean; SD= standard deviation; Rn10-Ln10 = inter-aural latency difference between right and left n10.

Table (4): oVEMPs data in control subjects.

Patient no.	right ear		left ear		AR	Rn10 - Ln10
	n10 latency (msec)	n10 amp (µv)	n10 latency (msec)	n10 amp (µv)		
1	9.8	1.40	9.8	1.48	0.03	0.00
2	9.6	1.40	9.9	1.43	0.01	-0.30
3	10.5	1.40	10.2	1.51	0.04	0.30
4	10	1.43	10	1.43	0.00	0.00
5	9.9	1.43	9.4	1.43	0.00	0.50
6	9.7	1.42	9.7	1.40	0.01	0.00
7	10.2	1.44	10.2	1.40	0.01	0.00
8	9.8	1.42	9.8	1.43	0.00	0.00
9	10	1.43	10	1.40	0.01	0.00
10	10.2	1.39	10.2	1.43	0.01	0.00
11	10.3	1.34	10.3	1.41	0.03	0.00
12	9.4	1.37	9.4	1.37	0.00	0.00
13	10.4	1.39	10.3	1.37	0.01	0.10
X	9.98	1.40	9.94	1.42	0.01	0.05
SD	0.33	0.03	0.31	0.04	0.01	0.19

AR = amplitude asymmetry ratio; X= Mean; SD= standard deviation; Rn10-Ln10 = inter-aural latency difference between right and left n10.

Table (5): Comparison between VM patients and control subjects as regards the latency and amplitude for cVEMPs (1st to 3rd rows) and oVEMPs (4th to 5th rows)in the right ears.

	VM group Mean ± S.D	Control group Mean ± S.D	t test value	*P*
p13 peak latency (msec)	14.69 ± 1.32	12.10 ± 0.58	0.000*	<0.05
n23 peak latency (msec)	21.84 ± 1.42	21.55 ± 1.25	0.6	>0.05
p13-n23 amplitude	18.09 ± 5.43	25.53 ± 5.45	0.000*	<0.05
n10 peak latency (msec)	11.19 ± 0.43	9.98 ± 0.33	0.000*	<0.05
n10 amplitude (µv)	0.86 ± 0.07	1.40 ± 0.03	0.000*	<0.05

* Statistically significant at p value less than 0.05

Table (6): Comparison between VM patients and control subjects as regards the latency and amplitude for cVEMPs (1st to 3rd rows) and oVEMPs (4th to 5th rows)in the left ears.

	VM group Mean ± S.D	Control group Mean ± S.D	t test value	*P*
p13 peak latency (msec)	14.94 ± 1.58	12.05 ± 0.54	0.000*	<0.05
n23 peak latency (msec)	21.99 ± 1.64	21.87 ± 1.13	0.17	>0.05
p13-n23 amplitude	17.49 ± 4.74	27.73 ± 5.50	0.000*	<0.05
n10 peak latency (msec)	11.52 ± 0.62	9.94 ± 0.31	0.000*	<0.05
n10 amplitude (µv)	0.82 ± 0.06	1.42 ± 0.04	0.000*	<0.05

* Statistically significant at p value less than 0.05

Table (7): Comparison between VM patients and control subjects as regards the inter-aural peak latency difference for the c VEMP (1st and 2nd rows) and o VEMP (3rd row).

	Control group Mean S.D	Study group Mean S.D	t test value	P value
p13 latency difference	0.05 ± 0.11	0.89 ± 0.97	0.04*	<0.05
n23 latency difference	0.56 ± 0.96	1.45 ± 1.22	0.08	>0.05
n10 latency difference	0.09 ± 0.17	0.54 ± 0.47	0.04*	<0.05

* Statistically significant at p value less than 0.05.

Table (8): The number of abnormal cVEMPs and oVEMPs responses in VM patients

	Right ears		Left ears		Total	
	>2.5	SD%	>2.5	SD%	>2.5	SD%
p13 peak latency (msec)	12/19	63	16/19	84	28/38	74
n23 peak latency (msec)	0/19	0	0/19	0	0/19	0
p13-n23 amplitude (µv)	5/19	26	6/19	32	11/38	29
n10 peak latency (msec)	15/19	79	19/19	100	34/38	89
n10 amplitude (µv)	19/19	100	19/19	100	38/38	100

Discussion

Vestibular migraine is an entity where neuro otologic manifestations of brainstem disorder is heterogenous. The results reflected the involvement of the vestibular system at any level from the brainstem connection down to the periphery with variable percentage. Like migraine itself, VM is diagnosed on the basis of clinical information as there are no specific biological markers. Several anatomic and functional interfaces between the vestibular system and mechanisms involved in migraine have been identified including spreading depression involving brainstem structures processing vestibular signals. Models of migraine pathophysiology acknowledge that the brainstem plays a key role in the genesis of the clinical features of migraine(17). In the present study, brainstem dysfunction was evident neuro otologically by means of videonystagmography where positional nystagmus was a common finding in more than half of the patients and unilateral hypo active caloric response. This results from dysfunction of vestibular structures in the brainstem or vestibulocerebellum presumably inhibitory fibers from the nodulus and uvula to the vestibular nuclei(18).

In the current study, latencies of cVEMP p13 and oVEMPs n10 responses were significantly prolonged in VM patients compared to the control group, while no significant prolongation of n23 latency of cVEMPs response was found. This has been reported in other studies(19-22), which found that prolonged p13 cVEMPs and oVEMPs n10 latency are the most common latency abnormality. The absence of significant prolongation of n23 in VM patients might be due to larger variation of n23 latency than that of p13. Latency prolongations have been reported in other pathologies affecting the brainstem such as multiple sclerosis, stroke and tumors(19,23). A number of possible explanations have been offered for the prolonged latencies as a hypothetical dopaminiergic dysfunction or abnormal brain metabolism. The most attractive one is a global brain disorder with substantial brainstem involvement leading to a secondary blood flow changes in the posterior circulation leading to ischemia(13,24).

In the present study, there were reduced amplitudes of both VEMP responses in VM group relative to the control group. Considering that the assessment VEMPs was performed during the symptom-free interval and the wide variation between the last migraine attack and the examination, the author postulates that the strong effect of reduced VEMP amplitudes in vestibular migraine lead not only to transient effects but also to permanent deficits. The reduced VEMP amplitude in VM patients may be hypoperfusion induced ischemia of labyrinthine structures. This would consequently lead to a disturbance of inner ear structures such as the saccule, which would have reduced VEMP amplitude as its peripheral vestibular electrophysiological correlate[25,26]. Alternatively, abnormal neurotransmitter modulation originated in the brainstem but also affecting peripheral vestibular structures might induce lesions of the saccule and/or sacculocollic pathways. Central actions of serotonin (5-HT) from the dorsal raphe nucleus (which has serotonergic afferents to the vestibular nuclei) during and between migraine attacks has led to the assumption that a low serotonin state facilitates activation of the trigeminovascular nociceptive pathways as induced by cortical spreading depression leading to a vasodilatation and plasma extravasation of the intracranial, dural and inner ear arteries[27]. The authors hypothesized that extravasation in turn induces local inflammatory processes (in particular in the stria vascularis) that might lead to an imbalance in the potassium homeostasis. Hence, changes in serotonin metabolism in patients with vestibular migraine might, therefore, not only have an impact on the central but also on peripheral vestibular mechanism[25,27]. While the majority of VM patients in the current study had abnormal VEMP responses, this did not agree with previous studies that reported normal VEMPs in VM patients[28]. This could be explained on the basis of some heterogeneity of findings or the heterogeneous nature of VM. Furthermore, despite the reduced VEMP amplitude, absence of VEMP response was not seen in this study. This did not agree with authors who found absent responses in a high propor-

tion of cases[29,30]. This could be explained by a higher intensity of stimulus used in this study protocol in comparison to their restricted lower intensity protocol.

Interaural latency differences and amplitude AR of VEMPs responses may be helpful when considered together with the absolute peak latency prolongations and amplitude reduction. In the current study, VM patients had longer interaural latency difference of cVEMPs p13 and oVEMPs n10, in addition to abnormal amplitude AR of both VEMPs responses compared to the control subjects. These results suggest VM patients had predominance of abnormality in the otolith brainstem pathways in one side relative to the other.

The present study showed that oVEMPs were more sensitive than cVEMPs in detecting abnormalities in VM patients. This could be attributed to either a technical or anatomical factor. Technically, oVEMPs have the advantage that the subjects do not contaminate the response with muscular activity (in cVEMPs, the patients have to contract the SCM muscle during recording) as it reflects the extra ocular EMG activity of the inferior oblique muscle with a more intra and inter-subject consistency of recording. Anatomically, cVEMPs descend via the vestibulospinal tract to the lower brainstem, while oVEMPs ascend through the MLF to the upper brainstem[14] which appears to be more involved in VM as evidenced by neuroimaging studies[31].

In conclusion the epidemiologic link between migraine and vestibular symptoms and signs suggests shared pathogenetic mechanisms. VEMPs response is affected in VM patients through abnormalities in the peripheral vestibular structures and/or the related brainstem pathways.

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OCULAR AND CERVICAL VESTIBULAR EVOKED MYOGENIC POTENTIALS IN PATIENTS WITH VESTIBULAR MIGRAINE

Elsaeid M. Thabet, MD

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CONTRALATERAL ACOUSTIC SUPPRESSION OF TRANSIENT EVOKED OTOACOUSTIC EMISSIONS IN VESTIBULAR MIGRAINE PATIENTS

Elsaeid M. Thabet, MD

Audiology Unit, ENT-HNS Deptartment, Mansoura University,

Mansoura, Egypt

Abstract

Background: *Otoacoustic emissions are low level sounds emitted by the outer hair cells in a healthy normal ear either spontaneously or in response to sound stimulation. OAEs now provides an important non-invasive method of auditory testing. Otoacoustic emissions suppression testing is a technique that enables easy and non invasive study of auditory efferent function. This pathway is thought to have a role in modulating the gain of auditory responses, and travels via the brainstem. The objective of this study was to explore this notion by examining auditory efferent function in patients with vestibular migraine, in whom significant brainstem dysfunction is thought to occur.* ***Materials and methods:*** *31 subjects (62 ears) were investigated (18 vestibular migraine patients "36 ears") and 13 age and gender matched control subjects "26 ears". Both groups were of bilateral normal hearing sensitivity at frequency range (250 - 8000 Hz). TEOAEs were obtained from all the tested 62 ears.* ***Results:*** *A significant difference was detected only at 1000 and 1414 Hz where TEOAEs amplitudes were higher than in controls ($p < 0.0001$). TEOAEs with contralateral acoustic suppression compared to TEOAEs in quiet in vestibular migraine patients was statistically insignificantly different ($p > 0.05$). Total otoacoustic suppression difference showed values below 1 dBSPL in migraineurs in contrast to the control group that showed values of 1 or more dBSPL.* ***Conclusion:*** *Dysfunctional central mechanisms modulating the cochlear activity could be one of the brainstem criteria that might be the earliest indicator of impending auditory involvement in migraine. The combination of CAS and linear TEOAE enables easy and noninvasive study of auditory efferent function.*

Key word: *auditory brainstem, Oto-acoustic emissions, Contralateral suppression, Vestibular migraine.*

Introduction

Otoacoustic emissions (OAEs) are low level sounds emitted by the outer hair cells (OHC) in a healthy normal ear either spontaneously or in response to sound stimulation. OAEs now provide an important non-invasive method of auditory testing (Gold, 1948; Kemp, 1978). OAEs suppression testing is a technique that enables easy and non invasive study of auditory efferent function. It is based on the principle that when OAEs are recorded with and without the presence of noise, recording in the noise condition shows reduced amplitude in comparison with the quiet condition (Murdin and Davis, 2008). The phenomenon of suppression of OAEs in humans by means of a contralateral acoustic stimulation (CAS) is due to activation of efferent synapses of OHC which would occur through the olivocochlear bundle (OCB) (Bonfils et al. 1987). The afferent arm of the reflex assessed by OAE suppression travels in the auditory nerve, and the efferent arm along the inferior vestibular nerve. The descending pathways originate from cortical and sub-cortical regions (Littman et al. 1991).

The olivocochlear neural pathway is comprised of efferent neurons that travel from the superior olivary complex in the brainstem to cochlear hair cells. The olivocochlear efferent fibres are of two types: medial olivocochlear (MOC) and lateral olivocochlear (LOC) efferents. LOC system arises from the lateral superior olive and travel via the vestibular nerve to the cochlea supplying the inner hair cells of the ipsilateral cochlea (Kimura and Wersall, 1962; War, 1975). MOC pathway originates in the medial part of the superior olivary complex. Portion of fibres cross the midline to the contralateral cochlea while others project to the ipsilateral cochlea via the vestibular nerves (Guinan, 2006). The MOC fibres innervate the OHCs; this is referred to as the medial olivocochlear system (MOCS). Activation of the medial olivocochlear (MOC) efferents attenuates cochlear gain and reduces the amplitudes of mechanical, electrical, and neural cochlear outputs (Mul-

ders and Robertson, 2000a; Mulders and Robertson, 2000b).

Vestibular migraine (VM) is a vestibular syndrome caused by migraine and presents with attacks of spontaneous or positional vertigo, lasting seconds to days, in conjunction with migrainous symptoms during the attack (Neuhauser, 2007). It is a clinically heterogeneous disorder that can present with any combination of recurrent spontaneous vertigo, positional vertigo, or head motion intolerance. At present the International Headache Society (IHS) classification does not include a specific category for VM. Neuhauser et al. (2001) proposed operational clinical criteria modeled on IHS headaches classification and defined two diagnostic categories for definite and probable VM. Unfortunately, the pathophysiology of VM is still a matter of speculation as neuro-otological investigation of vestibular migraine to date has failed to show any single abnormality which occurs with sufficient frequency to be of diagnostic utility as a biomarker. Several hypotheses have been put forward: Cortical spreading depression produce vestibular symptoms when the multisensory cortical areas that process vestibular signals located in the posterior insula and at the temporoparietal junction become involved (Fasold et al. 2002). Several neurotransmitters that are involved in the pathogenesis of migraine are also known to modulate the activity of central and peripheral vestibular neurons and could contribute to the pathogenesis of VM (Goadsby et al. 2009). Genetic defects of ion channels have been identified as the cause of various paroxysmal neurologic disorders. Many channelopathies exhibit migraine attacks as a part of the phenotype (Pantoni et al. 2010). Trigeminal nerve activation and subsequent changes in the cerebral vasculature are widely acknowledged to be key steps in the pathology of an attack associated with neurogenic inflammation (Goadsby et al. 2009).

Models of migraine pathophysiology acknowledge that the brainstem plays a key role in the genesis of the clinical features of migraine (Lafreniere et al. 2010). Brainstem activation has been shown on PET scanning, the acti-

vated brainstem areas encroach upon the location of the vestibular nuclei (Afridi et al. 2005). Few studies addressed the auditory characteristics during the headache free periods. Decreased pure tone hearing thresholds at high frequencies between 6 and 8 kHz was reported by Bayazit et al. (2001). Dysfunction in efferent system regulating cochlear outer hair cell at peripheral or central pathways was proposed in VM as reported by Bolay et al. (2008) who observed absence of suppression of the TEOAEs by contralateral sound stimulation. They postulated the presence of dysfunction either in the medial olivocochlear complex in the brainstem or at the synaptic transmission between olivocochlear efferents and OHCs in the cochlea. Dysfunctional central mechanisms modulating the cochlear activity could be one of the brainstem criteria that might be the earliest indicator of impending auditory involvement in migraine (von Brevern et al. 2005). Both ipsilateral and contralateral olivocochlear bundles and the vestibular nerve share anatomical relation, as they travel peripherally with the vestibular nerve to join the cochlear nerve at the vestibule-cochlear anastmosis of Oort (Arnesen and Osen, 1984). Due to this anatomical relevance, they might share similar pathological changes. The combination of TE-OAE with CAS enables easy and noninvasive study of auditory efferent function. The objective of this study was to explore this notion by detecting early asymptomatic cochlear dysfunction and its efferents in vestibular migraine patients using TEOAEs and contralateral suppression.

Materials and Methods

A total of 31 subjects (62 ears) were investigated (18 VM patients [36 ears] and 13 control subjects [26 ears]). VM patients were diagnosed as definite VM based on the criteria proposed by Neuhauser et al. (2001): recurrent episodic vestibular symptoms in the form of rotational vertigo of at least moderate severity to be present in a patient with current or a previous history of migraine diagnosed according to IHS criteria. Migraine symptoms (headache, photophobia, and phonophobia, visual or other auras) occur in at least two

of vertiginous attacks. This diagnosis was established after diagnostic imaging studies were done to rule out other diseases (magnetic resonance imaging of the brain with particular interest in the posterior cranial fossa using paramagnetic contrast enhancement) in all patients. The VM group consisted of 12 (67%) females and 6 (33%) males (age range 18 - 49; mean age 33 ± 8.3). The control group consisted of 13 subjects with age and gender matched to the study group and was of 8 (62%) females and 5 (38%) males (age range of 19 - 48; mean age 34 ± 7.4). There were no statistically significant differences between the VM and control group as regards their demographic data. Both groups were of bilateral normal hearing sensitivity at frequency range (250 - 8000 Hz). None of the studied subjects reported any other neurological problems, or exposure to ototoxic drugs. Audio-vestibular test battery included a detailed vertigo history, hearing evaluation and neuro otologic examination. All procedures were in accordance with the Helsinki declaration, and approved by Mansoura ethical research committee and all subjects and patients gave an informed consent.

Transient evoked OAEs (TEOAEs) were recorded using an ILO V6 system (Otodynamics, UK (Kemp, Ryan, and Bray 2005). The stimulus consisted of a standard set of linear clicks of intensity 75–85 dB SPL. The response amplitude was calculated by the ILO V6 hardware system over the frequency range 1000 Hz to 4000 Hz with a time window from 2.5 to 20.5 ms. Alternate responses were stored and averaged in 2 separate buffers A and B. The correlation between the 2 averages determined the reproducibility of the TEOAEs which was calculated by the device and expressed as percentage. The measurements were averaged after 260 sweeps and were only accepted when reproducibility was greater than 50%, stimulus stability was better than 70% and the difference between emission amplitude and associated noise floor was at least 3 dB (Lonsbury-Martin et al. 1991). During the test session, subjects sat in an acoustically untreated test chamber where the noise level was maintained between 20 and 40

dB SPL; this was in line with the American Speech Language Hearing Association standards of 50 dB SPL for background noise levels.

Contralateral acoustic suppression (CAS):

It is reported that the suppressive effect is greatest using binaural noise, with a lesser effect from ipsilateral noise and, in fact, contralateral noise results in the weakest suppression (Hurley et al. 2002). However, using ipsilateral or binaural noise creates problems in distinguishing signal from noise in the responses, and thus requires more complex analysis or the use of forward masking techniques. Hence, for simplicity, the use of contralateral noise is often preferred (Attias et al. 2005).

The ILO V6 software was set to Difference B on/off function. A uniform click was presented at 60 +/-3dB SPL via the ipsilateral probe. The threshold for white noise detection was ascertained in the contralateral ear via a second insert probe. During testing, white noise was presented contralaterally at 50dB SL. (The MOC can be activated by low (just audible) levels of noise, and the suppressive effect increases with higher intensities (Collet et al. 1990). The noise stimulus intensity must be less than 75 dB SPL for broadband noise for fear of eliciting middle ear muscle contraction. Previous studies have found that white or broadband noise at 30 to 40 dB SL is adequate (DeCeulaer et al. 2001). In each run, TOAEs were monitored for 35 s and the contralateral noise elicitor was turned on at 5 s and off at 25 s. There was a 10-s break between runs. 60 runs were averaged. The runs of 60 sweeps alternated between the quiet condition and noise condition (white noise presented contralaterally according to parameters defined above). The OAE suppression response was taken as the difference between the amplitude of the response with contralateral noise to the amplitude of the response in quiet. This technique provides a measure of suppression for each ear separately. An additional technique of analysis was also incorporated known as total suppression (TS). TS is defined as the sum of suppression values of the two ears in an individual, i.e. right ear

(TEOAE - TEOAE with CAS) + left ear (TEOAE - TEOAE with CAS). A suppression level below 1.0 dB SPL is often taken as abnormal (Prasher et al. 1994).

Statistical Analysis

The statistical analysis was done considering the results in ears. Commercial software (Graphpad Prism, v.5) was used. Descriptive statistics and non-parametric Mann–Whitney U test was used to compare TEOAEs test results between VM and control groups. Differences with p values less than 0.05 were considered to be statistically significant.

Results

TEOAEs were obtained from all tested 62 ears. Fix frequency TE-OAEs responses were extracted from the emission pattern by simply adding both ears responses and obtaining the mean value for each. TEOAEs in quiet and TE-OAEs with CAS were tested between 1000 and 4000 Hz. A statistically significant difference was detected only at 1000 and 1414 Hz where TEOAEs amplitudes were higher than in controls (table 1 and figure 1) ($p<0.0001$). The mean amplitudes of higher frequencies (2000 to 4000 Hz) were statistically insignificant between both groups ($p>0.05$). TEOAEs with CAS compared to TEOAEs in quiet in VM patients was of non significance statistically (f=1000 Hz, 0.91; 1414Hz, 0.7; 2000 Hz, 0.14; 2828 Hz, 0.58 and 4000 Hz, 0.43, $p>0.05$).

Applying analysis of the TS, the VM group showed values below 1 dBSPL in contrast to the control group that showed values of 1 or more dBSPL (table 2 and figure 2). To explore whether TEOAE in quiet level and TEOAE with CAS were related, additional correlation and linear regression analyses were undertaken. There was a significant correlation and linear predictive relationship between total OAE suppression and TEOAEs level in quiet (f= 0.04, $p<0.05$) for VM group (figure 3). There were neither significant correlations nor linear predictive relationships (f= 0.32, $p>0.05$) between total OAE suppression and TEOAEs level in quiet for control participants (figure 4). That is, there was a statistically significant association between TEOAE level and the degree of TEOAE suppression for VM patients only.

Table (1): Comparison of TEOAEs amplitude (dBSPL) without CAS (TEOAEQ) and with CAS (TEOAEN) of both groups.

Frequency (Hz)	TEOAE in quiet		Mann Whitney U test	P	TEOAE with CAS		Mann Whitney U test	P
	Study Group	Control Group			Study Group	Control Group		
	Mean SD	Mean SD			Mean SD	Mean SD		
1000	13.19 ±3.15	7.52±3.51	0.0001	*	12.90±3.82	6.35±4.67	0.0001	*
1414	12.51±3.97	8.67±2.12	0.0002	*	13.94±4.07	8.52±2.32	0.0001	*
2000	9.88± 3.72	9.60± 3.72	0.53	>0.05	11.46±4.13	10.44±1.76	0.44	>0.05
2828	6.61±7.71	8.28±1.83	0.77	>0.05	7.04±5.86	8.28±2.00	0.15	>0.05
4000	2.78 ±5.52	8.28±1.83	0.24	>0.05	2.43±5.87	3.81±2.77	0.10	>0.05

*= significant <0.05

Table (2): TEOAEs Total suppression (TS) results in both the study and control groups.

Study group total OAE response							Control group total OAE response						
A	B	A-B	C	D	C-D	TS (dBSPL)	A	B	A-B	C	D	C-D	TS (dBSPL)
14.8	14.7	0.1	17.8	17.3	0.5	0.6*	15.7	15.2	0.5	15.1	14.6	0.5	1
19	18.9	0.1	15.7	15.6	0.1	0.2*	14.6	14.1	0.5	13.8	13.2	0.6	1.1
19.3	19.2	0.1	16.4	16.2	0.2	0.3*	14.6	14.2	0.4	14.7	13.6	1.1	1.5
21.5	21.3	0.2	11.9	11.6	0.3	0.5*	15.6	15.0	0.6	14.1	13.2	0.9	1.5
26.9	26.6	0.3	19.3	19.2	0.1	0.4*	14.8	14	0.8	15.8	15.1	0.7	1.5
16.8	16.8	0	14.2	14	0.2	0.2*	14.7	14	0.7	14.4	13.8	0.6	1.3
22.4	22.3	0.1	18.3	17.9	0.4	0.5*	15.7	15.1	0.6	15.9	15	0.9	1.5
19.4	19.2	0.2	23.6	23.5	0.1	0.3*	14.6	13.8	0.8	14.3	13.8	0.5	1.3
22.6	22.4	0.2	18.5	18	0.5	0.7*	14.9	14	0.9	16.6	16	0.6	1.5
19.5	19.3	0.2	16.4	16.1	0.3	0.5*	15.7	15.0	0.7	15.1	14.4	0.7	1.4
19.8	19.8	0.0	21.1	21	0.1	0.1*	14.6	14.3	0.3	14	13.3	0.7	1
20.6	20.3	0.3	14.2	13.8	0.4	0.7*	14	13.6	0.4	15	14.4	0.6	1
20.8	20.5	0.3	20.3	20.0	0.3	0.6*	15.7	15	0.7	14.3	14	0.3	1
26.9	26.7	0.2	21.1	20.9	0.2	0.6*							
16.3	16.2	0.1	14.2	14.1	0.1	0.2*							
22.4	22.2	0.2	18.3	18.2	0.1	0.3*							
19.4	19.4	0	23.6	23.3	0.3	0.6*							
22.6	22.5	0.1	18.5	18.4	0.1	0.6*							

*= amplitude signal below 1 dBSPL.
A= right ear TEOAEs without suppression. B= right ear TEOAEs with suppression C = left ear TEOAEs without suppression D = left ear TEOAEs with suppression

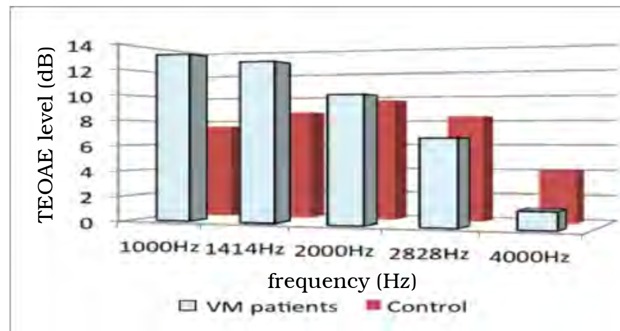

Fig. 1: TEOAE without suppression for VM & control groups.

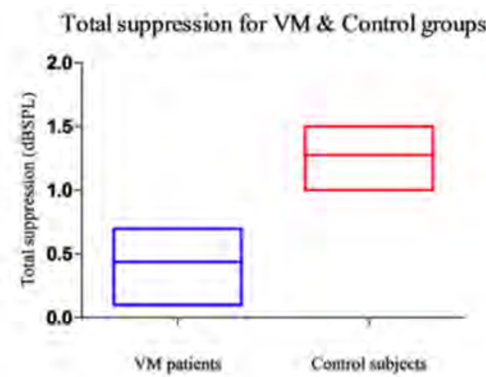

Fig. 2: Total suppression for VM & control groups.

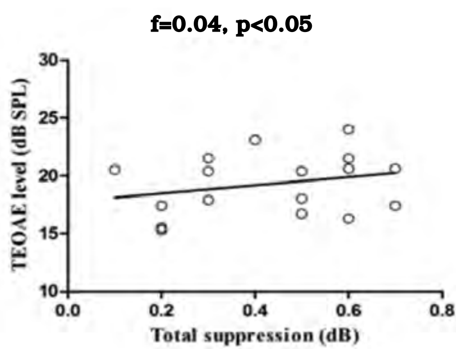

Fig. 3: Scatter plot of TEOAEs level and total suppression in VM patients.

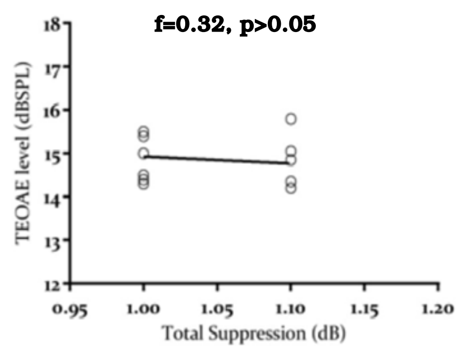

Fig. 4: Scatter plot of TEOAEs level and total suppression in control subjects.

Discussion

The principal focus of the present study was to evaluate subtle cochlea and its efferent pathway affection in VM patients looking for evidence of impaired sensory modulation in the auditory modality. Affection of cochlear efferent pathway in VM patients could occur because of anatomical relation of the ipsilateral and contralateral olivocochlear bundles with the vestibular nerve (Arnesen and Osen, 1984). All the studied patients on audiometric evaluation showed bilateral normal hearing sensitivity. This agreed with Battista, (2004) who speculated the source of the dysfunction was at the level of processing in the brainstem or higher structures, rather than occurring in the labyrinth.

In the current study, consistent with previous studies, the increased TEOAEs mean half octave bands level in VM patients, in contrast to normal participants, was statistically significant at low frequencies (1000 and 1414 Hz region) with insignificant differences at frequencies above. This agreed with Morand et al. (2000), who stated that the effect of CAS on emissions was more pronounced at lower frequencies and that TEOAEs are place specific, better below 1500 Hz. This may be explained by a decrease in tonic efferent activity to the OHC and reduction of tonic suppression of OHC electromotility, leading to larger OAEs amplitude. However, this did not agree with Bolay et al. (2008) who found that the mean amplitudes of TEOAEs were statistically insignificant at all frequency range in migraineurs. The author of the present study believed that methodological limitations may account for the difference as the eliciting stimulus was set at a higher level thus the role of middle ear reflexes cannot be excluded at this high level of sound intensity, so it is difficult to localize the lesion.

Interesting enough in this study was the finding that no significant difference in the absolute level of CAS of TEOAEs levels when compared to TEOAEs in quiet in VM patients. This agreed with Murdin et al. (2010) who found abnormal reduced OAE suppression in 11 out of 33 vestibular migraineurs. In addition, TS by contralateral noise assumed

a low value in VM patients than in controls. Reduced TS could occur due to problems anywhere along the reflex arc from the outer hair cells through the auditory nerve, central pathways via the cochlear nucleus, trapezoid body and superior olivary complex, through the crossed and uncrossed olivocochlear bundles and the efferent pathway via the inferior vestibular nerve; or, indeed, by affecting top down modulation via corticofugal pathways from the auditory cortex (Perrot et al. 2006).

There was a significant positive correlations and linear predictive relationships between TEOAE suppression and TEOAE level for VM patients only. These results imply that although there was a relative insignificant reduction in TEOAE suppression in VM, the MOC is still functional. Note that our results do not allow for any conclusions about the remaining efferent auditory system, which may still play a part in the impaired sensory modulation in the auditory modality of vestibular migraine. These observations can be construed as evidence in support of auditory efferent pathway dysfunction in vestibular migraine. The reduced CAS of TEOAEs in VM patients may be due to an altered balance between excitation and inhibition resulting in reduced activation of MOC in brainstem. This is supported by previous studies that pointed out general interictal cortical dysfunction in migraineurs (Antal et al. 2005; McColl and Wilkinson, 2000; Mulder et al. 2001). Another explanation may be a neuromuscular junction abnormality (at MOC efferent OHC synaptic junction) due to disruption of cholinergic synaptic transmission at the level of the cochlea due to deletion of nicotinic acetyl choline subunits thus abolishing the suppressor effect of MOC activation (Vetter et al. 1999).

Conclusion

Dysfunctional central mechanisms modulating the cochlear activity could be one of the brainstem criteria that might be the earliest indicator of impending auditory involvement in migraine. Both ipsilateral and contralateral olivocochlear bundles and the vestibular nerve share anatomical relation, as they travel peripherally

with the vestibular nerve to join the cochlear nerve at the vestibule-cochlear anastmosis of Oort. Due to this anatomical relevance, they might share similar pathological changes. The combination of CAS and linear TEOAE enables easy and noninvasive study of auditory efferent function. It can be speculated that those subgroup of migraineurs manifest more extensive lesion with longer duration affecting both the cochlea and the vestibular divisions. Studying effect of the use of prophylactic migraine therapy on OAEs changes in migraine patients deserves further investigation.

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CONTRALATERAL ACOUSTIC SUPPRESSION OF TRANSIENT EVOKED OTOACOUSTIC EMISSIONS IN VESTIBULAR MIGRAINE PATIENTS

Elsaeid M. Thabet, MD

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FREE TRANSVERSE RECTUS ABDOMINIS MYOCUTANEOUS (TRAM) FLAP FOR IMMEDIATE BREAST RECONSTRUCTION FOLLOWING MASTECTOMY, A CASE REPORT

Emad Eldeen Hamed Mottawee M.Sc*,
Samir Abd El Lateif Zidane MD, Adel Taha Denewer MD
and Ashraf Abd El Fattah Nasr khater MD
Surgical Oncology Unit, Oncology Centre-Mansoura University, Egypt

Abstract

Introduction: *The lower abdomen is often abundant source of tissue for autologus breast reconstruction.Blood supply to the lower abdominal tissue allows it to be used in a variety of techniques, pedicled transverse rectus abdominis myocutaneous flap (TRAM), free TRAM and deep inferior epigasteric perforator flap (DIEP).*

Aim of the work: *In this study we aimed to present and show feasibility of free transverse rectus abdominis myocutaneous (TRAM) flap for breast reconstruction.*

Case Study: *A 35 old years female patient with invasive duct carcinoma of the right breast planned for skin sparing mastectomy (SSM) and immediate breast reconstruction by free transverse rectus abdominis myocutaneous (TRAM) flap.*

Conclusion: *We'll continue to offer this technique of free transverse rectus abdominis myocutaneous (TRAM) flap as an ideal method for breast reconstruction. We believe that there is much needs to be done in the future in relation to the correct flap choice and better functional recovery.*

Key Words: *TRAM flap; breast reconstruction; free TRAM.*

Introduction

The use of the transverse rectus abdominis musculocutaneous (TRAM) flap in breast reconstruction was first described by Hartrampf in 1982. Since that time, the TRAM flap has undergone multiple modifications and refinements, yet it has remained a reliable option for post mastectomy breast reconstruction. It is an excellent option for an otherwise

healthy patient who has the adequate abdominal soft tissue redundancy to achieve the goal volume for the reconstructed breast. TRAM flap-based breast reconstruction has the potential advantage of recreating a breast mound without the need for an implant as well as essentially performing an abdominal lipectomy at the donor site.[1]

Free TRAM flap design provides a more direct and better blood supply to the TRAM skin island, which is one of the main advantages of the free TRAM flap over the pedicled flap. Relative indications for this procedure are similar to those for the pedicled TRAM flap. Unlike the pedicled TRAM flap, however, this technique can be used when the superior epigastric artery has been divided (e.g., in a patient who has had a previous open cholecystectomy.[2]

The free TRAM flap requires a reconstructive surgeon with expertise in microsurgery. Nowdays, the use of the internal thoracic artery and vein as recipient vessels in breast reconstruction is preferred all over the world. Although exposure of the recipient vessels requires resection of the costal cartilage and is therefore somewhat technically challenging, this is overweighted by the numerous benefits of this technique as regard to an excellent perfusion pressure and better ability to position the flap on the chest wall with superior reconstruction outcome.[3]

Case Report

A 35 years old female patient presented to the surgical oncology department, Mansoura Oncology Centre with right breast mass. Local examination showed right Retroareolar painless breast mass with nipple retraction and enlarged mobile right axillary lymph nodes. Mamography was performed and showed right breast retroareolar malignant mass with malignant axillary lymph nodes. Metastatic work up was performed which proved to be free.

Excisional biopsy under general anaethesia was performed and concluded invasive duct Carcinoma.

We performed skin sparing mastectomy (SSM) and axillary

clearance with preparation of the right internal mammary as a recipient vesseles, figure (1).

We planned reconstruction by free transverse rectus abdominis myocutaneous (TRAM) flap. The incision was outlined the day prior to the operation with markings of the inferior epigastric artery perforators utilizing the C/T angiography, figure (2).

The upper border of the incision extended medially from both anterior superior iliac spines(ASIS) to just above the umbilicus while the lower border passed between both ASIS through the lower abdominal crease, figure (2).

Skin and subcutaneous fatty tissue are harvested at the level of the aponeurosis from above downwards separating the umbilicus by avelar technique then elevation of the upper abdominal flap was performed till the costal margins to assess tension free closure of the abdomen prior to the lower incision of the flap.

The flap harvest continued from lateral to medial till identification of the marked perforators then acuff of muscle was taken around the perforators sparing the lateral part of the rectus abdominis muscle (muscle sparing TRAM), figure(3).

The deep inferior epigasteric vessels are dissected till the origin from the external iliac artery to harvest the desired length of the pedicle. figure(4).

Once the recipient vessels are ready for anastomosis, the pedicle is separated. Then the flap is taken to the recipient site. At that time 5000 unite heparin sulphate is injected IV. Trimming of the adventitia of the vascular pedicle was then performed. We started by temporary fixation of the flap to the surrounding breast flaps to ensure stability. Microvascular anastmosis is then done using 5.5 X magnification (Keeler surgical loop) and suturing using Prolene 7/0., figure (5). After completion of vascular anastmosis, the flap is fixed superiorly and medially to pectoralis major muscle by vicryl 2/0.

Abdominal wall was repaired by direct fascial closure without

mesh, figure (6).

The patient was kept in the ICU unit at Oncology Centre-Mansoura University for two days. Monitoring of the flap was performed using Doppler on the marked site of perforators. Monitoring was done every 2 hours for the first 2 days then every 4 hours for the next 3 days. Patient is put on heparin sulphate infusion 5000 units/12 hours for five days.

Patient is discharged ten days postoperative with imaging by C/T angiography to ensure vascular patency with good aesthetic result, figure (7&8).

Nipple areola complex reconstruction was performed two monthes postoperative using C/V flap technique, figure (9).

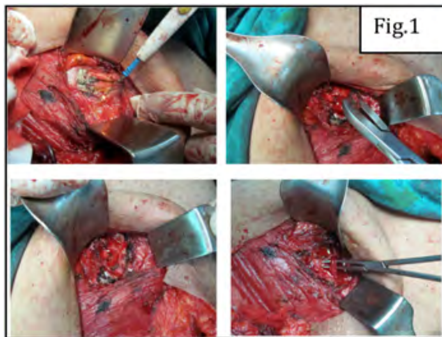

Fig. 1: Exposure of the internal mammary vessels.

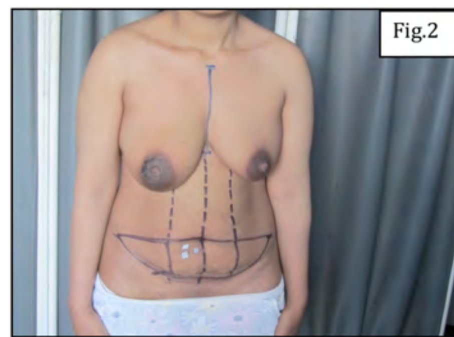

Fig. 2: Preoperative markings showing the perforators of the deep inferior epigasteric artery.

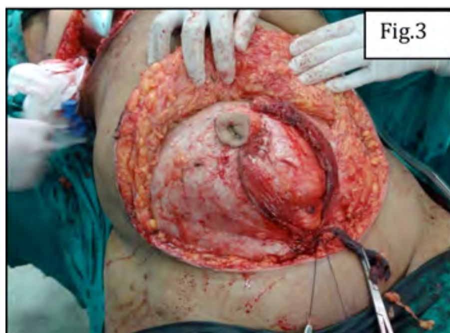

Fig. 3: Muscle sparing harvest, sparing the lateral part of the rectus abdominis.

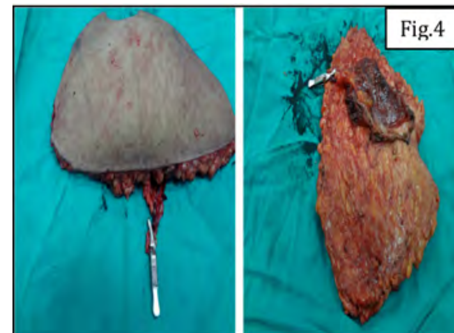

Fig. 4: Free TRAM flap harvested with its vascular pedicle.

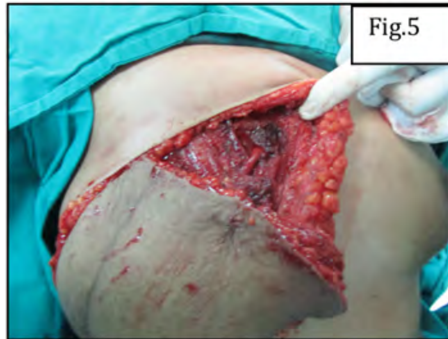

Fig. 5: Micro-vascular anastomosis with the internal mammary vessels.

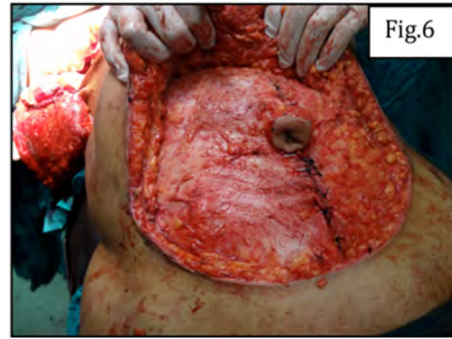

Fig. 6: Direct fascial closure of the abdomen.

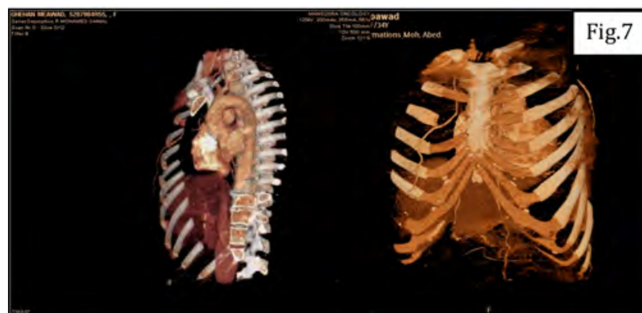

Fig. 7: Post operative C/T angiography showing patency of the anastomosis.

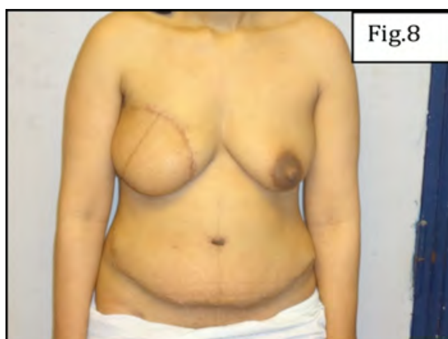

Fig. 8: Postoperative view of the reconstructed breast.

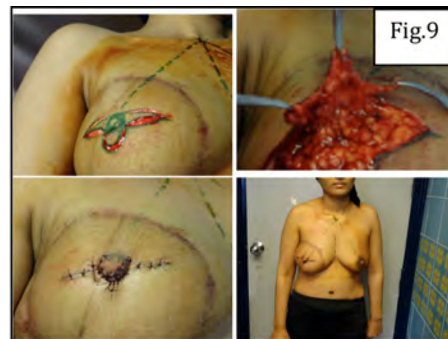

Fig. 9: C/Vflap for reconstruction of the nipple areola complex.

Discussion

In 1982, Hartrampf[4] and his colleagues introduced the pedicled TRAM flap with its greater ability to achieve a natural appearing, ptotic breast with secondary aesthetic improvements in the donor site. This flap quickly supplanted the latissimus as the first choice for pedicled autologous breast reconstruction. In 1989, Grotting[5] introduced modification on "free abdominoplasty flap" described by Holmstrom[6] in 1979, citing a better skin island blood supply, easier flap inset, improved contour in the absence of a tunneled pedicle, and decreased abdominal donor-site morbidity.

Free TRAM flap design provides a more direct and better blood supply to the TRAM skin island, which is one of the main advantages of the free TRAM flap over the pedicled flap. Relative indications for this procedure are similar to those for the pedicled TRAM flap. Unlike the pedicled TRAM flap, however, this technique can be used when the superior epigastric artery has been divided (e.g., in a patient who has had a previous open cholecystectomy.[7]

We presented this case of free TRAM flap for immediate breast reconstruction after skin sparing mastectomy, to show that it's a feasible and reliable technique in breast reconstruction with a very good outcome.

It was observed that microsurgical techniques alone appear to show better flap perfusion and less frequent herniation and bulging in patients in whom free TRAM flaps were used than in patients who underwent flap transfer using conventional bipedicled or augmented techniques[8].

In our presented case we did direct facial closure for donor site without mesh, which resulted in a good wound healing and without postoperative hernia.

Conclusion

We concluded that free TRAM is a feasible technique for breast reconstruction in a tertiary referral and teaching center (Oncology Centre-Mansoura University). We'll continue to offer this technique for patients with breast cancer willing breast reconstruction. Enhanced attention to patient se-

lection, preparation of patients for surgery, fluid balance, and coordination between experienced members of the microvascular team should increase effectiveness and safety even further.

We believe that there is much needs to be done in the future in relation to the correct flap choice and better functional recovery.

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FREE TRANSVERSE RECTUS ABDOMINIS MYOCUTANEOUS (TRAM) FLAP FOR IMMEDIATE BREAST RECONSTRUCTION FOLLOWING MASTECTOMY, A CASE REPORT

Emad Eldeen Hamed Mottawee M.Sc,
Samir Abd El Lateif Zidane MD, Adel Taha Denewer MD
and Ashraf Abd El Fattah Nasr khater MD

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COMPARATIVE STUDY OF DIFFERENT ANESTHETIC MODALITIES IN PEDIATRIC STRABISMUS SURGERY

Gamal Zakaria El Morsy MD, Enas Aly Abd El Motlb MD, Mostafa Mohammed Ali Said MD, Olfat Mostafa Mostafa Ismail MD and Fatma Lahloub M.Sc

Surgical Oncology Unit, Oncology Centre-Mansoura University, Egypt

Abstract

Objectives: *Occulocardiac reflex, postoperative vomiting and pain occurring during strabismus surgery require the use of effective anesthetic and analgesic planes. This study aims at comparing sevoflurane anesthesia with TIVA using propofol of ketamine infusion concerning hemodynamic stability, oculocardiac reflex, postoperative vomiting, emergence agitation and postoperative pain and analgesia.*

Methods: *60 ASA I & II patients with age 1-10 years submitted for elective correction of strabismus were randomly classified into 3 groups. All patients received fentanyl 1μg.Kg*$^{-1}$ *slowly I.V. Group K: induced with ketamine 1.0 mg.Kg*$^{-1}$ *I.V. and maintained at a rate of 1-3 mg.Kg*$^{-1}$*.h*$^{-1}$*. Group P: induced with Propofol 3 mg.Kg*$^{-1}$ *I.V. and maintained at a rate of 5-7 mg.Kg*$^{-1}$*.h*$^{-1}$*. Group S: induced with sevoflurane 8% in 100% oxygen and maintained with sevoflurane 3% in 100% oxygen.*

The lowest heart rate and OCR during traction were recorded. Postoperative vomiting was recorded using the Numeric Rank Score. Emergence agitation behavior score was used. FLACC observational pain score scale was used.

Results: *Oculocardiac reflex was was the highest in propofol group-followed by ketamine group and sevoflurane group. Postoperative vomiting (POV) score was significant among the tested groups. Emergence agitation (EA) score was significant among tested groups. FLACC score was significantly low in ketamine group when compared to sevoflurane and propofol group 1 hour after of recovery.*

Conclusion: *Sevoflurane induction maintenance anesthesia provides stable hemodynamic andlower inciedence OCR. POV wears out within one hour postoperatively. TIVA usind ketamine and propofol is effective and safe.*

Introduction

Pediatric strabismus surgery present a number of challenges for the anesthesiologist; mainly oculo-cardiac reflex (OCR) and postoperative vomiting (POV).[1] During strabismus surgery, incidence of OCR ranges from 14-90% according to type of anesthetic agents, the use of anticholinergic and the defining limit of OCR.[2-7] POV is one of the most common and serious complication following general anesthesia. Manipulations of the eye or pain are assumed to cause POV. Consequences of POV include dehydration and electrolyte imbalance, subconjunctival hemorrhage, unplanned and prolonged hospital admission and decrease patient satisfaction.[8] Antiemetics are used to control POV. In addition to its limited value; arrhythmias following the administration of anti-emetics limit its use.[9] Emergence agitation and pain are serious problems following anesthesia in children and require planning and care.[10]

Sevoflurane is the cornerstone of pediatric anesthesia and gaining wide and universal acceptance among anesthesiologists regarding hemodynamic stability and tolerance. However; emergence agitation and postoperative vomiting are prominent limitations.[11] Propofol TIVA is an anesthetic regimen for pediatric population that is devoid of sevoflurane side effects[12], though it reportedly increases OCR. Ketamine TIVA, although assumed to decrease oculocardiac reflex and maintain stable hemodynamic in children, is scarce in literature and was not compared to sevoflurane induction maintenance anesthesia. This study aims at comparing sevoflurane induction maintenance anesthesia with total intravenous anesthesia using propofol of ketamine infusion concerning hemodynamic stability, oculocardiac reflex, postoperative vomiting, emergence agitation and postoperative pain and analgesia.

Patients and Methods

After obtaining approval of the local ethical committee; this controlled single blinded randomized study was conducted on 60 ASA I & II patients with age ranging from 1-10 years who were submitted for elective correction of strabismus in Mansoura University

Ophthalmology Center. Guardians of all patients singed written informed consent before participating in this study. Randomization was done by closed envelop method. All cases were done by same surgeon.

Patients were classified into 3 groups after routine laboratory investigation and insertion of intravenous cannula after application of (EMLA) cream; 20 patients each; as follows: group S (n=20) [sevoflurane group], group K (n=20) [ketamine group], and group P (n=20) [propofol group].

After pre-oxygenation, at a rate of 6 L/min, all patients received fentanyl 1μgKg^{-1} slowly I.V. thereafter; anesthesia was induced and maintained as follows: group S; anesthesia was induced with sevoflurane 8% in 100% oxygen facemask and maintained with sevoflurane 3% in 100% oxygen. Group K; anesthesia was induced with ketamine 1.0mgKg^{-1} I.V. and maintained at a rate of 1-3mgKg^{-1}h^{-1}. Group P; anesthesia was induced with propofol 3mgKg^{-1} I.V. and maintained at a rate of 5-7 mgKg^{-1}h^{-1}. Tracheal intubation was facilitated by rocuronium 0.6mgkg^{-1} I.V. Ventilation was done using pressure controlled ventilation (PCV) mode maintain SpO_2 more than 95% and $EtCO_2$ 35-40 mmHg.

Upon completing surgery, anesthetics were discontinued. When spontaneous breathing and gag reflex returned the endotracheal tube was removed and the patient was transferred to recovery room. Neostigmine 0.05mgkg^{-1} and atropine 0.01mgkg^{-1} were used to reverse neuromuscular blocker.

Traction time, recovery time (the time elapsed between cessation of anesthetics till the time where verbal communication was regained) and duration of surgery were reported.

Heart rate [HR] and arterial blood pressure [MBP] were recorded 5minutes after induction of anesthesia [R I], at conjunctival incision [R II], lowest heart rate during traction [R III] and at the end of surgery [R IV].

During traction of extra-ocular muscles, The lowest heart rate

was recorded; OCR was identified if HR decreases more than 20% of basal HR. Occurrence of OCR was recorded in the following manner 1=OCR and 2= no OCR. The surgeon was asked to release traction. If bradycardia persisted; atropine 0.05mgKg^{-1} was given intravenously and recorded as follows; 1= required atropine and 2 = not required atropine. Any arrhythmia was recorded.

POV was recorded 10 min after extubation, after one hour and after 6 hours interval using the Numeric Rank Score (NRS) 0, 1 and 2 referring to no vomiting, vomiting once and vomiting twice or more respectively.(10,13,14) POV was treated with Granisteron HCl 10 μg.Kg^{-1} given intravenously when POV score was ≥ 1 and recorded.(15)

Emergence agitation EA was recorded at 10 min and 1 hour after arrival to recovery room. An emergence agitation score 1, 2, 3, 4, and 5 referred to sleeping, awake and calm, crying but consolable , inconsolable crying, and agitation and thrashing respectively.(16)

Evaluation of postoperative pain using FLACC scale is accepted for this age group (table 1), and was done 10 min after arrival to recovery room, one hour and 6 hours intervals. Ketorlac 0.5mgkg-1 was given intravenously if FLACC was ≥ 4and recorded.(17)

Statistical Analysis

Power analysis using G*Power 3.01 was done comparing mean ± SD of heart rate 5minutes after induction of anesthesia [R I] and lowest heart rate during traction [R III]. Effect sample size was 12 when α=0.05, actual power=0.97 and effect size d=2.215.

Statistical analysis was done using SPSS program version 18.0. Data was expressed as number, percent, median, range and mean±SD as appropriate. Test of normalization was done using Shapiro-Wilk W test of normality. Changes in quantitative values among and within tested groups were done by one way analysis of variance ANOVA followed by LSD post hoc test or repeated measures ANOVA with Bonferroni adjustment. Changes in qualitative data were compared using Chi

square test followed by Kruskal Wallis or by Kruskal Wallis followed by Mann whitney test. Correlation was done using Spearman correlation test. p value ≤0.05 was considered statistically significant.

Results

Demographic data in table 2 showed no significant differences were found between the 3 groups considering age, gender and body weight. Recovery time was significantly longer in ketamine group when compared to both sevoflurane (p=0.00) or propofol (p=0.00). Traction time was significantly short in sevoflurane group when compared to ketamine (p=0.002) or propofol (p=0,029). Duration of surgery showed no significant difference between study groups.

Changes in HR and MBP [RI], [RII], [RIII] and [RIV] among study groups are shown in figure 1 and 2. There was no significant difference as regard HR (p=0.484) while MBP showed significant changes (p=0.000) among studied groups; sevoflurane group were significantly less than both ketamine and propofol groups and propofol group was significantly less when compared to ketamine group.

Incidence of dysrhythmias and requiring atropine were insignificant among the three groups, in contrast, incidence of OCR among the tested groups was significantly low in sevoflurane group compared to propofol group (p=0.041) and of no significance when compared to ketamine group (p=0.190) or when ketamine group was compared to propofol group (p=0.435) (Table 3).

Incidence of OCR (table 3) was highest in propofol group; 85% (p=0.002) followed by ketamine group; 75% (p=0.025) and sevoflurane group 55% (p=0.655).

POV score within the first 10 minutes of recovery from anesthesia was significant among the studied groups compared to POV score after 1h and after 6 hours as shown in table 4. (p=0.026, p= 0.067, p=0.601 respectively). Requiring antiemetic treatment was also significant among tested groups (p=0.016).

Sevoflurane group showed significantly more POV when com-

pared to propofol group after the first 10 min and 1 hour of recovery and required significantly more antiemetic treatment than propofol group. Ketamine group was significant compared to propofol group concerning antiemetic treatment and POV after 1 hour of recovery from anesthesia.

Emergence agitation (EA) score was significant among tested groups at 10 minutes of recovery from anesthesia (p=0.000) but was insignificant after 1 hour of recovery from anesthesia (p=089) (table 4). Sevoflurane group revealed significantly high EA score 10 min and 1h after recovery from anesthesia compared to propofol group. Ketamine group showed significantly lower EA score compared to sevoflurane group 10 minutes after recovery; however there was no significant difference between both groups after 1hour of recovery. Ketamine group revealed significantly high EA score compared to propofol group 10min after recovery but no significant difference after 1hr of recovery.

FLACC was significant among groups 10 minutes and 1hour after of recovery from anesthesia (p=0.013 and p=0.008 respectively) (table 4). Requiring treatment for pain was highly significant among tested groups (p=0.002). FLACC score was significantly low in ketamine group compared to sevoflurane group 10 minutes after recovery from anesthesia and both sevoflurane and propofol group 1 hour after of recovery from anesthesia. There was no significance between sevoflurane group and ketamine group or propofol group 6 hours of recovery from anesthesia. Requiring treatment for pain was significantly high in sevoflurane and propofol groups when compared to ketamine group.

Weak correlation was found between OCR and traction time 0.012. POV and traction time revealed moderate correlation 0.301 as did POV and duration of anesthesia 0.374. There was no correlation between OCR and POV.

Table (1): Details of FLACC scale.[18]

Behavioral categories	Score	Interpretation
Face	0	No particular expression or smile
	1	Occasional grimace/frown; withdrawn or disinterested; appears sad or worried
	2	Consistent grimace or frown; frequent/constant quivering chin, clenched jaw; distressed-looking face
Legs	0	Normal position or relaxed; usual tone & motion to limbs
	1	Uneasy, restless, tense; occasional tremors
	2	Kicking, or legs drawn up; marked increase in spasticity, constant tremors or jerking
Activity	0	Lying quietly, normal position, moves easily; Regular, rhythmic respirations
	1	Squirming, shifting back and forth, tense or guarded movements; mildly agitated (e.g. head back and forth, aggression); shallow, splinting respirations, intermittent sighs
	2	Arched, rigid or jerking; severe agitation; head banging; shivering (not rigors); breath holding, gasping or sharp intake of breaths, severe splinting
Cry	0	No cry/verbalization
	1	Moans or whimpers; occasional complaint; occasional verbal outburst or grunt
	2	Crying steadily, screams or sobs, frequent complaints; repeated outbursts, constant grunting
Consolability	0	Content and relaxed
	1	Reassured by occasional touching, hugging or being talked to. Distractible.
	2	Difficult to console or comfort; pushing away caregiver, resisting care or comfort measures

Table (2): Demographic data, body weight (kg), traction time (TT) (min), recovery time (RT) (min), and duration of anesthesia (D) (min) in the studied groups. Group S (sevoflurane) n=20, group K (ketamine) n=20 and group P (propofol) n=20. Data are expressed as mean ± SD or number & (%).

		Group S	**Group K**	**Group P**	***p***	***r***
Age (years)		4.84 ± 2.24	4.70 ± 2.55	4.49 ± 2.34	0.897	
Gender (no.)	Male	8 (40%)	10 (50%)	12 (60%)	0.485	
	Female	12 (60%)	10 (50%)	8 (40%)		
Body weight (kg)		18.70 ± 4.67	19.80 ± 6.02	17.85 ± 5.44	0.524	
TT (min)		27.05 ± 19.41*†	11.55 ± 9.41	16.4 ± 14.55	0.006	0.301
T (min)		4.5 ± 1.76*	8.9 ± 3.78‡	5.15± 1.35	0.000	
D (min)		67.2 ± 23.06	57.15 ± 22.28	62.25 ± 20.77	0.080	0.374

* $p \leq 0.05$ is significant when group S is compared to group K.
† $p \leq 0.05$ is significant when group S is compared to group P .
‡ $p \leq 0.05$ is significant when group K is compare to group P.
r value of correlation between traction time and POV, duration of surgery and POV.

Table (3): OCR (no.), atropine required (no.) and incidence of dysrhythmia in the studied groups. Group S (sevoflurane) n=20, group K (ketamine) n=20 and group P (propofol) n=20. Data are expressed in number n (%).

	Group S	Group K	Group P	*p*	*r*
OCR (no.)	11 (55%)†	15 (75%)	17 (85%)	0.104	0.012
OCR significance within each group (*p*)	0.655	0.025[§]	0.002[§]		
Atropine required (no.)	4 (20%)	2 (10%)	4 (20%)	0.624	
Dysrhythmia (no.)	5 (25%)	3 (15%)	2 (10%)	0.438	

† $p \leq 0.05$ is significant when group S is compared to group P .
[§] *p* value is significant when $p \leq 0.05$ for OCR within each group
r value represents correlation between OCR and traction time.

Table (4): POV, EA, FlACC scores and requiring treatment for POV and pain in studied groups. Group S (sevoflurane) n=20, group K (ketamine) n=20 and group P (propofol) n=20. Data are expressed as median (range) or number n (%).

	Group S	Group K	Group P	*p*
POV 10 min (score)	1 (0-1) †	1 (0-1)	1 (0-1)	0.026
POV 1 h (score)	1 (0-1) †	1 (0-1) ‡	0(0)	0.067
POV 6 h (score)	1 (0-1)	1 (0-1)	0(0)	0.601
POV require ttt (no.)	9(45%) †	7 (35%) ‡	1 (5%)	0.016
EA 10 min (score)	3.5 (2-5) * †	1 (0-2) ‡	2.5 (1-4)	0.000
EA 1 h (score)	2 (1-3)	2 (1-3)	2.5 (2-4)	0.089
FLACC 10 min (score)	6 (3-9) *	3 (1-5)	3 (1-5)	0.013
FLACC 1 h (score)	3 (2-4) *	2 (1-3) ‡	2.5 (1-5)	0.008
FLACC 6 h (score)	2 (0-4)	2 (1-3)	2 (0-5)	0.505
Pain require ttt (no.)	13 (65%)*	2 (10%) ‡	8 (40%)	0.002

* $p \leq 0.05$ is significant when group S is compared to group K.
† $p \leq 0.05$ is significant when group S is compared to group P .
‡ $p \leq 0.05$ is significant when group K is compare to group P.

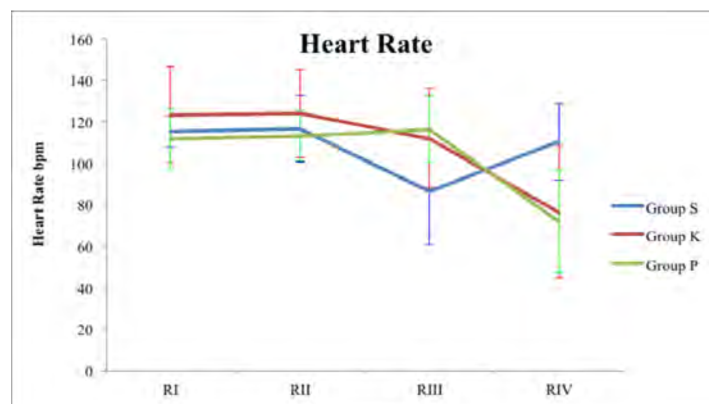

Fig. 1: HR changes recorded as **[R I], [R II], [R III]** and **[R IV]** among studied groups. Data are expressed as mean ± SD.

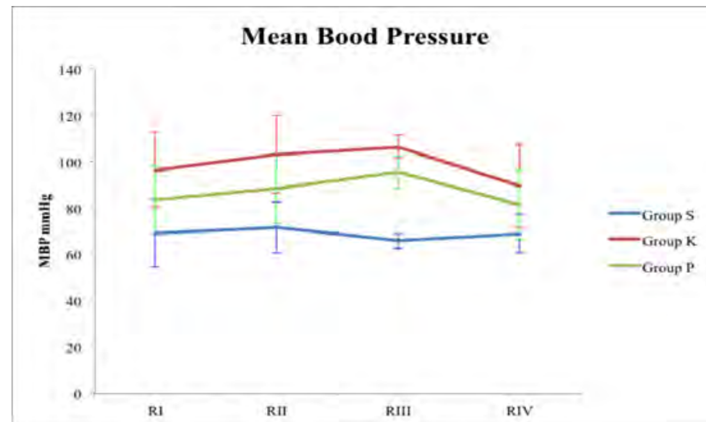

Fig. 2: MBP changes recorded **[R I], [R II], [R III]** and **[R IV]**. Data are expressed as mean ± SD.
* $p \leq 0.05$ is significant when group S is compared to group K.
† $p \leq 0.05$ is significant when group S is compared to group P .
‡ $p \leq 0.05$ is significant when group P is compare to group K.

Discussion

The main findings of this study concluded that sevoflurane anesthesia was associated with lowest incidence of OCR followed by ketamine then propofol. Atropine required and incidence of arrhythmias were insignificant between study groups. Heart rate changes were not significant among study groups. Mean arterial blood pressure readings were lower in sevoflurane group compared to ketamine and propofol groups. Pharmacodynamic properties of sevoflurane, ketamine and propofol explain these findings.

In contrast of our study, Hahnenkamp et al.[5] presented a significantly lower incidence of OCR when ketamine was used for maintenance of anesthesia (21%) when compared to sevoflurane (90%) or propofol (100%). However, all groups were induced with propofol which by its own increases the possibility of OCR, thus the effect of ketamine and sevoflurane is obscured. Alfentanil was added only to propofol group, knowing that short acting opioid increases incidence of OCR.[3]

Choi et al.[2] concluded that a single bolus induction dose of ketamine decreases the incidence of OCR when compared to single bo-

lus induction dose of propofol which is similar to our results. Although Schaller[19] questioned the use of ketamine infusion for prevention of OCR with regards of its experimental hazards on developing brains and neuronal apoptosis. Further studies are still to be designed to prove its effect on developing human brains.

In accordance with this study, Mizrak et al.[14] compared TIVA with ketamine or propofol and concluded that incidence of OCR was lower in ketamine group than propofol group. However, recovery time was shorter in ketamine compared with propofol group. The author offered no explanation for his finding.

In accordance with this study, several authors found no significant difference concerning atropine requirements and arrhythmias.[2-5,10,14,20-26] They concluded that propofol is accompanied with lower heart rate thus the possibility to use atropine is higher.[3,5,22,27]

Gurken et al.[22] reported higher incidence of POV in sevoflurane group compared with propfol group; although not significant; similar to our study. Chung et al.[28] compared sevoflurane in N2O 66% with propofol remifentanyl TIVA. POV was higher in sevoflurane compared to propofol group with no significance. Sevoflurane group experienced higher incidence of POV during first hour compared to propofol group.

In contrast to this study, Mizrak et al.[14] reported a lower incidence of POV in ketamine group (3%) compared to propofol group (12%). The author related POV to total dose of ketamine than $7 mgkg^{-1}$.

Few trials have tested the association between OCR and POV in pediatric strabismus surgery with uncertain results.[4,29-31] Karanovic et al. and Welters et al.[4,31] found no correlation between POV and OCR similarly, although both used inhaled anesthesia; halothane; which increases the occurrence of OCR and POV.

Allen et al.[29] found significant association between OCR and PONV though he used propofol for

induction which both increases incidence of OCR and decreases POV. Deb et al.[32] concluded that the occurrence of OCR and POV were related. Chung et al.[21] also concluded an increase in occurrence of both OCR and POV without measuring degree of correlation, thus not conclusive.

Kanaya et at.[33] proved that EA is less frequent when propofol is compared to sevoflurane. Nakayma et al.[34] concluded that propofol TIVA reduced EA in children in comparison with sevoflurane. Pieters et al.[35] on the other hand found no significant difference between propofol and sevoflurane anesthesia when fentanyl was added as an analgesic. Literature comparing EA in children when ketamine TIVA is used in comparison with other anesthetics are lacking. Most available literatures demonstrate the effect of inducing anesthesia with ketamine or adding a small dose of ketamine on EA with sevoflurane. Mizrak et al.[14] compared TIVA with either propofol or ketamine and administrating 1.0µgkg^{-1} fentanyl at induction. Higher EA score in ketamine was reported when compared to propofol though not significant. Treston et al.[36] showed that ketamine used for emergency department pediatric procedural sedation caused little if any emergence phenomenon; similar to the present study, and that its recovery was rarely associated with delirium.

Choi et al.[2] demonstrated that a single dose of ketamine at induction led to reduced analgesic requirements in postoperative recovery area when compared to propofol. However, he did not clarify how pain was evaluated. Mizrak et al.[14] similarly reported lower pain scores when ketamine TIVA was compared to propofol TIVA 10 minutes and one hour after recovery. Fentanyl was administered at induction to both groups. However facial pain score scale used in his study is a subjective score and thereby inappropriate for his age group (1-10 years).[37]

Using heart rate as a sign for OCR is a limitation in the present study. Non invasive blood pressure monitoring use was a limiting factor; however invasive arterial

blood pressure monitoring is not justified for its risks in short procedures especially when pediatric patients are concerned. Sevoflurane induction in children older than 7 years was demanding. Rare technical difficulties were faced to assure a maintained intravenous infusion flow.

In conclusion, Sevoflurane induction maintenance anesthesia provides stable hemodynamic, lower incidence OCR and thereby anticholenergic requirements are minimized. Although it increases the incidence of POV, this effect wears out within one hour postoperatively and is readily avoidable. Both ketamine TIVA and propofol TIVA are equally effective and safe. Although propofol TIVA increase incidence of OCR, it did not prove to be significant. Ketamine provides good postoperative analgesia, pleasant and quiet recovery inspite of longer recovery time. It is well tolerated by pediatric patients. Further studies are need to to compare sevoflurane induction maintenance anesthesia with ketamine TIVA in short surgical procedure in pediatric patientsand to shed more light of benefits and hazards of ketamine total intravenous anesthesia with relevance to its pharmacodynamic and pharmacokinetic properties to develop an appropriate target controlled infusion regimens in children as those developed for propofol for effective anesthesia.

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COMPARATIVE STUDY OF DIFFERENT ANESTHETIC MODALITIES IN PEDIATRIC STRABISMUS SURGERY

Gamal Zakaria El Morsy MD, Enas Aly Abd El Motlb MD, Mostafa Mohammed Ali Said MD, Olfat Mostafa Mostafa Ismail MD and Fatma Lahloub M.Sc

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STRUCTURAL AND LABORATORY CHANGES IN THE AORTA OF ADULT FEMALE ALBINO RATS IN CASES OF EXPERIMENTAL HIGH FAT DIET AND UNDER EFFECT OF SOME MEDICINAL PLANTS

Ezz-Eldin E. Abdalla MD

Histology Department, Faculty of Medicine, Al-Azhar University (Damietta)

Abstract

Background: *This study was designed to evaluate the effect of high fat diet on histological structure of the aorta and if medicinal plants have a protective role towards these changes or have an effect as hypolipidemic agents.*

Material and Methods: *Forty four adult female albino rats were used and divided into nine equal groups. The first group was considered as a control group. The second group was fed with high fat diet (25% fat and 2% cholesterol) for 3 weeks. Groups 3, 4 and 5 were similar to the second high fat diet group but treated with Oat, Fennel and Triphala respectively. The sixth group was also fed with high fat diet for 6 weeks. Groups 7, 8 and 9 were treated with same medicinal plants as groups 3, 4 and 5. Rats were exposed to histological study of the aorta sections and laboratory evaluation of lipid profile.*

Results: *The main changes in the tunica media were in the form of increased thickness, splitting of smooth muscles and vaculation. The changes in the endothelial cells were in the form of disfigurement, detachment, swelling, pyknotic nuclei, sub-endothelial swelling and appearance of foam cells. These changes appeared clearly in high fat diet group especially in group 6. With the use of plants, these changes improved in different degrees especially with oat as structure of the aorta tended to be normal. Also, oat has a potent hypolipidemic agent if compared with other plants.*

Conclusion: *Used plants have a role in the prevention or correction of the adverse effects of cases with high fat diet.*

Key words: *Albino rat, High fat diet, Medicinal plants.*

Introduction

High fat diet and hyperlipidemia represent an important social and clinical problem (Mori et al., 2010). High fat diet has negative consequences such as type II diabetes (Samaras and Campbell, 2000), dyslipidemia (Novelli et al., 2007) and cardiovascular disease (Diniz et al., 2008).

High fat diet induced hyperlipidemia, which is a heterogeneous disorder involving multiple etiologies. It is characterized by elevation in serum levels of free fatty acids, triglycerides (TG), total lipid (TL), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), a polipoprotein B and reduced serum high-density lipoprotein-cholesterol (HDL-C) concentration (Feng et al., 2011; Zhang et al., 2011).

Atherosclerosis is a syndrome affecting arterial blood vessels and defined as degenerative changes in the intima of medium and large sized arteries as a result of the accumulation of fatty materials such as cholesterol, triglyceride, complex carbohydrates, blood, blood products and cellular waste products, and accompanied by the formation of fibrous tissues and calcium deposition in the intima of blood vessels (Dogne et al., 2005; Cohn, 2008 and Gopichandchinta et al., 2009). Endothelial dysfunction, inflammation, fibrosis, and calcification of the arterial intima are the proposed mechanisms in response to retained low-density lipoprotein (LDL) molecules (Williams and Tabas, 1995). Another mechanism leading to reduction in the arterial elasticity is arteriosclerosis, associated with degradation of elastin, proliferation of collagen, and deposition of calcium in the media and adventitia in contrast to changes in the intima in atherosclerosis (Safar, 2007; Soljanlahti et al., 2008).

Hyperlipidemia promotes functional abnormalities and structural vascular wall injury with increased lipid peroxidation which is a major factor in the vascular damage (Napoli & Lerman 2001; Adaramoye et al., 2005 and Hu et al., 2006).

Antioxidant and cholesterol lowering activities of plants extracts or isolated components can be effectively utilized to reduce the development of obesity and associat-

ed vascular damage (Choudhary et al., 2005 and Chung et al., 2008).

The use of medicinal plants for health started from thousands of years and still a part of the medical practice in Egypt and other developed countries (Cosge et al., 2008). Herbal remedies or food supplements have increasingly become attractive alternatives to prevent or treat hyperlipidemia, especially for those with cholesterol at the borderline levels (Deng, 2009).

Many medicinal plants can be used as hyperlipidemic lowering agents in Egypt as Oat, Fennel and Triphala.

Oat (Avena sativa) is a light-green annual herb: contain high concentration of proteins, lipids, vitamins, antioxidants and minerals (Panfili et al., 2003 and Brindzová et al., 2008). It is distinct among other cereals by its multifunctional characteristics and nutritional profile (Butt et al., 2008). Advanced study in food and nutrition revealed that oat bran is a rich source of soluble fiber in the form of â-glucan and well-balanced carbohydrates, proteins, fats, vitamins (thiamine, pantothenic acid, niacin, folic acid and á-tocopherol) and minerals (calcium, phosphorus, potassium, magnesium, iron, zinc and copper), beside a wide spectrum of bioactive phenolic compounds contributed to its using as an alternative food for the human (Ötles & Cagindi, 2006 and Nijjar et al., 2010).

Fennel (Foeniculum vulgare) is commonly used in household remedy for various medicinal purposes (Sandhu and Heinrich, 2005). Fennel seed has been known as a medicinal and aromatic herb, commonly used to flavor breads, fishes, salads and cheeses (Kaur and Arora, 2010). Fennel has an antioxidant activity, hypocholerolaemic activity and has lipid lowering effect (Choi & Hwang., 2004; Liu et al., 2004; and Birdane et al., 2007).

Triphala meaning "three fruits", is made from fruits of three trees that grow throughout India and the Middle East (Terminalia chebula, Terminalia belerica and Emblica officinalis) It has a capacity to reduce blood lipid and inhibit hepatic cholesterol biosynthesis and increasing local bile acid ex-

cretion. (Khanna et al., 1996). Triphala is used to promote appetite and digestion, increase the number of red blood cells and aid in removal of undesirable fat in the body when dissolved in the mouth. Triphala is used to clear congestion and headaches; other claimed benefits include helping to maintain normal blood sugar levels, as well as improvement in skin tone. Triphala prevents aging, imparts immunity and improves mental faculties. it also helps to detoxify the liver and purify blood (Jagetia et al., 2004 and Pfundstein et al., 2010).

Material and Methods

A- Material:

Forty four adult female albino rats were used in this study and divided into nine equal groups. Average weight of rats was 150±20 gm.

The first group was considered as a control group and received normal diet.

The second group was fed with high fat diet by administration of 25% fat and 2% cholesterol for 3 weeks. Rats received no doses of medicinal plants.

The next 3 groups (Third, Fourth and fifth groups) fed with high fat diet and were treated with medicinal plants Oat, Fennel and Triphala respectively for 3 weeks.

The sixth group was fed with high fat diet by administration of 25% fat and 2% cholesterol for 6 weeks. Rats received no doses of medicinal plants.

The next 3 groups (Seventh, Eighth and Ninth groups) fed with high fat diet and were treated with medicinal plants Oat, Fennel and Triphala respectively for 6 weeks.

The plants were added with diet and oral intake by the following dose:

- Oat (Avena sativa): It was added to diet (20gm/100gm B.WT) (Sadiq et al., 2008).

- Fennel (Foeniculum vulgare) (10mg /100gm B.WT): The aqueous extract of F. vulgare was prepared by boiling 10gm of the plant seeds with 100 ml distilled water for 10 min. After cooling at room temperature, the extract was filtered and stored in the refrigerator and added to diet (Brinker, 1998).

- Triphala (Terminalia chebula) (25mg/100gm B.WT): The aqueous extract of triphala was prepared by boiling 10 gm of fruits of triphala with 40 ml distilled water for 10 min. After cooling at room temperature, the extract was filtered and stored in the refrigerator and added to diet (Juss, 1997).

B- Methods:

A) Microscopic study: Sections from the abdominal aorta were prepared from each group and stained by Hx & E, Mallory trichrome stain and Orcin stain (Drury and Wallington, 1980).

B) Laboratory tests: Collection of rats' serum was taken for estimating the following parameters:

1- Cholesterol level (Henry et al., 1974).

2- Triglyceride level (Fossati and Prencie, 1982).

3- HDL (high density lipoprotein) (Burstein, 1970).

4- LDL (low density lipoprotein) (Friedewald et al., 1972 & Puavilai and Laoragpongse, 2004).

C) Statistically evaluation: All of the statistical analyses were performed by SPSS version 12 (SPSS Inc., USA). Descriptive statistics were shown as arithmetic mean±SD. Differences between the control group, the high fat diet groups and treated groups were tested using T-test and P was significant at <0.05 (Altman, 1991).

Results

A) Histological results of aorta sections:

1- Control group (Fig 1-4) revealed normal structure of its three layers, tunica intima of smooth regular endothelia arrangement, normal sub-endothelial layer and inner elastic limiting membrane, tunica media composed of concentric layers of smooth muscle enclosing layers of elastic fibers and normal adventitia.

2- Second group (high fat diet group 3 weeks) (Fig 5-10) revealed disfigurement, detachment, swelling of endothelial cell with pyknotic nuclei and lost of its regularity and smooth appearance. Also, there were increased thickness, splitting of smooth muscle in the media and vacuolation due to fat deposition. Elastic fibers were separated and collagen fibers were proliferated.

3- Treated group 3weeks with Oat (group 3) (Fig 11, 14 and 15) revealed that the wall of the aorta returned to its normal arrangement with mild thickness in the smooth muscle in the media. Also collagen fibers were normally distributed with mild separation of elastic fibers.

4- Treated group 3weeks with Fennel (group 4) (Fig 12) revealed vacuolated thickened tunica media and detachment of endothelial cells.

5- Treated group 3weeks with Triphala (group 5) (Fig 13 revealed increased thickness, splitting of smooth muscle in the media with loss of regular arrangement of endothelial cells and smooth surface.

6- High fat diet group for 6 weeks (high fat diet group 6 weeks) (Fig 16-21) revealed desquamated detached swollen endothelial cells with pyknotic nuclei, sub-endothelial swelling and loss of its regularity and smooth appearance. Also there were marked increased thickness, splitting of smooth muscle in the media and vacuolation due to fat deposition. Elastic fibers were separated and destructed with proliferated collagen fibers.

7- Treated group 6weeks with Oat (group 7) (Fig 22, 25 and 26) revealed that the wall of the aorta returned to its normal arrangement with mild vacuolation and mild thickness in the smooth muscle in the media. Also collagen fibers were mild increased with mild separation of elastic fibers.

8- Treated group 6weeks with Fennel (group 8) (Fig 23) revealed vacuolated thickened media.

9- Treated group 6weeks with Triphala (group 9) (Fig 24) revealed vacuolation, increased thickness of the media and pyknotic nuclei of endothelial cells.

B)- Laboratory tests and Statistical analysis:

The different laboratory data performed in this study were tabulated and statistically analyzed in the table and histogram (1-5).

P- value compared to the control group (P>0.05: Non significant P<0.05: Significant P<0.01: Highly significant.

1- The level of cholesterol (mg/dl):

Table (1): The level of cholesterol in female albino rats after induction of high fat diet (hyperlipidemic) groups and treating with (Oat, Fennel, and Triphala) shows a significant (P-value<0.05) increase in total cholesterol between control and high fat diet (hyperlipidemic) groups (3weeks & 6weeks). Also shows a significant (P-value<0.05) decrease in total cholesterol between treated groups by Oat, Triphala and Fennel respectively and high fat diet (hyperlipidemic) groups.

Groups	Mean ± SD	P- value
Control (G1)	75.20 ± 6.53	
Hyperlipidemic 3 week (G2)	145.80± 6.30	< 0.05
T3 with Oat 3weeks (G3)	109.80 ±7.40	< 0.05
T4 with Fennel 3weeks (G4)	126 .00±3.39	< 0.05
T5 with Triphala 3weeks (G5)	118.80 ±4.15	< 0.05
Hyperlipidemic 6 week (G6)	167.80 ±5.63	< 0.05
T7 with Oat 6weeks (G7)	119.40±7.30	< 0.05
T8 with Fennel 6weeks (G8)	148.20±6.06	< 0.05
T9 with Triphala 6weeks (G9)	131.60±4.83	< 0.05

2- The level of triglycerides (mg/dl):

Table (2): The level of triglycerides in female albino rats after induction of high fat diet (hyperlipidemic) groups and treating with (Oat, Fennel, and Triphala) shows a significant (P- value<0.05) increase in triglycerides between control and high fat diet (hyperlipidemic)groups (3weeks & 6weeks). Also shows a significant (P-value< 0.05) decrease in triglycerides between treated groups by Oat, Triphala and Fennel respectively and high fat diet (hyperlipidemic) groups.

Groups	Mean ± SD	P- value
Control (G1)	85.20 ± 6.26	
Hyperlipidemic 3 week (G2)	153.60± 5.59	< 0.05
T3 with Oat 3weeks (G3)	121.80 ±5.89	< 0.05
T4 with Fennel 3weeks (G4)	134.00 ±6.20	< 0.05
T5 with Triphala 3weeks (G5)	126.80 ±5.12	< 0.05
Hyperlipidemic 6 week (G6)	175.20 ±3.96	< 0.05
T7 with Oat 6weeks (G7)	147.40±4.39	< 0.05
T8 with Fennel 6weeks (G8)	162.40±5.73	< 0.05
T9 with Triphala 6weeks (G9)	153.80 ± 4.76	< 0.05

3-The level of HDL-cholesterol (mg/dl):

Table (3): The level of HDL-cholesterol in female albino rats after induction of high fat diet (hyperlipidemic) groups and treating with (Oat, Fennel, and Triphala) shows a significant (P- value<0.05) decrease in HDL-cholesterol between control and high fat diet (hyperlipidemic) groups (3weeks & 6weeks). Also shows a significant (P-value< 0.05) increase in HDL-cholesterol between treated groups by Oat, Triphala and Fennel respectively and high fat diet (hyperlipidemic) groups.

Groups	Mean ± SD	P- value
Control (G1)	30.20 ± 1.92	
Hyperlipidemic 3 week (G2)	21.00± 2.74	< 0.05
T3 with Oat 3weeks (G3)	27.80 ±3.11	< 0.05
T4 with Fennel 3weeks (G4)	23.80 ±2.28	< 0.05
T5 with Triphala 3weeks (G5)	25.20 ±1.30	< 0.05
Hyperlipidemic 6 week (G6)	16.60 ±2.07	< 0.05
T7 with Oat 6weeks (G7)	25.80±2.59	< 0.05
T8 with Fennel 6weeks (G8)	19.80±2.59	< 0.05
T9 with Triphala 6weeks (G9)	23.80 ± 1.92	< 0.05

4- The level of LDL-cholesterol (mg/dl):

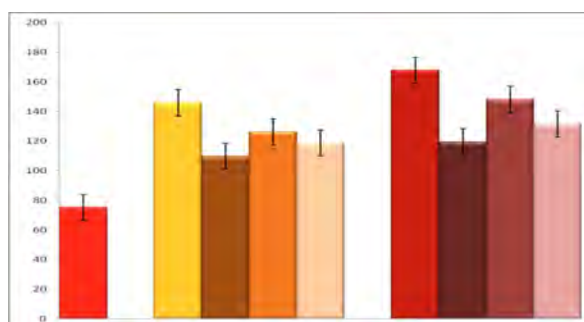
Table (4): The level of LDL-cholesterol in female albino rats after induction of high fat diet (hyperlipidemic) groups and treating with (Oat, Fennel, and Triphala) shows a significant (P- value< 0.05) increase in LDL-cholesterol between control and high fat diet (hyperlipidemic) groups (3weeks & 6weeks). Also shows a significant (P- value< 0.05) decrease in LDL-cholesterol between treated groups by Oat, Triphala and Fennel respectively and high fat diet (hyperlipidemic) groups.

Groups	Mean ± SD	P- value
Control (G1)	37.96 ± 1.57	
Hyperlipidemic 3 week (G2)	94.08± 4.21	< 0.05
T3 with Oat 3weeks (G3)	57.64± 2.26	< 0.05
T4 with Fennel 3weeks (G4)	75.4 ± 3.54	< 0.05
T5 with Triphala 3weeks (G5)	68.24 ± 5.45	< 0.05
Hyperlipidemic 6 week (G6)	116.1± 5.10	< 0.05
T7 with Oat 6weeks (G7)	64.12± 5.49	< 0.05
T8 with Fennel 6weeks (G8)	95.91± 4.46	< 0.05
T9 with Triphala 6weeks (G9)	77.03± 2.92	< 0.05

5- Thickness of tunica media (*mu*):

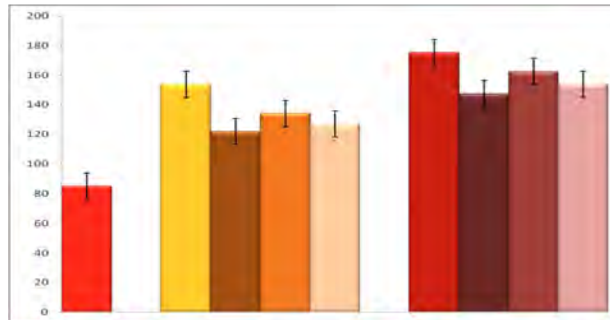
Table (5): The thickness of tunica media in female albino rats after induction of high fat diet (hyperlipidemic) groups and treating with (Oat, Fennel, and Triphala) shows a significant(P- value<0.05) increase in thickness of tunica media between control and high fat diet (hyperlipidemic) groups (3weeks & 6weeks). Also shows a significant (P- value<0.05) decrease in thickness of tunica media between treated groups by Oat, Fennel and Triphala respectively and high fat diet (hyperlipidemic) groups.

Groups	Mean ± SD	P- value
Control (G1)	65.75 ± 0.56	
Hyperlipidemic 3 week (G2)	70.90± 0.83	< 0.05
Treated with Oat 3weeks (G3)	66. 95 ±0.70	< 0.05
Treated with Fennel 3weeks (G4)	69.17 ±0.77	< 0.05
Treated with Triphala 3weeks (G5)	70.03 ±0.65	< 0.05
Hyperlipidemic 6 week (G6)	73.99 ±0.63	< 0.05
Treated with Oat 6weeks (G7)	70.66±0.90	< 0.05
Treated with Fennel 6weeks (G8)	72.08±0.65	< 0.05
Treated with Triphala 6weeks (G9)	72.61±0.53	< 0.05



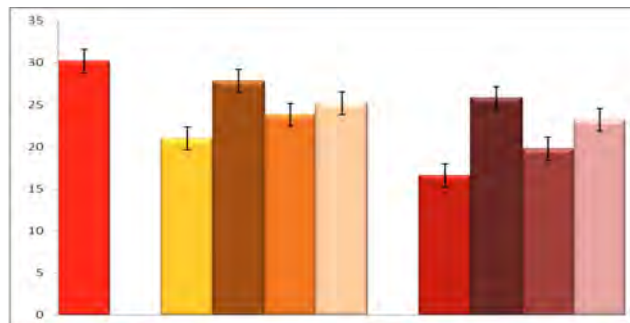
Histogram 1

Control · Hyperlipidemic 3w · T3 with oat 3w · T4 with Fennel 3w · T5 with Triphala 3w
Hyperlipidemic 6w · T7 with oat 6w · T8 with Fennel 6w · T9 with Triphala 6w



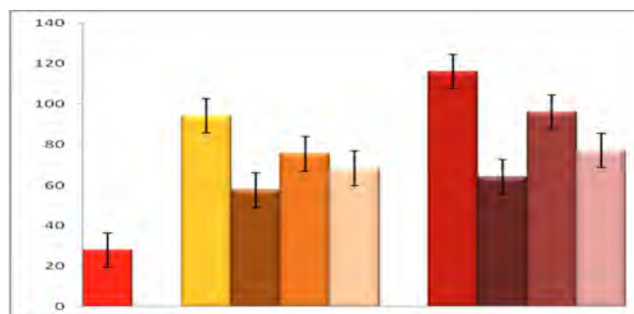
Histogram 2

Control | Hyperlipidemic 3w | T3 with oat 3w | T4 with Fennel 3w | T5 with Triphala 3w
Hyperlipidemic 6w | T7 with oat 6w | T8 with Fennel 6w | T9 with Triphala 6w



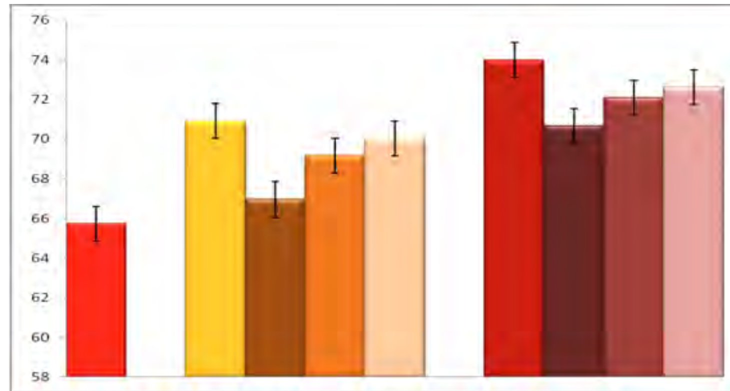
Histogram 3

Control | Hyperlipidemic 3w | T3 with oat 3w | T4 with Fennel 3w | T5 with Triphala 3w
Hyperlipidemic 6w | T7 with oat 6w | T8 with Fennel 6w | T9 with Triphala 6w



Histogram 4

Control | Hyperlipidemic 3w | T3 with oat 3w | T4 with Fennel 3w | T5 with Triphala 3w
Hyperlipidemic 6w | T7 with oat 6w | T8 with Fennel 6w | T9 with Triphala 6w



Histogram 5

Control | Hyperlipidemic 3w | T3 with oat 3w | T4 with Fennel 3w | T5 with Triphala 3w
Hyperlipidemic 6w | T7 with oat 6w | T8 with Fennel 6w | T9 with Triphala 6w

1- Control group

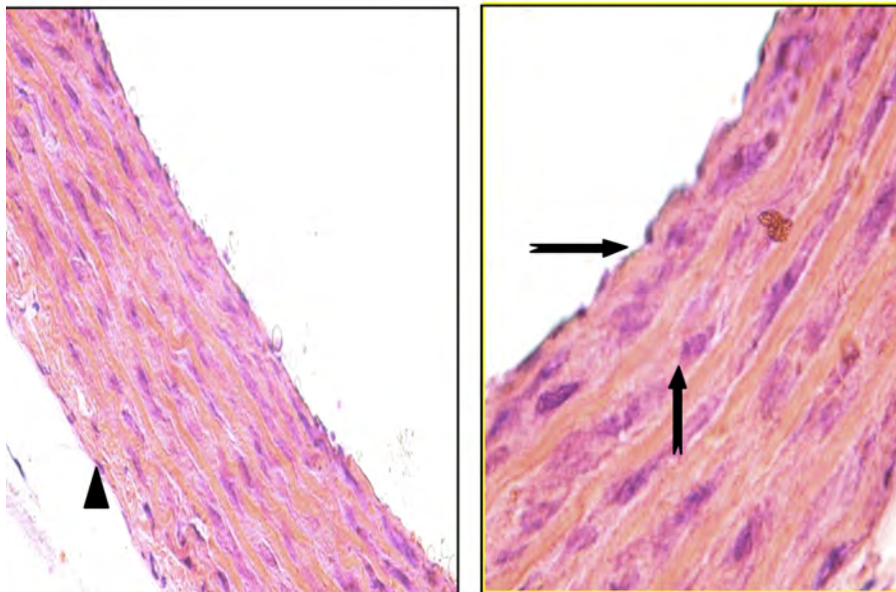


Fig. (1&2): Photomicrograph of control rat aorta showing normal appearance of three layers intima (Transverse arrow), media (Vertical arrow) and adventitia (arrow head) (Hx & E x400 and x1000 respectively).

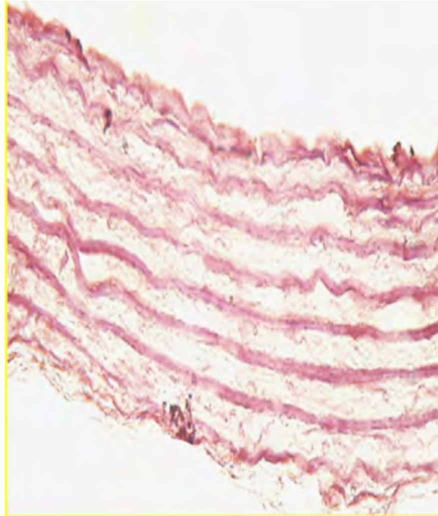


Fig. 3: Photomicrograph of control rat aorta showing normal distribution of elastic fibers (Orcin stain x400).

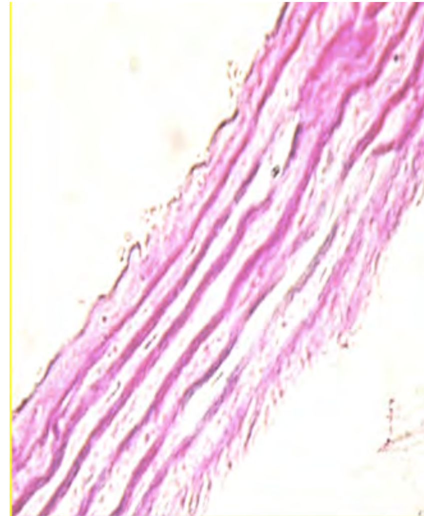


Fig. 4: Photomicrograph of control rat aorta showing normal distribution of collagen (Mallory trichrome stain x400).

2- High fat diet group 3 weeks

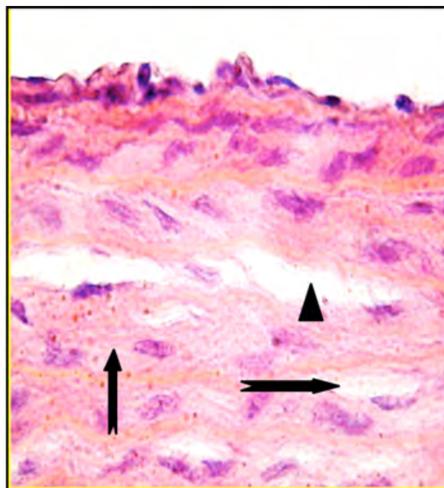


Fig. 5: Photomicrograph of second group high fat diet 3weeks rat aorta showing increased thickness (Vertical arrow), splitting of smooth muscle (Arrowhead) and vaculation (Transverse arrow) in the media (Hx &E x1000).

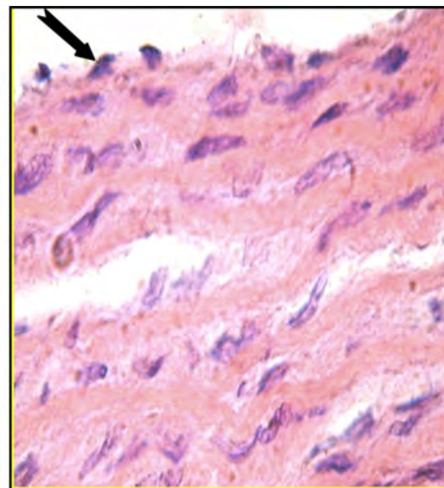


Fig. 6: Photomicrograph of second group high fat diet 3weeks rat aorta showing pyknotic nuclei of endothelial cells (Arrow) (Hx &E x1000).

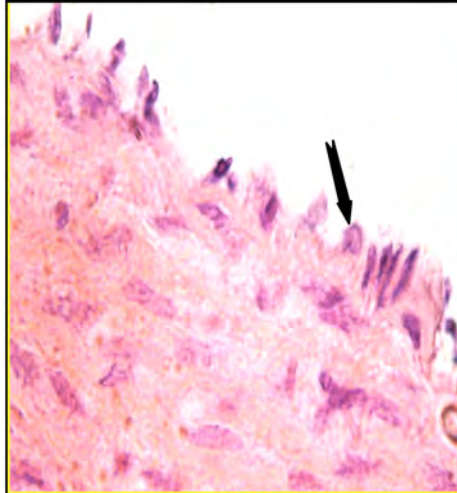


Fig. 7: Photomicrograph of second group high fat diet 3weeks rat aorta showing disfigurement and detachment of endothelial cells (Arrow) (Hx &E x1000).

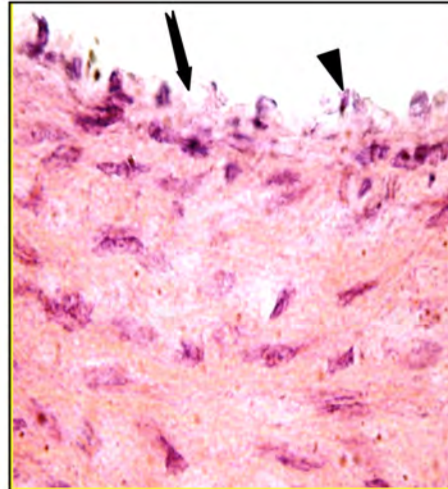


Fig. 8: Photomicrograph of second group high fat diet 3weeks rat aorta showing swelling of endothelial cell forming foam cell (Arrow) with loss of regular arrangement and smooth surface (Arrowhead) (Hx & E x1000).

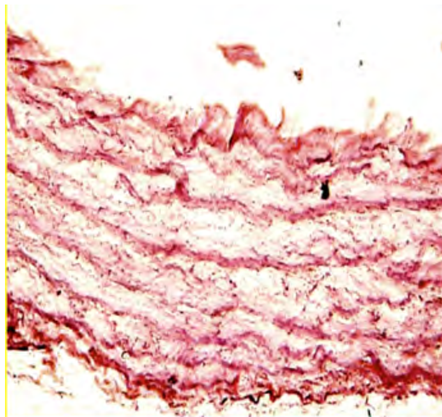


Fig. 9: Photomicrograph of second group high fat diet 3weeks rat aorta showing separation of elastic fibers (Orcin stain x400).

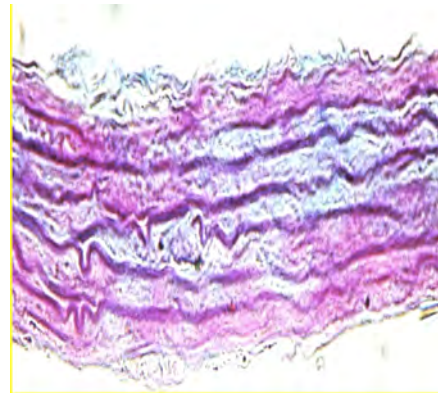


Fig.10: Photomicrograph of second group high fat diet 3weeks rat aorta showing increased and proliferation of collagen (Mallory trichrome stain x400).

3- Treated group 3weeks with Oat

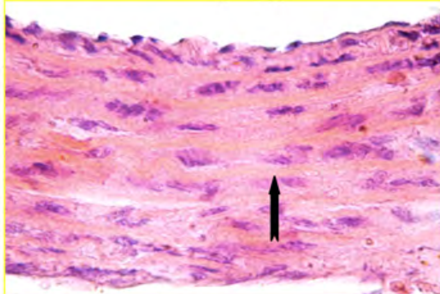


Fig.11: Photomicrograph of third group Oat treating 3weeks rat aorta showing normal arrangement of three layers with mild thickness in the media (Arrow) (Hx &E x1000).

4- Treated group 3weeks with Fennel

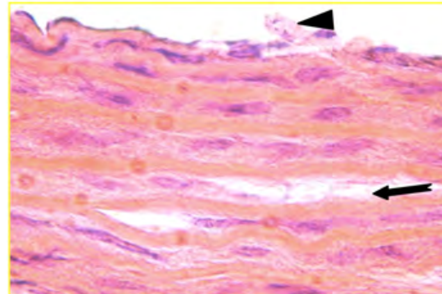


Fig.12: Photomicrograph of third group Fennel treating 3weeks rat aorta showing vaculation (Transverse arrow) in the media with detachment of endothelial cells (Arrowhead) (Hx &E x1000).

5- Treated group 3weeks with Triphala

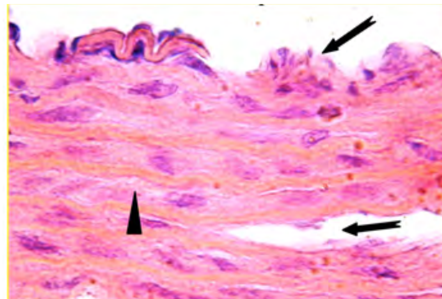


Fig.13: Photomicrograph of third group Triphala treating 3weeks rat aorta showing increased thickness (Arrowhead), splitting of smooth muscle (Transverse arrow) in the media with loss of regular arrangement of endothelial cells and smooth surface (Oblique arrow) (Hx & E x1000).

3- Treated group 3weeks with Oat

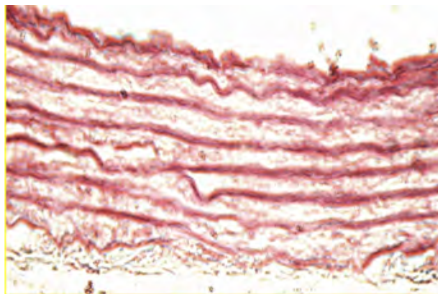


Fig.14: Photomicrograph of third group Oat treating 3weeks rat aorta showing mild separation of elastic fibers (Orcin stain x400).

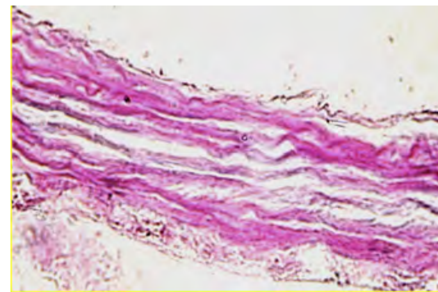


Fig.15: Photomicrograph of third group Oat treating 3weeks rat aorta showing normal distribution of collagen (Mallory trichrome stain x400).

6- High fat diet group 6 weeks

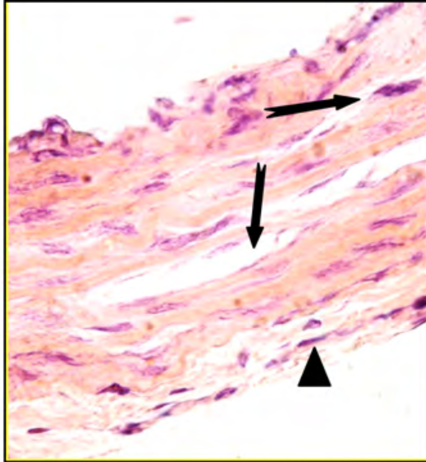


Fig.16: Photomicrograph of sixth group high fat diet 6weeks rat aorta showing marked splitting of smooth muscle (Vertical arrow), vaculation (Transverse arrow) in the media with increased cellularity in the adventitia (Arrowhead) (Hx &E x1000).

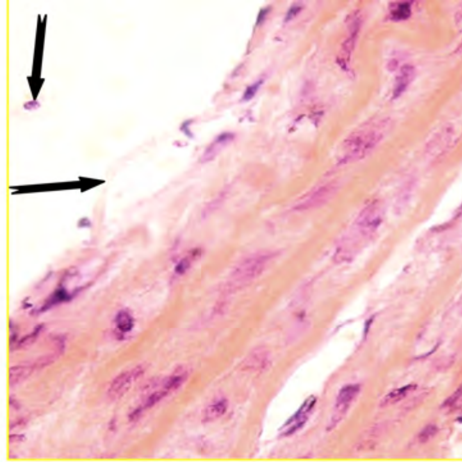


Fig.17: Photomicrograph of sixth group high fat diet 6weeks rat aorta showing detachment of swelling endothelial cells(foam cells) (Transverse arrow) and desquamated cells (Vertical arrow) (Hx &E x1000).

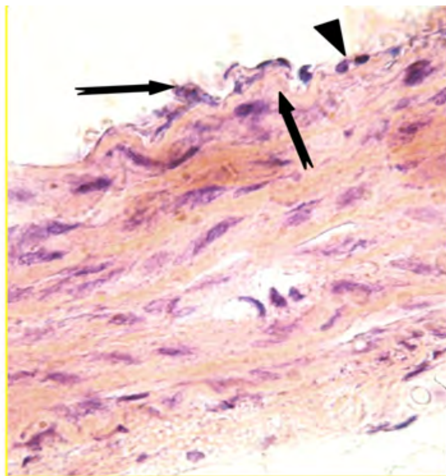


Fig.18: Photomicrograph of sixth group high fat diet 6weeks rat aorta showing loss of regular arrangement and smooth surface (Transverse arrow), pyknotic nuclei (Arrowhead) and sub-endothelial swelling (Vertical arrow) (Hx &E x1000).

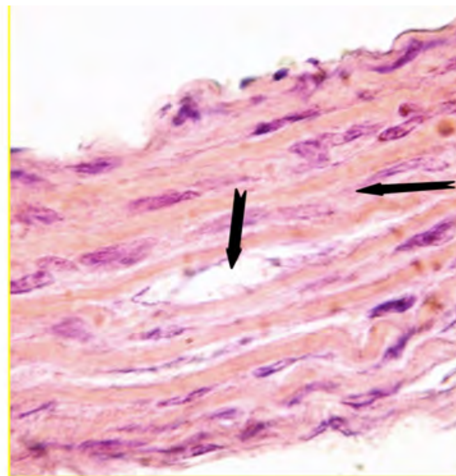


Fig.19: Photomicrograph of sixth group high fat diet 6weeks rat aorta showing vaculation (Vertical arrow), increased thickness of smooth muscle in the media (Transverse arrow) (Hx &E x1000).

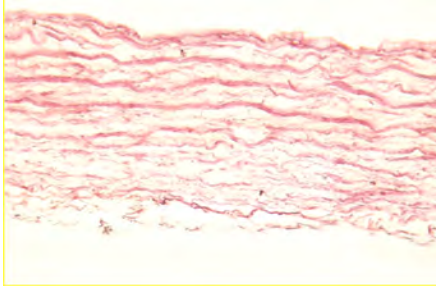


Fig.20: Photomicrograph of sixth group high fat diet 6weeks rat aorta showing marked separation and destruction of elastic fibers (Orcin stain x400).

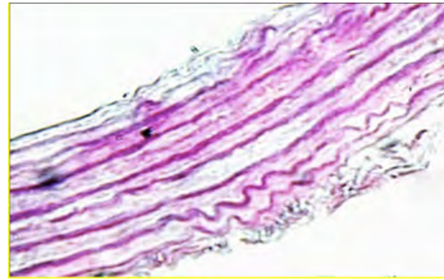


Fig.21: Photomicrograph of sixth group high fat diet 6weeks rat aorta showing increased and proliferation of collagen (Mallory trichrome stain x400).

7- Treated group 6weeks with Oat

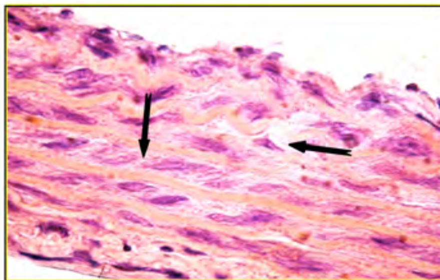


Fig.22: Photomicrograph of seven group Oat treating 6weeks rat aorta showing normal arrangement of three layers with mild thickness in media (Vertical arrow) with mild vaculation (Transverse arrow) (Hx &E x1000).

8- Treated group 6weeks with Fennel

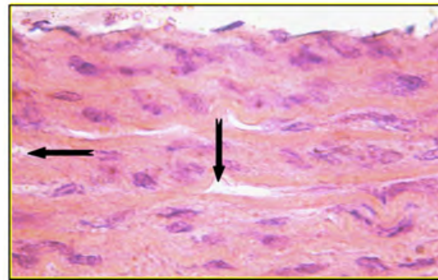


Fig.23: Photomicrograph of eight group Fennel treating 6weeks rat aorta showing vaculation (Vertical arrow), increased thickness (Transverse arrow) of the media (Hx &E x1000).

9- Treated group 6weeks with Triphala

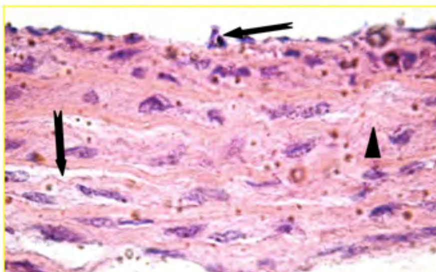


Fig.24: Photomicrograph of nine group Triphala treating 6weeks rat aorta showing vaculation (Vertical arrow), increased thickness (Arrowhead) of the media and pyknotic nuclei of endothelial cells (Transverse arrow) (Hx &E x1000).

7- Treated group 6w with Oat

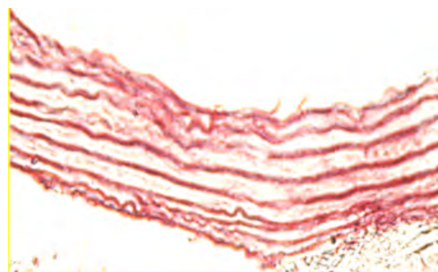


Fig.25: Photomicrograph of seven group Oat treating 6weeks rat aorta showing mild separation of elastic fibers (Orcin stain x400).

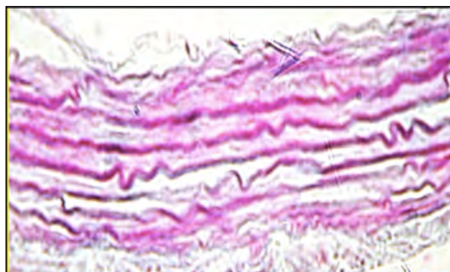


Fig.26: Photomicrograph of seven group Oat treating 6weeks rat aorta showing mild increase of collagen (Mallory trichrome stain x400).

Discussion

Epidemiological and experimental studies have revealed that a high-fat diet might induce oxidative stress and lipid oxidation that contributes to the high risk of cardiovascular disease (CVD) (Mansour et al., 2009).

Lipid and lipoprotein abnormalities are regarded as a highly modifiable risk factor for cardiovascular disease due to the influence of cholesterol, one of the most clinically relevant lipid substances in atherosclerosis (Zhang et al., 2011).

Epidemiological data strongly suggest that diets rich in plant derived food are generally asasociated with a preventive role against the development of chronic diseases (Kris-Etherton et al., 2002). According to the oxidation hypothesis, Steinberg and Witztum (2010) proposed that LDL, in its oxidized form, is crucial to cellular uptake to form macrophages derived from cells in early development of atherosclerotic lesions. Oxidized LDL has many atherogenic properties.

In the present study, the aorta of high fat diet (hyperlipidemic) groups showed proliferation of smooth muscles of tunica media associated with thickening and vaculations of tunica media which might due to accumulation of fatty infiltration in the tunica media which leads to narrowing of the lumen of aorta. Also there were swelling and desquamation of endothelial cells with pyknotic nuclei.

In accordance, a previous study indicated that hyperlipidemia causes swelling of endothelial cells to form foamy cells, perivascular hemorrhage, vaculation in

the cells of tunica media , accumulation of plaque of fatty deposits in the arteries and minor increase in the thickness of vessel wall as a result of smooth muscle proliferation and migration from the tunica media into the intima and aggravate narrowing of the arterial diameter, which restricts blood flow to vital organs (Rioufol and Finet, 2002).

In agreement with the present results, (Nakagawa et al., 2009) showed that feeding of albino rats with high fat diet increased atherogenic indices and induces vascular endothelial dysfunction in isolated aorta of atherogenic-diet rats. Adekunle et al., (2013) reported that there were large number of smooth muscle like cells and focal aggregation of foam cells resulting in intima thickness. Lipid droplets were found in the smooth muscle cells and foam cells in the thickened intima in the aorta and brachiocephalis of the atherogenic diet fed rabbits.

From the obtained results in this study, it was observed that splitting of elastic fibers even destruction occur in group which continued on high fat diet for 6 weeks. Also there was proliferation of collagen fibers as a result of hyperlipidemia. In accordance to Rioufol and Finet (2002) who reported that the hyperlipidemia leads to splitting of elastic fibers.

Chen et al., (2009) studied the mechanical properties of aorta in rats with atherosclerosis (AS), where the relationship between mechanical measurements and collagen concentration was evaluated. A close relationship between the mechanical constants and the percentage of elastin and collagen content was observed. It was concluded that mechanical remodeling in aortic artery of AS might be related to histological remodeling.

Vijayabaskar et al (2008) proved that the significantly elevated levels of plasma cholesterol in rats fed with high fat diet might induce damage to the endothelial cell membrane lining the large arteries such as aorta and might be the initial events in the etiology of atherosclerosis. Moreover, Prado et al., (2008) provide evidence that hypercholesterolaemia has been extensively associated with endo-

thelial cell dysfunction.

Buil-Cosiales et al., (2009) demonstrated that dietary fiber intake is inversely associated with carotid media thickness.

In respect to the fact that HDL cholesterol is inversely related to total body cholesterol and a reduction of plasma HDL cholesterol concentration may accelerate the development of atherosclerosis leading to ischemic heart diseases, by impairing the clearing of cholesterol from the arterial wall (Kanungo et al., 2007).

From the obtained results in this work, it was noticed that the aorta of Oat treated rats revealed that prevention in the diet-induced histopathological lesions in the aorta tissues as compared with those of hyperlipidemic rat.

Oat inhibits vascular smooth muscle cell proliferation (Nie et al., 2006). The potential antiatherogenic activity of oats was tested by evaluating their effects on adhesion of monocytes to human aortic endothelial cells mono-layers expression of adhesion molecules and production of pro-inflammatory cytokines and chemokines by the endothelial cells (Liu et al., 2004).

In this study, it was noticed that the aorta of Fennel treated rats' revealed vaculations In the tunica media and slight thickening in its wall but in the Triphala treated group the aorta showed no evidence of histological changes when was compared with hyperlipidemic group.

These results were in adverse with Shaila et al., (1998) who reported that the aorta of Triphala (Terminalia chebula) treated animals showed decreased atthermatous plaque formation.

In this study, it was observed that keeping the animal on high fat-diet resulted in dyslipidemic changes as illustrated by the significant increase in serum Total cholesterol (TC), Total Triglycerides (TG), and low density lipoprotein-cholesterol (LDL-C), as well as a significant reduction in serum high density lipoprotein-cholesterol (HDL-C) level compared to the rats on normal diet.

These results were confirmed with histological changes of feeding rats with high fat diet which showed vaculations of tunica media and narrowing in the lumen of aorta sections. These results were in accordance with Farmer and Gotto (1992) who reported that plasma levels of LDL are associated with the occurrence of atherosclerosis and impairment of endothelial function of arteries in hyperlipidemic patients.

It was also observed that keeping the animal on medicinal plants Oat, Fennel and Triphala respectively resulted in changes in lipid profile as illustrated by the significant decrease of TL, TG, TC, LDL-C, as well as a significant increase in serum HDL-C level especially with using Oat. Also Triphala and Fennel showed significant decrease in lipid profile but in lesser extent than Oat respectively.

Kerckhoffs et al., (2003); Kelly et al., (2007); Aly, (2012) and Sullivana et al., (2013) reported that oat and oat bran contained factors which could reduce the levels of serum total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) both in animals and human.

Peterson and Dimberg, (2008) suggested that a wide variety of bioactive phenolic compounds and their derivatives have been identified from oats as hydroxycinnamic acids. Also Brindzová et al., (2008) reported that the potential health-promoting properties because of their antioxidant activities.

The present data agreed with Kirby et al., (2004) who reported that administration of Triphala leads to reduction of cholesterol, LDL-C, TG levels and increase of HDL-C level. These results correlated with Sanjay et al., (2010) who reported that the Triphala extract fractions rich in phenolic content were found to have more antioxidant activity than that of crude extracts and moreover the principle antioxidant molecules are non flavonoid phenolic compounds.

The present data agreed with Fatiha et al., (2011) who reported that hyperlipidemic rats treated with fennel extract had a decrease

in plasma levels of TG, TC, LDL-C, and an increase in HDL-C level. This result suggested that cholesterol-lowering activity of the fennel can result from a rapid catabolism of LDL-C through its hepatic receptors for final elimination in the form of bile acids as demonstrated by Guimaraes et al., (2000). Also, Garg et al., (2009) who reported that one of the dietary plants used as hyperlipidemia-lowering factor is fennel herb (Foeniculum vulgare).

Conclusion

Oat supplement was an effective non-pharmacological agent for reverse aortic structural changes and laboratory changes in cases of high fat diet. Also Triphala and Fennel reduced lipid levels but had a minimal effect on structural changes.

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STRUCTURAL AND LABORATORY CHANGES IN THE AORTA OF ADULT FEMALE ALBINO RATS IN CASES OF EXPERIMENTAL HIGH FAT DIET AND UNDER EFFECT OF SOME MEDICINAL PLANTS

Ezz-Eldin E. Abdalla MD

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URINE ANALYSIS SCREENING AMONG PRESCHOOL CHILDREN FOR DETECTION OF URINARY ABNORMALITIES

Abd El-Hamed S. Al-Hamshary MD, Osama S. Al-Shaer MD*, Effat H. Assar MD and Manal M. Al-Ebshihy M.B.B.Ch

Benha Pediatrics Department and Clinical Pathology Department, Benha, Egypt.*

Abstract

Background: *To determine the prevalence of abnormal urinary findings by screening healthy children from 2-5 years by dipstick test for asymptomatic urinary abnormalities.*

Patient and Methods: *This cross sectional study, was carried out in Al-Behera Governorate, from February 2013 to April 2013. One thousand healthy children included in this study. They subjected to urine examination by dipstick for hematuria, proteinuria, glucosuria, nitrite and leucocytes. A second test was done after 10-14 days. Positive children in the second dipstick was subjected to microscopic urine analysis. Positive cases was subjected to urine culture, serum creatinine and ultrasound. All data was analyzed by using statistical method.*

Results: *Urinary screening was performed with the dipstick test to the studied children, and they were re-examined again after 2 weeks. 152 children (15.2%) had urinary abnormalities in the first screening. Hematuria was found in 57 children (5.7%), proteinuria in 35 children (3.5%), combined hematuria and proteinuria in 4 children (0.4%), nitrite in 10 children (1%), pyuria in 46 children (4.6%) and no one had glucosuria. Of these 152 children only 39 (3.9%) had persistent urinary abnormalities in the second test. Hematuria was found in 12 children (1.2%), Proteinuria in 10 children (1%), pyuria in 12 children (1.2%), combined proteinuria and hematuria in 2 children (0.2%), nitrite in 3 children (0.3%) and no one had glucosuria.*

Conclusions: *Asymptomatic urinary abnormalities might be detected by the preschool screening program, a way for early management of some renal diseases.*

Keywords: *Urine analysis sreening, preshool children, urinary abnormalities.*

Introduction

Early identification and treatment of kidney diseases in children and adolescents are important initial steps in prevention of chronic kidney diseases (CKD)[1]. To reduce the number of patient with both End Stage Renal Diseases (ESRD) and cardiovascular disease, effective screening and treatment methods for CKD should be established[2].

Data from the United States Renal Data System (USRDS) show that incidence of kidney failure is rising among adults and is commonly associated with poor outcomes and high cost. Moreover in the past 20 years the incidence of chronic kidney disease in children has steadily increased[3].

Urine analysis, a simple and inexpensive test, is the cornerstone in the evaluation of the kidney function. Proteinuria as well as hematuria may be the only eary signs of renal diseases[4]. Also the presence of detectable nitrites in urine has been used to diagnose urinary tract infection. Urinary tract infection is very common in children with severe consequences on the kidney function leading to chronic kidney disease and hypertension if left untreated[5]. The level of proteinuria is one of the strongest predictors for renal function deterioration[6].

There is evidence that a screening program may open that way for the early management of these diseases, especially where treatment is already established[7].

Nowadays dipsticks tests are widely used as the simplest and cheapest method for detecting urinary abnormalities. when Urine tests are positive in preschool screening test, a second test is preformed in the same manner, when urinary abnormalities are found again, urine analysis is taken under the microscope. So urine analysis plays an important role in the detection and diagnostic work up of patients with renal diseases[8]. Several studies have used reagent strips and have documented their effectiveness in detecting urinary abnormalities[9].

Subjects and Methods

Subjects: This cross sectional study, was carried out in Al-Behera Governorate, from February 2013 to April 2013. It comprised (1000) preschool children of

both sexes. Ages of the children included in the study ranged from 2 to 5 years and they were 520 males (52%) and 480 females (48%).

Methods:

All children enrolled in this study were subjected to:

1- Full history taking and Clinical examination.

2- Urine analysis:

Urine specimen obtained from each child was tested with urine dipstick for protein, blood, glucose, nitrite and leukocyte. Children with abnormal results, had another urine examination by dipstick after 10-14 days.

Those who have positive results twice were subjected to microscopic urine examination. A red blood cell count of five or more per high power field (HPF), one or more plus protein, one or more plus glucose, pus cells count of five or more per high power field (HPF) and crystals were considered as abnormal urine findings.

Those with positive results in microscopic urine analysis were subjected to:

1-Urine culture:

Urine culture was done for children with pus cells count of 20 or more per high power Field[10].

2- Abdominal ultrasound:

Abdominal ultrasound was done to all children with positive results in microscopic urine examination.

3- Serum creatinine was done to all children with positive results in microscopic urine examination.

Statistical methods: The collected data were tabulated and analyzed by the suitable statistical tests using the SPSS computer software.

Results

One thousand children were included in this study, 520 boys (52%) and 480 girls (48%), the age of the children ranged from 2 to 5 years.

Urinary screening was performed with the dipstick test to the studied children, and they were re-examined again after 2 weeks. 152 children (15.2%) had urinary abnormalities in the first screening. Hematuria was found in 57 children (5.7%), proteinuria

in 35 children (3.5%), combined hematuria and proteinuria in 4 children (0.4%), nitrite in 10 children (1%), pyuria in 46 children (4.6%) and no one had glucosuria.

Of these 152 children only 39 (3.9%) had persistent urinary abnormalities in the second test. Hematuria was found in 12 children (1.2%), Proteinuria in 10 children (1%), pyuria in 12 children (1.2%), combined proteinuria and hematuria in 2 children (0.2%), nitrite in 3 children (0.3%) and no one had glucosuria.

There is statistically significant difference between the results of the first and 2nd dipstick. This explained by that most cases of hematuria and or proteinuria in children are transient and hence the importance of second dipstick tests in detecting the real prevalence of these abnormalities. The differences between prevalence of 1st dipstick and 2nd dipstick can be explained also by the possibility of false positive results of dipstick being a good negative and not good positive test.

Complete urine examination was performed to 39 children (3.9%) with persistent abnormal finding by dipstick test. Only 24 children (2.4%) had urinary abnormalities. Among them 7 children (0.7%) were positive for RBCs (RBCs ≥5/HPF), one had combined hematuria and proteinuria (protein ≥+1) (0.01%), 12 children (1.2%) had pyuria (pus cells ≥5/HPF), and 4 children (0.4%) were positive for crystals.

There was highly significant difference observed in the prevalence of urinary abnormalities between boys and girls as girls more affected than boys, but there was not significant difference in prevalence of urinary abnormalities according to age.

Urine culture was performed to 12 children with puria (Pus cells >20WBCs/hpf and it was positive in 8 children (0.8% from total studed children 1000), 4 of them the organism was E.coli, one was Klebsiela, two was proteus and one was pseudomonas.

7 cases of positive culture was girls while there was only one boy affected. Statistical analysis re-

vealed that positive culture was more common in girls than in boys due to more affection of girls by urinary tract infection.

Ultrasonographic examination revealed the presence of abnormalities in 3 cases out of 24 cases subjected to ultrasonographic examination, one of them showed bilateral hydroureter, the other showed bilateral hydronephrosis, and the last showed signs of cystitis.

Voiding cystourethrogram was done to the case of hydroureter and showed that it is grade 1 vesicoureteral reflux and also was done to the case of hydronephrosis and showed that it is grade 2 vesicoureteral.

Table (1): Results of the initial and the second dipstick tests of the studied children.

Positive results	Initial dipstick (at the start) n=152 (15.2%)		2nd dipistick (2 weeks later) n=39 (3.9%)	
Hematuria	57	5.7%	12	1.2%
Proteinuria	35	3.5%	10	1%
Mixed hematuria and proteinuria	4	0.4%	2	0.2%
Glucose	0	0.0%	0	0.0%
Nitrite test	10	1%	3	0.3%
Leucocyte	46	4.6%	12	1.2%

Discussion

To reduce the number of patients with both end stage renal diseases (ESRD) and cardiovascular diseases, effective screening and treatment methods for chronic kidney disease (CKD) should be established[2]. Screening the general population may decrease the incidence of ESRD resulting from glomerulonephritis[11].

In our study the prevalence of urinary abnormalities detected by 1st dipstick test was (15.2%). This comes in accordance with studies as Badeli et al[12] who screened 1520 children in Iran aged from (4-6) years and reported that urinary abnormalities were (13.6%) at the first screening. On the contrary Parakh et al[13] screened 2243 children in India aged from (2-6) years and reported that urine abnormalities were detected in (6.3%) in the first screening test. The differences in the prevalence rates may be explained by sociodemografic character or geographic causes.

In our study the prevalence of persistent urinary abnormalities detected by 2nd dipstick test was (3.9%). Other studies as Badeli et al[12] reported that urine abnormalities were (4.7%) at the second screening test. On the contrary Parakh et al[13] repoted that urinary abnormalities were (1.9%) at the second screening which is lower than our study. This can be due to geographic cause.

In our study isolated hematuria (IH) in 1st dipstick was found in 57 children (5.7%). This agrees with other studies as Badeli et al [12] reported hematuria in (5.8%) of children, On the other hand, Plata et al[14] reported hematuria in (3.4%).

In our study 2nd dipstick test was done after 2 weeks, isolated hematuria was found in 12 children (1.2%) which agrees with the study of Plata et al[14] who found hematuria in (1.6%) of children and (1%) in Shajari et al[15]. Other studies as Badeli and colleagues [12] reported hematuria in (2.4%) of children and (0.13%) by Zainal et al[16].

In our study isolated proteinuria by 1st dipstick was found in 35 children (3.5%) of our study

population in accordance with Badeli et al[12] who reported that isolated proteinuria was (2.9%). On the other hand Plata et al(14) reported proteinuria in (5.9%) of children.

In our study isolated proteinuria by 2nd dipstick was found in 10 children (1%) which agrees with some studies as Badeli et al[12] who reported proteinuria in (1.3%) of children and Hanif et al[17] who reported (1.6%). However other studies as Shajari et al[15] reported proteinuria in (3.6%) children. Isolated proteinuria was (3.3%) in Jafar et al[18]. This can be due to difference in the age of the screenend children by this studies (school age).

As regards the prevalence of persistent urinary abnormalities detected by complete urine examination in our study it was (2.4%). This agrees with Badeli et al[12] who reported a percentage of (2.3%). However in other studies as Shajari et al[15] it was (1.2%). This may be due to the prevalence of crystaluria (0.4%) in our results which were not included in other studies.

In our study complete urine examination showed that 7 children (0.7%) had hematuria, this agrees with the study of Park et al[19] who reported hematuria in (0.9%) of children and with Zainal et al[16] who reported hematuria in (0.6%).

In our study isolated proteinuria was not found in any child (0.0%) which disagrees with Hanif et al[17] who reported proteinuria in (0.2%) of children and agrees with Zainal et al[16] who did not detect any proteinuria in microscopic urine analysis. one of our children (0.1%) had combined hematuria and proteinuria which agrees with the study of Park et al [19] who reported (0.1%) of children and with Shajari et al[15] who reported (0.24%) of children.

In our study pyuria was detected in 12 children (1.2%) which agrees with Shajari et al[15] who found it in (1%) in the microscopic urine analysis. Our results showed the prevalence of crystaluria was (0.4%), All other screening studies as (Shajari et al in Iran, Park et al in Korea) did not examine the presence of crystaluria by confirmatory urine examination.

In our study there is a statistically significant difference between the affected cases as regards sex where females were more commonly affected than males which disagrees with Badeli et al[12] and who reported that there was no significant difference according to sex among their patients. This is agrees with Shajari et al[15], who showed that microscopic urinary abnormalities were more common in girls than boys in Nigeria. Also our study agrees with Hanif et al[17].

In our study there is no significant difference between the affected cases as regards their age which agrees with Badeli et al[12] and who reported that there was no significant difference according to age. This disagrees with Hanif et al[17] who found significant difference as younger age more affected.

In our study urine culture was performed to 12 children with pyuria. It was positive in 8 only of them (0.8% from total studed children). The causative organisms were E.coli in 4 of them (33.4%) of children, proteus in 2 children (16.6%), pseudomonas in one child (8.3%) and klebsiella in one child (8.3%) and no growth in 4 children (33.4). In our study there is statistically significant difference between the affected cases as regard sex as the number of males affected was only one and the number of females was 7 females. This difference between both sexes can be explained by the anatomical difference in urethra between boys and girls as short urethra in girls lead to increased risk to infection and high rate of recurrences.

This is agrees with Durmisvec et al[20] who screened 1768 children and detect 22 cases (1.24%), 3 boys and 19 females (1:6.3). The causative organism was E.coli in (43.3%), Klebsiella in (17.3%) and Proteus in (15.1%). Also agrees with Kumar et al[21] who screened 502 children India and detect 7 cases (1.39%). The causative organism was E.coli in (57.1%), Klebsiella in (14.28%), Enterococcus fecalis in (14.28%) and Proteus in (14.28%).

This is disagrees with Elo et al [22] who screened 792 children in Nigeia and detect 31 cases (4%).

The causative organism was Staph. aureus in (40.6%), Streptcoccus fecalis in (28.1%), E.coli in (15.6%), Klebsiella in (9%) and Pseudomonus in (7%). This can be due to difference in the age as infants included in this study. All these study detected statistically significant difference between the affected cases as regard sex.

Conclusion

• Asymptomatic urinary abnormalities might be detected by the preschool screening program, a way for early management of some renal diseases.

• Screening tests must be done twice with 2 weeks interval to improve the specificity of the dipstick test, avoid the interference of exercise or emotional stress and detect persistent urinary abnormalities.

• Follow up of patients with confirmed abnormal urine examination, as some of these diseases may be reversible, recurrent or progressive.

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Abd El-Hamed S. Al-Hamshary MD,
Osama S. Al-Shaer MD, Effat H. Assar MD
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AUDITORY INVOLVEMENT IN HYPERURICEMIA

Ahmed Hussien MD*, Sherifa A. Hamed MD**
and Essam Eldin A. Mahmoud MD***

ENT Department, Banha University Hospital. Departments of Neuroscience**,*
*Audiology Unit***, Assiut University Hospital.*

Abstract

Hyperuricemia is suggested as risk of sensorineural hearing loss. This study aimed to evaluate cochlear function in group of patients with hyperuricemia. We studied the auditory function in 25 patients with primary hyperuricemia using pure-tone audiometry and transient evoked otoacoustic emission (TEOAEs) analysis. We hypothesized that vascular compromise, is in part, could be responsible; hence carotid dopplex ultrasongraphy was done to assess the common carotid artery intima-media thickness (CCA-IMT), an early marker for atherosclerosis and transcranial doppler ultrasonography (TCD) was also done to the basal intracranial vessels. Compared to control subjects, the mean hearing threshold and pure tone audiometery were within normal age-dependent ranges. Patients reported significant reduction in the amplitude of TEOAEs at 4 kHz (P<0.01). Significant correlation was identified between TEOAE and uric acid level, patients' age, duration of illness, CCA-IMT, MFVs of the middle cerebral arteries and vertebral arteries. These data imply that TEOAEs is a useful method for detection of sub-clinical cochlear compromise in patients with hyperuricemia. It is possible that hyperuricemia could be accompanied by increased stiffness and/or compromise the blood supply of the outer hair cells, which will impair their electromotile response. The human data should be supplemented with animal data.

Keywords: *Cochlea, hearing impairment, hyperuricemia, transient otoacoustic emission, Transcranial Doppler.*

Introduction

Idiopathic sudden sensorineural hearing loss (ISSNHL) represents an acute inner ear disorder. No clear causes for this disease have been found so far, but cochlear ischemia has been hypothesized as one of the etiopathological mechanisms (Aimoni et al., 2010).

Measurement of OAEs is a rapid, reproducible and objective method of evaluating hearing, and a non-invasive measurement of cochlear function. The clinical utility of OAEs has been extensively described in both normally hearing subjects and those with sensori-neural hearing loss (Kepplere et al., 2010).

The primary clinical applications of these emissions appear to be in neonatal screening and ototoxic monitoring (Mcfadden et al., 1993). Audiologic examinations suggest that sudden deafness, tinnitus and impairment of sound localization are usually due to dysfunction of the cochlea resulting from ischaemia to the inner ear and central auditory pathways (Przewozny et al., 2008). Moreover, anatomo- pathologic changes such as thromboembolic events or vessel constriction in pathologic findings of the inner ear are identical and responsible for the ischaemia, but ischemic focuses can also damage auditory pathways and centres (Helleman et al., 2010, Gorga et al., 1993). Cochlear microcirculatory disorders associated with impaired local oxygenation have been considered to be a major pathogenetic factor in hearing impairment, and most therapeutic strategies are aimed at improving cochlear blood flow and oxygenation (Morgenstern et al., 1994).

Otoacoustic emission (OAE) permits sensitive and objective monitoring of dynamic changes in cochlear responsiveness before functional and significant hearing loss. It offers objective and repeatable information and is substantially less time consuming than pure-tone audiometry (Marshall and Heller, 1996). It might be very valuable in scientific research and clinical practice (Reyes et al., 2001).

In the last decade, OAE has been utilized for assessment and

monitoring of hearing impairment of many systemic diseases that bearing risk for atherosclerotic and arteriosclerotic diseases including hyperchlosterolemia /dyslipidemia and hyperglycemia, (Ravecca et al., 1998, Suzuki et al, 2000, Marcucci et al., 2005). Furthermore, several studies found that atherosclerotic related diseases including hyperchlosterolemia (Martin Villares et al., 2005) and diabetes mellitus (de León-Morales et al., 2005) have been found to aggravate hearing loss related to age. However, as far as our knowledge, this is the first study in which OAE is utilized for evaluation of patients with hyperuricemia. Hyperuricemia is a known metabolic disorder. It is a known risk factor for cerebral and cardiac vascular diseases. Increased uric acid level has been suggested to result in a complex interaction with other local vascular substrate toxicities resulting in increased arterial endothelial damage (Kanellis and Kang, 2005). Hyperuricemia has also been found to aggravate age related hearing loss (Adam, 2001). It is conceivable that a similar pathomechanism could be attributed to hyperuricemia-related hearing impairment.

Aim of The Work

As far as our knowledge, this is the first study for assessment of the possible subclinical hearing involvement in group of patients with hyperuricemia utilizing TEOAEs. We hypothesized that vascular compromise, is in part, could be responsible; hence carotid dopplex ultrasongraphy was done to assess the common carotid artery intima-media thickness (CCA-IMT), an early marker for diffuse cerebral atherosclerosis and transcranial doppler ultrasonography (TCD) was also done to the basal intracranial vessels; middle cerebral, posterior cerebral, vertebral and basilar arteries; to detect the perfusion condition of the brain.

Methods

Patients:

Included in this study were 25 patients with hyperuricemia, (mean age, 46.41±7.42; range 27-55 years), (male/female: 17/8). Patients were recruited from the departments of Neuroscience and internal medicine, Saudi German Hospital-Aseer, Saudia Arabia. Thirty-five healthy volunteers were chosen as control subjects for

comparison (mean, 46.18±6.34; range 27-55 years). The demographic characteristics of the studied groups were summarized in table 1. This study was accepted by the regional Ethical Committee. Detailed information of the study was given to all participants and all gave their written consent to attend the study.

Non of the patients nor control subjects were smokers, chronic alcoholic abusers, reported exposure to unsafe noise, use of ototoxic drugs, history of metabolic disease associated with hearing loss, history of otological, central nervous system, labyrinthine disorder or systemic disease as renal insufficiency, gout, diabetes mellitus, hypertension, hypercholesterolemia/dyslipidemia, Cushing's syndrome, hypothyroidism, active gastrointestinal disease, family history of hearing loss, family history of vascular disease or risk for vascular disease.

Data collected per participant were biological variables as age, gender, systolic and diastolic blood pressures, weight, height, body mass index (BMI), calculated using the following formula (BMI=weight (kg)/height $(m))^2$. Overweight was defined as a BMI between 25-30kgm^{-2} and obesity as a BMI over 30kgm^{-2}. All participants were subjected to full audiological, medical and neurological history and examination.

Specimen collection and analysis:

After an overnight fast, blood samples were drawn at (8.00-10.00 a.m.). Laboratory blood tests included complete blood count, lipid profile (total cholesterol or TC, triglycerides or TG, low density lipoprotein-cholesterol or LDL-c and high density lipoprotein-cholesterol or HDL-c), liver and kidney function tests, fasting blood glucose and uric acid levels. Serum levels of TC, TG, HDL-c and LDL-c were measured by enzymatic colourimetric method using the autoanalyzer Hitachi 911 (Boehinger, Mannheim, USA). Serum uric acid was determined by colorimetric US plus kit, supplied by Roche diagnostics, (GmbH, D-68298 Mannheim, USA).

Audiological evaluation:

All patients and controls under-

went basic audiological evaluation that included initial otoscopic examination, standard pure-tone audiometries, speech discrimination, tympanometry and acoustic reflex to exclude any pathologic condition of the external or middle ear (American Speech and Hearing Association Committee on Audiometric evaluation, 1988, Soliman, 1976 and Silman and Gelfand, 1982). Hearing threshold were determined in decibel (dB) hearing level (HL) by a commercially available clinical audiometer for frequencies of 0.25, 0.5, 1, 2, 4 and 8 kHz (two channel clinical Audiometer, interacoustics model AC 40 using software 1.28, 1998, Denmark).

In the same session TEOAEs was recorded and analyzed from both ears. Subject selection was based on the hearing levels from 0.5 to 4 kHz being better than 25 dB HL, normal tympanograms and stapedial reflexes and presence of TEOAEs in at least one ear. TEOAEs was recorded using a computer-based Otodynamic analyzed Ltd, ILO92, Otodynamic Ltd., Hatfield, England, U.K. Nonlinear clicks were used as a stimulus (duration, 80 μs; amplitude, 80±2 dB sound pressure level (SPL); repetition rate, 25/s). Each recording was terminated as soon as 260 responses at least 3 dB SPL above noise level were obtained. Our criterion for the presence of TEOAEs was based on a cut-off at overall wave reproducibility more than 55% when stimulus stability was better than 80% or overall response level 4 dB SPL. Changes in response amplitude and reproducibility in broadband and frequency-bands (1, 1.4, 2, 2.8, 4 kHz) were compared between patients and control subjects (Sisto and Moleti, 1999).

Carotid color duplex examination:

Examination of common carotid artery intima-media thickness (CCA-IMT) was manually performed using a 5MHZ linear transducer of a color duplex flow imaging system (Acuson 128 XP, Acuson Corporation, Mountain View, CA, USA), which operates in several modes: real time B, color doppler and spectral doppler modes. We investigated IMT occurrence in the CCA using B-mode image (Widder et al., 1990).

Measurement were made over the right and left carotid arteries and average IMT (mean of the two sides) was recorded.

Transcranial Doppler Ultrasonography (TCD):

TCD recordings were performed using a multi dopX4 machine (DWL Elektronische system GmbH, Sipplingen, Germany). We used a standard method of insonation according to the published standard (Otis and Ringelstein, 1996). The middle cerebral (MCAs), posterior cerebral (PCAs) and basilar arteries (BA) were insonated through temporal window just above the zygomatic arch through the transcranial approach. The intracranial segments of the vertebrobasilar system were studied trans-temporally for evaluation of the PCAs and suboccipitally for evaluation of the terminal part of the verterbral arteries (VAs) and the origin of the BA. The mean flow velocities (MFVs) (Cm/sec) and pulstility indexes were used for analysis.

Statistical analysis:

Results are presented as mean ± SD. Calculations were done with the statistical package SPSS for windows, version 12.0 (SPSS Inc., Chicago, IL, USA). Statistical comparison among different groups was evaluated using Student's t-test or one-way ANOVA unless otherwise stated. Parametric and nonparametric statistical analyses were utilized for comparison. Significance was determined by p-value of <0.05. Hearing loss was calculated for each ear separately as the amount of threshold shift above the standard audiometric zero. TEOAEs values were considered abnormal when their amplitudes were 2 standard deviations (SD) below the mean of the control group.

Results

Table (1) showed the demographic and laboratory characteristics of the studied groups. The mean systolic and diastolic blood pressure, BMI, fasting blood glucose, lipid profile (TC, TG, LDL-c and HDL-c) of patients showed no significant difference compared to control subjects. All participants were chosen as non-smokers.

Normal middle ear status was confirmed by otoscopy and standard aural procedures. All pa-

tients had type (A) tympanograms with normal middle ear pressures and normal static compliance. The acoustic reflex levels were consistent with pure tone thresholds of these subjects. In Basic audiological evaluation, all patients reported auditory thresholds of ≤25 dB hearing level at all standard audiometric frequencies (0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 kHz). Comparison between left and right hearing thresholds for patients and control subjects did not show any statistical difference and all were within normal age-dependent ranges (Spoor, 1967) (table 2). Compared to the control subjects, 72% of the patients (n=18) reported significant amplitude reduction of the TEOAEs observed at frequency of 4.0 kHz ($p<0.01$) (table 3). When analyzed for gender, males demonstrated marked amplitude reduction of TEOAEs compared to females (4.4±1.6 for male vs. 6.1±0.9; $p<0.01$).

Compared to control group, the mean values of the common carotid artery intima-media thickness (CCA-IMT) and the mean flow velocities (MFVs) of the basal cerebral arteries (middle cerebral or MCAs, posterior cerebral or PCAs, vertebral or VAs and basilar arteries or BA) were not statistically significant (table 4). But we identified a significant correlation between the changes in TEOAEs and uric acid level ($r=-0.435$; $p<0.01$), patients' age ($r=-0.399$; $p<0.05$), duration of illness ($r=-0.638$, $p<0.001$), common carotid artery intima-media thickness ($r=-0.385$; $p<0.05$), MFVs of the MCAs (r=-0.450.; $p<0.05$) and VAs (r=-0.472.; $p<0.01$).

Table (1): Demographic characteristics of the studied groups.

Data	Patients (n=25)	Controls (n=35)	P-value
Age, mean±SD (range); years	46.4±7.4 (27.0-55.0)	46.2±6.3 (30.0-55.0)	p>0.05
Sex; number (%) Male Female	 17 (68%) 8 (32%)	 26 (74.3%) 9 (25.7%)	-
Duration of illness, mean±SD (range); years	2.9±1.7 (0.5-7.0)	-	-
Systolic blood pressure; mmHg	136.0±3.3	140.0±6.5	p>0.05
Diastolic blood pressure; mmHg	79.0±5.2	70.0±6.5	p>0.05
BMI (kg/m^2)	25.5±4.1	26.6±2.8	p>0.05
Fasting blood glucose level (mg/dl)	104.0±6.5	101.0±6.1	p>0.05
Uric acid, mean±SD; mg/dl	8.1±1.2 (6.4-11.9)	4.2±0.8 (2.80-5.2)	**P<0.0001**
TC, mean±SD; mg/dl	155.1±43.9	139.0±36.1	p>0.05
TG, mean±SD; mg/dl	115.0±30.8	92.6±35.7	p>0.05
LDL-C, mean±SD; mg/dl	92.9±17.9	90.4±38.3	p>0.05
HDL-C, mean±SD; mg/dl	47.7±7.2	53.7±7.1	p>0.05

Data are expressed as # (%), BMI; Body mass index, TC; Total serum cholesterol, TG; Triglycerides, LDL-c; Low density lipoprotein-chloesterol, HDL-c; High density lipoprotein-cholesterol.

Table (2): Pure-tone audiometry thresholds for all ears: comparison between patients and control subjects.

Frequency (kHz)	Patients (n=25) mean±SD (range)	Control (n=35) mean±SD (range)	P-value
0.25			
RT ear	20.0±4.4 (10.0-30.0)	21.8±4.1 (15.0-30.0)	p>0.05
LT ear	20.6±3.6 (15.0-30.0)	22.1±6.0 (10.0-35.0)	p>0.05
0.5			
RT ear	18.2±4.4 (10.0-25.0)	17.2±4.8 (10.0-25.0)	p>0.05
LT ear	19.4±3.6 (10.0-25.0)	17.5±5.4 (10.0-30.0)	p>0.05
1.0			
RT ear	16.0±4.7 (10.0-25.0)	18.0±4.3 (10.0-25.0)	p>0.05
LT ear	19.6±4.3 (10.0-25.0)	19.0±4.2 (15.0-30.0)	p>0.05
2.0			
RT ear	17.1±5.7 (10.0-25.0)	17.65±4.64 (10.0-25.0)	p>0.05
LT ear	18.5±3.8 (10.0-25.0)	17.65±4.96 (10.0-25.0)	p>0.05
4.0			
RT ear	18.1±4.9 (10.0-25.0)	18.7±5.4 (10.0-30.0)	p>0.05
LT ear	18.5±4.4 (10.0-25.0)	18.2±4.9 (10.0-25.0)	p>0.05
8.0			
RT ear	24.7±4.9 (15.0-35.0)	24.9±5.2 (10.0-35.0)	p>0.05
LT ear	23.7±4.1 (15.0-35.0)	24.3±4.6 (15.0-35.0)	p>0.05

p>0.05: non-significant

Table (3): TEOAEs echo level (amplitude changes in dB SPL) for all ears.

Frequency (kHz)	Patients (n=25) mean±SD (range)	Control (n=35) mean±SD (range)	P-value
Overall echo level			
RT ear	18.6±1.0 (16.6-20.5)	19.1±1.5 (14.6-22.4)	p>0.05
LT ear	18.7±1.9 (14.3-22.5)	19.2±1.9 (14.8-22.4)	p>0.05
1.0			
RT ear	7.0±1.0 (5.2-8.7)	7.1±0.8 (5.1-8.5)	p>0.05
LT ear	6.7±1.2 (4.1-8.2)	6.8±1.5 (3.5-11.4)	p>0.05
1.4			
RT ear	12.0±1.7 (8.9-17.3)	12.2±1.7 (9.3-17.4)	p>0.05
LT ear	12.4±2.1 (7.4-17.7)	12.6±1.4 (9.5-15.9)	p>0.05
2.0			
RT ear	12.1±2.0 (7.8-18.0)	12.6±1.6 (10.1-17.2)	p>0.05
LT ear	12.2±2.0 (7.8-17.2)	12.5±1.3 (9.2-16.4)	p>0.05
2.8			
RT ear	11.8±1.7 (7.4-16.5)	11.7±1.4 (8.5-14.2)	p>0.05
LT ear	11.9±2.1 (6.0-17.8)	11.9±1.3 (8.7-13.5)	p>0.05
4.0			
RT ear	6.0±1.4 (-5.6-16.2)	7.5±1.6 (4.8-9.8)	**p<0.01**
LT ear	5.0±1.0 (-4.2-13.1)	7.3±1.3 (4.7-9.2)	**p<0.01**

p>0.05: non-significant, p<0.01: highly significant

Table (4): The mean values of the Intima-media thickness and flow velocities of the basal cerebral arteries: comparison between patients and control subjects

Variable	Patients (n=25) mean±SD (range)	Control (n=35) mean±SD (range)	P-value
CCA-IMT			
RT	0.62±0.1	0.58±0.4	p>0.05
LT	0.59±0.2	0.58±0.1	p>0.05
MFV; Cm/sec			
MCA			
RT	76.5±3.0	79.5±5.1	p>0.05
LT	76.3±3.5	85.5±7.2	p>0.05
VA			
RT	36.7±7.1	39.6±9.5	p>0.05
LT	36.7±7.2	40.3±11.0	p>0.05
PCA			
RT	36.1±5.8	36.5±5.8	p>0.05
LT	34.8±5.7	38.1±6.7	p>0.05
BA	39.5±8.7	42.6±11.5	p>0.05

CCA-IMT, common carotid artery-intima-media thickness; MFV, mean flow velocity; MCA, middle cerebral artery; VA, vertebral artery; PCA, posterior cerebral artery; BA, basilar artery, p>0.05: non-significant.

Discussion

Otoacoustic emission (OAE) is a sensitive objective and reliable method to measure and monitor cochlear hair cell function (Probst et al., 1991 and Marshall and Heller, 1996). As far as our knowledge, this is the first to study auditory function in patients with hyperuricemia without any neurological, medical or otological affection using TEOAEs. Our findings showed that patients with hyperuricemia demonstrated subclinical hearing impairment at high frequencies of TEOAEs (4 kHz). There are several possible explanations regarding hyperuricemia-related hearing impairment.

It is known that the electromotile function of the cochlea is responsible for the nonlinear mechanical amplification which depends on the fluidity, lipid composition, and stiffness of the OHCs of the lateral wall membrane of the cochlea (Dallos, 1992 and Oghalai et al., 2004). The results of the TEOAEs in which affection started in high frequencies, indicate that cochlear compromise in hyperuricemia could be due to outer hair cells (OHCs) affection along the basal cochlear region (Gates et al., 2002).

Several experimental, human studies and many literatures contain several causal relationships between that is in accordance with the commonly reported high frequencies hearing impairment affection in many systemic vascular diseases as hyperchlosterolemia/dyslipidemia (Oghalai et al., 1999, Suzuki et al., 2000 and (Evans et al., 2006), diabetes mellitus (de León-Morales et al., 2005) and age-related hearing loss (Gates et al., 2000, Adam et al., 2001, Alam et al., 2001, Jang et al., 2006). This is commonly attributed to functional changes related to ischemic complications associated atherosclerosis-related microcirculatory disturbances of the cochlear vasculature (Schmolke and Hormann, 1990, Ravecca et al., 1998 and Evans et al., 2006). Also, (Aimoni et al., 2010) suggest that diabetes mellitus, hypercholesterolemia and a high burden of cardiovascular risk factors are associated with the risk of ISSNHL. High frequency loss has been found to be related to OHCs degeneration along base-

to-apex gradient that follows reduced function of the stria vascularis due to hair cell loss mainly in the basal turns (Gates et al., 2002 and Marcucci et al., 2005). Many epidemiological and experimental studies suggested that hyperchlosterolemia/dyslipidemia may result in hyperviscosity of the blood and atherosclerosis resulting in reduced blood perfusion of the cochlea and increase susceptibility to noise, thus trigger disorders of hearing dysfunction as presbycusis and noise-induced hearing loss (Axelsson and Lindgren, 1985). Several experimental studies revealed that the main histopathological finding in the inner ear affection in hypercholesterolemia/dyslipidemia is the strial marginal cells throughout the cochlea and in the OHCs of the apical turn (Nguyen and Brownell, 1998) with compromise of the stria vascularis of the basal cochlear turns (Hidaka, 1997). Studies about the relationship between diabetes mellitus and hearing impairment have shown that the most frequent finding is a high frequency sensorineural hearing loss (Lisowska et al., 2001, Kakarlapudi et al., 2003 and de León-Morales et al., 2005). In the study of de León-Morales et al. (2005), the authors found that progressive hearing loss affecting the high frequencies was proportionately related to patients' age and duration of diabetes mellitus and was independent to peripheral neuropathy, retinopathy or nephropathy. Hyperuricemia is a known risk for cerebral and cardiac vascular diseases (Kanellis and kang, 2005) so; reduction of TEOAEs could possibly follow the decreased function of the stria vascularis and hair cell loss.

Schmolke and Hörmann (1990) reported the frequency of the vascular risk factors (overweight, hypertension, hypercholesterinaemia, hypertriglyceridaemia, hyperuricaemia, hyperglycaemia and smoking) in patients with sudden hearing loss and stated that only hyperuricaemia and hyperglycaemia are found more often in patients suffering from sudden deafness than in the normal population. There was a negative correlation between hearing improvement and the number of risk factors. Also the number of risk factors increased proportionally to the age of the patients. The patient's age and late treatment were

the only unfavourable prognostic factors for hearing improvement.

Studies have shown that the inner ear has high metabolic demands and the cochlea is a vascular region provided with terminal capillary bed. It is not able to form collateral vessels which could restore blood flow in the ischemic regions (Mom et al., 2002). Because cochlea has high sensitivity to minimal blood flow reduction, occlusions at this level can lead to clinical manifestations, even before evidence of microvascular complication. The high metabolic demands of the inner ear and the auditory pathway could make them a target of the disease (Lalanne et al., 1992 and Einer et al., 1994). Although we did not identified an evidence of atherosclerotic changes in the main cerebral vessels by measuring the carotid artery intima-media thickness (CA-IMT) using color duplex ultrasonography, as early marker for atherosclerosis (Raitakari, 1999) and by assessing the cerebral perfusion of the major intracerebral basal arteries using transcranial doppler ultrasongraphy (Elmere et al., 2003), we identified significant inverse correlation between the changes in TEOAEs and CCA-IMT and MFVs of the middle cerebral arteries (MCAs) and vertebral arteries (VAs). However, this does not exclude the vascular compromise as a possible etiology of hearing impairment in our patients. The poorer auditory function with male sex observed in this study could be attributed to the effect of androgens (Jerger et al., 1993 and Evans et al., 2006).

Another explanation is that hyperuricemia could possibly induce hearing impairment through aggravation of age related hearing loss (Adam 2001) as we identified a significant correlation between the changes in TEOAEs and uric acid level, patients' age and duration of illness. Hyperuricemia has been implicated as another risk factor for otoconial degeneration in old age and linking filaments with otoconial fragments (Adam et al., 2001). The otoconia, is the accessory extracellular superstructure covering the utriclular and saccular sensory epithelia, the two otolithic organs containing sensory hair cells and supporting cells (Lins et al., 2000). Several studies

found that atherosclerotic related diseases including hyperchlosterolemia (Martin Villares et al., 2005) and diabetes mellitus (de León-Morales et al., 2005) can aggravate hearing loss related to age. Hyperuricemia could be accompanied by an increased stiffness of the cells and this will impair the electromotile response of the OHCs added to the vascular compromise caused by hyperuricemia.

Finally, we cannot exclude a hidden complex hereditary metabolic disorder of inborn error of purine metabolism as a cause of auditory impairment and hyperuricemia (Simmonds et al., 1985, Mavrikakis et al., 1990) although none of the patients experienced a manifest otological, medical or neurological disorder.

Conclusion

As far as we know, our study is the first to demonstrate that patients with hyperuricemia have an increased vulnerability to develop subclinical hearing impairment. The study clearly shows that TEOAEs are reduced at high frequency region. This data indicates that impaired cochlear function due to high uric acid levels, more precisely may affect the outer hair cells (OHCs). It is possible that hyperuricemia could be accompanied by increased stiffness and/or compromise the blood supply of the OHCs at the basal cochlear region and this will impair their electromotile response. Whether the observed effect is caused by direct incorporation of uric acid into the OHCs or an indirect functional impairment of the OHCs as by reduction of the endocochlear potential due to vascular malfunction of stria vascularis, mandates further research.

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Ahmed Hussien MD, Sherifa A. Hamed MD and Essam Eldin A. Mahmoud MD

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ASYMPTOMATIC BACTERIURIA IN CATHETERIZED AND NON-CATHETERIZED YOUNG CHILDREN IN INTENSIVE CARE UNIT

Abdel-Hamid El Hamshary MD*, Ali. El shazley MD, Osama. El Shaier MD***, Yahia Hamed MD* and Marwa Nassar MB.B.ch******

Pediatrics, **Urology, ***Clinical pathology Departments Benha University and Pediatric Department, Benha children Hospital***

Abstract

Background: *Urinary tract infections (UTIs) are among the most common infection diseases of humans, with Escherichia coli being responsible for more than 80% of all cases. Asymptomatic bacteriuria (ASB) occurs when bacteria colonize the urinary tract without causing clinical symptoms and can affect both catheterized patients (catheter-associated ASB (CA-ASB) and non- catheterized patients.* ***Objectives:*** *To determine the prevalence of abnormal urinary findings by screening children in I.C.U by dipstick test for asymptomatic urinary abnormalities.* ***Patients and methods:*** *This cross sectional study was carried out on 60 children. Age of the children ranged from 2 to 6years old. From Nov 2012 to March 2013, they were classified into 2 groups: 30 cases are catheterized and 30 cases are non-catheterized).* ***Result:*** *Urine culture and Dipstick were done for both catheterized and non-catheterized children.*

Conclusion: *There are several rapid and simple tests for the detection of asymptomatic bacteruria in catheterized and non-catheterized children presented with acute illness in I.C.U. These include: Leukocyte esterase, nitrite test, catalase, enhanced urine analysis and Gram stain. Single screening test has high specificity but combinations of three screening tests have high sensitivity.*

Introduction

Urinary tract infections (UTIs) are among the most common infection diseases of humans, with Escherichia coli being responsible for more than 80% of all cases. Asymptomatic bacteriuria (ASB) occurs when bacteria colonize the urinary tract and can affect both catheterized and non-catheterized patient(1). There are several rapid tests for the detection of UTI in children, leukocyte esterase dipstick urine test was considered as a more recent review of primary care based pediatric studies using urine culture as the reference standard found that no individual symptom or sign, or any combination of symptoms or signs, was sufficient to rule in a diagnosis of UTI.[1] UTI may be missed in as many as 50% of young children presenting to primary care. The clinical diagnosis of UTI in young children is difficult because: pre-verbal (predominantly under 3 years), non-specific symptoms (e.g. fever, irritability, vomiting and poor feeding) when suffering from a wide range of illnesses(2).

Catheter-related urinary tract infection (UTI) occurs because urethral catheters inoculate organisms into the bladder(4). and promote colonization by providing a surface for bacterial adhesion and causing mucosal irritation.(3)

Aim of The Work

To determine the prevalence of abnormal urinary findings by screening children in I.C.U by dipstick test for asymptomatic urinary abnormalities in catheterized and non-catheterized children presenting with acute illness in I.C.U.

Patients and Methods

The students included were randomly selected ages of the catheterized and non-catheterized pediatric patient. The students included were randomly selected 60 cases, (30 cases are catheterized and 30 cases are non-catheterized. Age of the children ranged from 2 to 6 years old.

Exclusion criteria

1. Feverish child.
2. Child complained from any UTI symptom (dark urine,

dysuria, frequency, renal pain, changes of urine volume and suprapubic pain).

3. Child administrated antibiotic last 48h.

Statistical analysis: The program used was SPSS version 16. Qualitative data was summarized using frequency and. percentage. Fischer exact test was used .

Results: Data were collected from 60 children present in primary and Intensive Care Unit in Benha children hospital.

Results

Figure 1: shows the sensitivity and specificity of Nitrite, leukocyte esterase and or catalase test. Positive results were found in (19) cases (5) cases of whom were positive by culture as illustrated in figure (5). And sensitivity = 5/6 x 100 = 83.3% and negative results in (41) cases (40) cases whom were negative by culture (figure (5) with specificity = 40/54 x 100 = 74.4%. Predictive value of positive = 5/ 19=26.3%. Predictive value of negative =40/41= 97.

Figure 2: shows prevalence of asymptomatic bacteruria (10%) urine specimens were positive for significant bacteriuria by culture in both catheterized and non-catheterized with predominance in females (13.3%) compared to males (6.7%).

Table: Types of bacterial isolates in cases of asymptomatic bacteriuria in catheterized children

Table 1: Illustrated bacteruria in related to short time catheter (less than 6 days), E-coli and co-agulase staphylococci are the most common organism.

Catheterization / *Urine culture*	*Positive*		*Negative*		*Total*	
	No	*%*	*No*	*%*	*No*	*%*
E-coli	2	6.7	1	3.3	3	5.0
Co-agulase+ve staphylococci	2	6.7	0	0.0	2	3.3
Enterobacter	0	0.0	1	3.3	1	1.7
Negative	26	86.7	28	93.3	54	90.0
Total	30	100	30	100	60	100

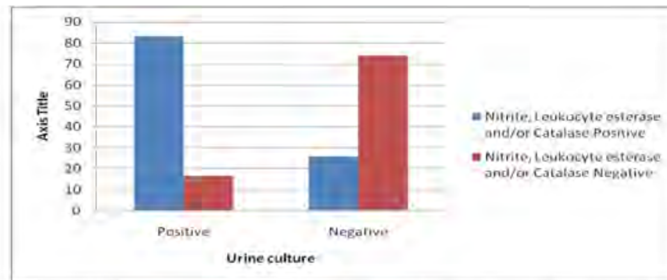

Figure 1: Shows Sensitivity and Specificity of Nitrite, Leukocyte esterase and or Catalase Test in both catheterized and non-catheterized.

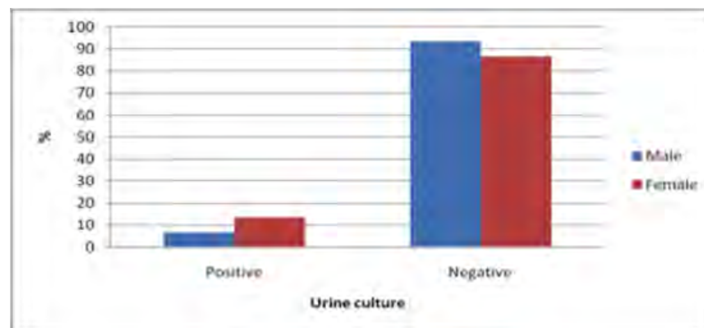

Figure 2: Shows Prevalence of asymptomatic bacteruria in male and female.

Discussion

Urinary tract infection (UTI) in children is a relatively common diagnosis encountered in general pediatric practice. Because of the potential severity of such infections, the workup is an important part of management. Protocols for evaluation of any urinary tract abnormalities, appropriate management is essential. Urine analysis is a simple and inexpensive test, which remains to be the cornerstone in evaluation of the kidneys. It can be easily employed in screening of renal abnormalities[5].

In our study the prevalence of asymptomatic bacteruria among the children in this study was found to be (10%). This was agree with[2] UTI) in children consulting for any acute condition vary wide-

ly (from 2% to 20% depending on setting and inclusion criteria.

Conducted in India by[6] it was shown that asymptomatic bacteruria was predominant in females (7.6%) which is higher than that of males (2.9%).[7] in Pokhara valley reported that the ratio of asymptomatic bacteruria prevalent among femal This comes in accordance with the study of asymptomatic bacteruria. Also, in the study of asymptomatic bacteruria, the Study also agrees with[9].

Asymptomatic bacteruria in children in India stands at (7.5%) [10]. Most probably the reasons for this high prevalence (10%) in our study that most of the samples collected from low socioeconomic.

Bacteruria was predominant in the age group (2-6) years old. This result comes in accordance with[8] in Nigeria who found that the prevalence of asymptomatic bacteruria predominant in children aged 6 years old was (13%) [11], class and high prevalence of pinworm infestation in children. Both[12] and[13] reported.

The relationship between asymptomatic bacteruria and type of bacteria: present that the E. coli are the main bacteria which cause asymptomatic bacteruria in catheterized and non-catheterized. The E. coli isolates constituted about (50%) of the total bacterial isolates.

This result agrees with[14] in Iran who found that the main isolated pathogen in the study of asymptomatic bacteruria was E. coli (50%), the study is in accordance with (6)who found that the main isolated bacteria in the study of asymptomatic bacteruria was E. coli (32.8%), and[15] in Jordan who found that the main isolated bacteria in the study of asymptomatic bacteruria was E. coli (72%). In[16] in Egypt urine culture was performed to 13 children (1%) with pyuria. It was positive in all of them. The causative organisms were staphylococci in 4 children (30%).

The results of manually per-

formed dipsticks and the strip analysis in the laboratory were the same[17]. The combination of different urine tests gives more accurate result[18].

In this study the three screening tests used are nitrite, catalase and leukocyte esterase. They were used separately and combined and to assess the sensitivity and specificity and the results were compared with the results of urine culture.

The results of our study agree with[19]. When nitrite, catalase and leukocyte esterase were combined urinalysis and urine dipstick for sensitivity was (73.63%) and the specificity was (83.16%).

The presence of a urinary catheter is the most important risk factor for bacteriuria[3].

Once a catheter is placed, the daily incidence of bacteriuria is 3-10%. Between 10% and 30% of patients who undergo short-term catheterization (ie, 2-4 days) develop bacteriuria and are asymptomatic[3], it matches with our study that incidence of catheterized more than non-catheterized.

Escherichiacoli are most commonly responsible, but Staphylococcus aureus, coagulase-negative staphylococci and Enterobacter species also are known to cause infection. Proteus and Pseudomonas species are the organisms most commonly associated with biofilm growth on catheters (4).it matches with our study that E-choli is common but also Coagulase.

Recommendations:

Screening programs for asymptomatic bacteruria should be applied to all primary care children in both catheterized and non-catheterized, especially girls, no single screening test is sufficient and it is better to use combinations of two or three tests.

Conclusion

In summary, this is a study in primary care, involving obtaining clinical samples from children, and will help guide management

of the acutely unwell child, which is a common and important aspect of primary health care delivery.

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ASYMPTOMATIC BACTERIURIA IN CATHETERIZED AND NON-CATHETERIZED YOUNG CHILDREN IN INTENSIVE CARE UNIT

Abdel-Hamid El Hamshary MD, Ali. El shazley MD, Osama. El Shaier MD, Yahia Hamed MD and Marwa Nassar MB.B.ch

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HIGH INSULIN RESISTANCE IS ASSOCIATED WITH LOW RAPID VIROLOGICAL RESPONSE IN CHRONIC HCV PATIENTS

Mohamed A. Metwally MD*, Roshdy M. Khalaf Allah MD,**** and Inas A. Ahmed MD*****

Hepatology, Gastroenterology, and Infectious Diseases Department, Internal Medicine Department**, Biochemistry and Molecular Biology Department***, Benha University, Benha, Qualubia, Egypt. Kafr El-Shikh liver research center (KLRC)****, Kafr El-Shikh, Egypt*

Abstract

AIM: *To study the prevalence of insulin resistance in chronic HCV patients and it's relation to rapid virological response (RVR).*

Patients and Methods: *This prospective study included 50 chronic HCV patients who were candidates for treatment with interferon therapy. Baseline variables including; demographic, clinical, laboratory, and histological variables were collected. Insulin resistance was calculated using Homeostasis Assessment Model for Insulin Resistance (HOMA-IR). Rapid virological response (RVR) was identified by 2 log decrease of viral load or more at 4 weeks of therapy. Baseline variables were tested for association with IR. Univariate and multivariate analysis were done to identify independent predictors of RVR.*

Results: *We included 50 patients with mean age 40.7±9.7 years, 22/50 (44%) were males. Out of 50 patients 32 (64%) had IR with HOMA-IR >2.5 and 13 (26%) had HOMA-IR>4. From baseline variables, IR was associated with higher level of ALT (P=0.03) and had significant correlation with ALT (Pearson correlation= 0.30, P=0.04), tendency of significant correlation with age (Pearson correlation = -0.25, P=0.07) and AST (Pearson correlation = 0.26, P=0.06). RVR was achieved in 39/50 (78%). Failure to have RVR was associated with insulin resistance with HOMAIR >4 (P=0.03), low serum albumin (P=0.005), higher TSH (P=0.01), higher BMI (P=0.04) and grade of inflammatory activity (P=0.08). In logistic regression analysis HOMA-IR >4 still an independent negative predictor for RVR.*

Conclusions: *Insulin resistance is a common phenomenon in Egyp-*

tian patients with CHCV. High insulin resistance is an independent negative predictor for RVR.

Key Words: *Egypt, Insulin resistance, HOMA-IR, Hepatitis C, Interferon, RVR, Treatment.*

Introduction

Hepatitis C virus infection is a serious worldwide problem. It has been estimated that there are over 150 million with HCV infection worldwide, with an increasing incidence of new infections (3-4 million every year)[1]. Egypt has the highest worldwide prevalence of HCV (10-20%) where it infects about 15% of the general population, more than 90% of them are due to genotype 4 variants[2,3]. It is the leading cause of liver cirrhosis, hepatocellular carcinoma, and liver transplantation in the country[4].

Several extra hepatic diseases, including dermatologic, hematologic, autoimmune and metabolic disorders have been associated with chronic HCV infection[5]. Moreover, chronic HCV was considered as an entity of metabolic syndrome that increases insulin resistance, which is defined as a condition in which normal amounts of insulin are inadequate to produce a normal insulin response from fat, muscle and liver cells and measured mainly by calculating a homeostasis model of assessment insulin resistance (HOMAIR)[6]. Greater insulin resistance is more prevalent among patients with HCV infection compared with those with other liver diseases and with the general population[7]. It is still not clear whether HCV replication directly increases insulin resistance, or whether hyperinsulinemia stimulates viral replication. It is also unclear whether fat accumulation in hepatocytes results in insulin resistance (IR) due to liver disease or whether it is directly caused by HCV infection. It has been demonstrated that insulin resistance may occur at an early stage of chronic HCV infection, before steatosis and fibrosis develop, suggesting viral mechanisms of IR development irrespective of steatosis[8].

In patients with HCV infection, insulin resistance is involved in progression of hepatic fibrosis and development of hepatocellular car-

cinoma[9]. The degree of IR is independently associated with the degree of liver fibrosis regardless of the presence or absence of steatosis[10]. The pathogenic linkage between IR and fibrosis is still unclear, but it can be hypothesized that HCV itself, by interfering with insulin signaling processes, can cause a hyper-insulinemia state which has a fibrogenic potential.

As host and viral factors contributing to the response to antiviral treatment are still being sought, IR became intensively studied as a possible predictor of poor response in chronic hepatitis C[8]. In a few studies it was shown that it can be an independent predictor of poor response to antiviral treatment together with some other factors, such as genotype not 1 or 4 or advanced fibrosis[11]. It was also indicated that the effect of IR on responsiveness was independent of BMI and hepatic steatosis, although these two factors are also believed to be associated with poorer response[12]. There is debate whether IR directly makes patients resistant to antiviral treatment or only distinguishes patients who are difficult to treat because they are obese or have more advanced fibrosis/cirrhosis.

An early virological response (EVR) is defined as a ≥2 log reduction or complete absence of serum HCV RNA at week 12 of therapy compared with the baseline level. Failure to achieve an EVR is the most accurate predictor of not achieving an SVR. The absence of an EVR is the most robust means of identifying non-responders[13]. The positive predictive value for rapid virologic response and early virologic response was 88% and 83%, respectively. After controlling for predictors, low baseline histological grade and stage, low baseline HCV RNA ($P<0.001$), and low baseline body mass index ($P=0.013$) were associated with SVR[14].

Aim of Work

The aim of this study was to determine prevalence of insulin resistance, measured by homeostasis model assessment-insulin resistance (HOMA-IR), and its relation to rapid and early virological response in patients with CHC undergoing combination antiviral therapy with pegylated interferon and ribavirin.

Patients and Methods

This prospective study was conducted in Kafr El-Shikh liver research center (KLRC) and included 50 consecutive HCV RNA-positive patients with biopsy-proven chronic hepatitis C (CHC) who were a candidate for treatment with pegylated interferon and ribavirin.

The study protocol was approved by the Institutional Ethical Committee and informed consent was obtained from each patient before involvement in the study.

Patients who had the following criteria were recruited; 18 years or older, has white blood cell (WBC)>4,000/μL, neutrophil count >2,000/μL, platelets>100,000/μL, Hb> 13 gm/dL in males and 12 gm/dL in females, albumin > 3.5gm/L, serum cratinine<1.2, positive anti-HCV and HCV-RNA PCR, and evidence of chronic hepatitis on liver biopsy performed within the previous 12 months.

Patients were excluded from the study if they have any of the following criteria; HBS Ag positive, ANA≥1:80, history of DM or with fasting glucose >110mg/dL, any other condition of liver disease other than HCV: autoimmune liver disease, alcoholic liver disease, decompensated liver disease, chronic kidney disease, ischemic cardiovascular disease, patients with organ transplant, substance abuse, sever pre-existing psychiatric conditions, pregnancy or breast feeding, known history of hemolytic anemia, antiviral, or immunosuppressive therapy within the last 6 months.

Patients who gave informed consents were included in the study and all patients were subjected to the following:

1. Thorough history taking.

2. Clinical examination including measurement of the height and weight for every patient. BMI was calculated as the weight in kilograms divided by the height in meters squared. According to the WHO classification, BMI was considered normal when it ranged between 20 and 25 kg/m2, overweight ranged between 25 and 35 kg/m2, and obesity was defined as BMI >35 kg/m2.

3. Laboratory investigation in-

cluding alanin trasferase (ALT), aspartat transferase (AST), alkaline phosphatase, gama glutamyl transpeptidase (GGT), total and direct bilirubin, serum albumin, prothrombin time (PT), international normalized ratio (INR), blood urea, serum creatinin, fasting blood sugar, T3, T4, thyroid stimulating hormone (TSH), C reactive protein (CRP), alfa fetoprotein (AFP), anti-nuclear antibody (ANA), anti bilhazial antibody and fasting insulin. Hepatitis C viral load was determined using real-time polymerase chain reaction.

4. Pre-treatment insulin resistance, expressed by the homeostasis model assessment-insulin resistance (HOMA-IR) method, was calculated according to the formula: HOMA-IR= (fasting insulin concentration [mU/L] x fasting glucose concentration [mmol/L])/ 22.5. HOMA-IR was considered altered and patient has IR when it was greater than 2.5 as this cutoff value was chosen by recent study (Hao et al., 2011)[15].

5. Liver biopsies by true cut needle were taken under guided ultrasonography. Chronic hepatitis was evaluated on a semi-quantitative scale (METAVIR) for grading of activity from grade 0 to grade 4, staging of fibrosis from stage 0 to stage 4, and degree of steatosis from no, mild, moderate, or severe.

Patients follow up: Follow up of patients in the Hepatology Outpatient Clinic was conducted weekly during the first month and monthly thereafter along the course of therapy to check for safety. Throughout the study, patients were monitored for vital signs, weight, adverse events, medication compliance, thyroid function, hematologic parameters, blood chemistry and serum HCV-RNA levels. Quantitative HCV-RNA PCR was checked before, 4 weeks and 12 weeks of therapy.

Statistical Methods

Patients with insulin resistance were compared with patients with insulin sensitive regarding baseline variables and rapid and early virological response. Chi square test or Fisher's exact test were used to compare dichotomous or categorical variables and Student *t*-test or Mann-Whitney U test was

used to compare continuous variables depending on distribution of data and homgenity of variance. Correlation between HOMA-IR level and continuous variable was tested using Pearson's correlation analysis. Patients with virological response rapid or early were compared with patients without response by using the previous tests. Variables that were associating with virological response in univariate analysis were included in logistic regression analysis to determine the role of insulin resistance in predicting response after adjustment for other variables. All tests are two tailed and p value ≤ 0.05 is considered significant. All tests were done by using SPSS software version 19.

Results

In this cross sectional study, we recruited 50 consecutive patients with chronic HCV who fulfilled criteria for treatment with pegylated interferon and ribavirin. The mean age of patients was 40.7 ± 9.7 years, and 22 (44%) of treated patients were males.

Insulin resistance:

After calculation of HOMA-IR to our patients, it appeared that the mean HOMAIR was 3.2±1.5 with minimum 0.97 and maximum 6.73. Considering HOMA-IR more than 2.5 as insulin resistance, 32 patients (64%) had insulin resistance. Insulin resistance was higher in male gender 15/22 (68.2%) than female 17/28 (60.8%) but with no statistical significant difference. There is a statistically significant association between level of ALT and insulin resistance (P=0.04). Table 1 is showing the relation between Insulin resistance and baseline variables, and virological response.

We found that the upper quarter for distribution of HOMA-IR (75 percentile) is 4. We considered HOMA-IR above 4 as high Insulin resistance, 13 patients (26%) had high insulin resistance. Male gender was found in 9/13 (69.2%) of insulin resistance, and 13/37 (35.1%) (P=0.03). Rapid virological response was found in 6/13 (46.2%) of Insulin resistance and in 29/37 (78.4%) of patients with insulin sensitivity (P=0.03).

Test of correlation was done between HOMAIR and all continuous

variables. A significant statistical correlation was found between HOMAIR and ALT level (Pearson correlation= 0.30, P=0.04), tendency of significant correlation with age (Pearson correlation = -0.25, P=0.07), AST (Pearson correlation = 0.26, P=0.06). We had 4 patients with moderate steatosis, 3 (75%) of them had insulin resistance.

Rapid virological response:

A rapid virological response (decrease of viral load with 2 log or more from baseline after 4 weeks of therapy) was found in 35/50 (70%). Same patients who achieved a rapid virological response achieved also early virological response. Table 2 shows the relation between baseline variables and rapid virological response. Insulin resistance with HOMAIR >4 was found to be associated with failure to treatment response (P=0.03). Other variables which had a significant or tendency for significant association with virological response (serum albumin, P=0.005, TSH, P=0.01, BMI, P=0.04, and inflammatory activity grade p=0.08) were included with insulin resistance variable in a logistic regression model. Table 2 is showing that after adjustment for other variables, still IR an independent predictor of failure to achieve rapid or early virological response.

Table (1): Univariate analysis for baseline variables and rapid virological response association with insulin resistance.

Baseline variables	Total patients 50	Insulin sensitive 18 (36%)	Insulin resistant 32 (64%)	P
Age, years	40.7±9.7	43.7±10.1	38.9±9.2	0.09
Gender, Male	22(44%)	7 (38.9%)	15 (46.9%)	0.6
BMI (Kg/m²)	24.2±1.1	24.2±0.9	24.2±1.2	0.4
ALT (IU/L)	56.9±9.1	49.5±18.3	61.1±18.5	0.04
AST (IU/L)	65.5±62.9	52.5±22.8	72.8±76.3	0.3
Total bilirubin (mg/dL)	0.86±0.23	0.95±0.27	0.82±0.2	0.05
Alkaline phosphatase (IU/L)	151.7±58.2	148.7±56.6	153.4±59.9	0.8
Serum albumin (gm/L)	4.5±0.4	4.5±0.5	4.5±0.4	0.7
Prothrombin concentration (%)	90.4±7.4	91.7±6.5	89.6±7.9	0.3
AFP (ng/ml)	21.4±38.1	12.5±18.7	26.4±45	0.2
Hemoglobin (gm/dL)	13.5±1.6	13.7±1.6	13.6±1.4	0.7
WBCsx1000/μL	6.1±1.8	5.7±1.7	6.3±1.8	0.3
Platelet x 1,000/μL	215.2±56.6	233.7±56.8	204.9±54.7	0.08
TSH (pg/mL)	1.5±0.7	1.6±0.6	1.5±0.7	0.9
HCV RNA PCR/IU	285636±317693	272326±262455	292707±347240	0.8
Activity grade, >1	12 (24%)	5 (27.8%)	7 (21.9%)	0.7
Fibrosis stage, >1	23 (46%)	10 (55.6%)	13 (40.6)	0.4
Steatosis grade, >0	27 (54%)	11 (61.2%)	16 (50%)	0.6
Rapid virological response	35 (70%)	13 (72.2%)	22 (68.8%)	0.8

Table (2): Univariate analysis for baseline variables association with rapid virological response

Baseline variables	Total patients 50	Rapid virological responders 35 (70%)	Rapid virological non-responders 15 (30%)	P
Age, years	40.7±9.7	40.8±10.3	40.3±8.4	0.9
Gender, Male	22(44%)	17 (48.6%)	5 (33.3%)	0.3
BMI (Kg/m²)	24.2±1.1	24±1.1	24.7±0.9	0.04
ALT (IU/L)	56.9±9.1	54.9±19.3	61.7±18.4	0.2
AST (IU/L)	65.5±62.9	55.1±23.9	89.7±107.6	0.07
Total bilirubin (mg/dL)	0.9±0.2	0.9±0.2	0.9±0.3	0.9
Alkaline phosphatase (IU/L)	151.7±58.2	144.5±58.2	168.6±56.5	0.2
Serum albumin (gm/L)	4.5±0.4	4.4±0.3	4.7±0.5	0.005
Prothrombin concentration (%)	90.4±7.4	90.6±6.5	89.9±9.5	0.8
AFP (ng/ml)	21.4±38.1	21.1±28.9	22.2±55.1	0.9
Hemoglobin (gm/dL)	13.5±1.6	13.6±1.6	13.6±1.7	0.9
WBCsx1000/mL	6.1±1.8	6.1±1.8	5.9±1.6	0.6
Platelet x 1,000/mL	215.2±56.6	216.7±61	211.9±46.5	0.8
TSH (pg/mL)	1.5±0.7	1.4±0.5	1.9±0.9	0.01
HCV RNA PCR/IU	285636±317693	265232±303375	336645±357807	0.5
Activity grade, >1	12 (24%)	6 (17.1%)	6 (40%)	0.08
Fibrosis stage, >1	23 (46%)	15 (42.9%)	8 (53.3%)	0.6
Steatosis grade, >0	27 (54%)	21 (60%)	6 (40%)	0.1
HOMAIR >4	13 (26%)	6 (17.1%)	7 (46.1%)	0.03

Table (3): Logistic regression analysis for prediction of rapid virological response:

Predictors	OR	Lower 95%CI	Upper 95%CI	P
BMI (Kg/m²)	0.04	0.003	0.6	0.02
Serum albumin (gm/L)	0.029	0.001	0.587	0.021
IR (HOMAIR>4)	17.78	1.13	280.03	0.04
BMI>25 Kg/m²	0.02	0.0	1.51	0.076
TSH (pg/mL)	0.17	0.025	1.21	0.077
Activity grade, >1	8.52	0.57	128.62	0.12
AST (IU/L)	0.97	0.93	1.01	0.17

Discussion

IR has been reported to be associated with HCV infection more often than with other chronic liver diseases[16]. It was estimated in one study that CHC patients have a 3-fold increased risk of IR[17]. In addition, different sources have indicated that roughly 50% of patients with CHC exhibit some evidence of IR[18,12,19,20,21].

Although the most common method for measuring IR is HOMAIR, The cutoff value for insulin resistance in HOMA is a matter of debate. Taniguchi at al. defined the value >2.5 as an insulin-resistant state based on the HOMA-IR value[22]. While Sihoon et al. in their study, found that the cutoff point for defining insu-

lin resistance is a HOMAIR = 3.04 [23], Michalzuk et al. defined 2.71 as a cutoff value for insulin resistance in HOMAIR score[24] and Moucari et al. defined IR as HOMAIR >3[18], and Esteghamati et al. defined IR at a cutoff value of 1.77 and 4.33 for Iranian non diabetic and diabetic respectively[25].

Prevalence of IR among CHCV patients: In our study, we found that 32 out of 50 patients 64% (95% CI 51-77) had HOMAIR>2.5. Bakir et al. studied the prevalence of IR among Egyptian CHCV patients and found a prevalence of 46.7% (95% CI 28.8-64.6) with HOMAIR=2 and these results overlap and comparable to our results[26]. Hao et al. found that the prevalence of IR (HOMAIR>2.5) in CHCV G1 and G2 is 39.2% (95% CI 31.7-46.7%), with HOMAIR =3 the prevalence of IR was 54%[15]. Moucari et al. studied IR in 226 chronic HCV genotype 4 patients and found that 105 of them (46%) had IR with HOMAIR >3[18]. This result is comparable to our results where we found that 54% of our patients had HOMAIR >3. A prevalence of 56.1% was reported by Hsu et al. [19]. In the latter study, subjects with cirrhosis were not excluded. The different composition of the patient population might explain this discrepancy, as many studies have shown that IR is more prevalent in subjects with advanced fibrosis or cirrhosis[27,28,29]. It appears from the previous studies that the prevalence of IR is around 50% with no difference among different genotypes which is comparable to our results.

The relationship between IR and HCV infection is complex and bidirectional, and the exact pathogenesis is still unknown. Some clinical observations support a fat independent mechanism in the development of IR in HCV - infected subjects[21]. Koike et al. demonstrated in an animal model that HCV can induce IR itself by disturbing the insulin signaling pathway[6]. The author found that an elevated level of tumor necrosis factor, might inhibit tyrosine phosphorylation of insulin receptor substrate-1 in the liver, suppress intracellular transduction of insulin signals, and lead to IR in mice transgenic for the HCV core gene. HCV has also been reported to mediate dysfunction of insulin

signaling pathways by up regulating the expression of suppressor of cytokine signaling 3 or attenuating signal transducer and activator of transcription[30,31,32].

Relation between baseline variables and HOMAIR: Two previous studies have claimed a dose-response relationship between the serum HCV RNA titer and IR [18,19]. However, a recent multicenter study provided contradictory data[20]. Our data, consistent with the latter, indicated that the serum HCV RNA level was not a predictor of IR in patients with genotype 4. We found that there is no significant difference between mean level of HCV RNA viral load in insulin resistant and insulin sensitive patients at different cutoff values for HOMAIR (P=0.8 at cutoff 2.5 HOMAIR). We found also that there is no correlation between HOMAIR index and viral load (R=0.01, P=0.9). Same finding was reported by Hao et al. in genotype 1 and 2 HCV infection[15].

This disconcordance raises important questions regarding the pathogenesis of IR in CHC, and suggests that the exact pathways need further exploration. The regulation of insulin is a complex interplay between the liver, adipose tissue, and muscle[33]. Many factors affect insulin resistance, such as age, sex, obesity, cytokines, hepatic fibrosis and steatosis. HCV infection is just one of the contributing factors. Perhaps, intrahepatic replication, not the serum viral load, is a more sensitive HCV replication marker to predict the development of IR.

Regarding the relation between liver function tests and HOMAIR, we found that patients with insulin resistance have significantly higher ALT than patients with insulin sensitivity (P=0.03). We found also a significant correlation between HOMAIR and ALT (R2=0.3, P=0.03) and AST levels (R2=0.26, P=0.06). The same finding was reported by Mohamed et al.(34) who studied IR in a large number of CHCV Egyptian patients and found a correlation between HOMAIR and ALT (R2=0.21, P=0.0002) and AST (R2=0.16, P=0.02). However in a study by Hao et al.[15], there was no association between IR and ALT or AST in CHCV genotype 1 and 2 patients.

This disconcordance could be due to variations in HCV genotype behavior. Whether the increase in ALT reflects increase in inflammatory activity in liver biopsy or not is not known. However, we failed to find a significant association between grade of inflammatory activity in liver biopsy and HOMAIR (P=0.7). Failure to find this association could be due to absence of grade III and IV inflammatory activity from our study group.

Regarding fibrosis and steatosis, we could not find a significant association between IR and steatosis or fibrosis (P=0.6 and 0.4 respectively). This failure to find association can be due to having all our patients with no or mild steatosis except for 4 (8%) patients with moderate steatosis. We have only one patient with stage III fibrosis and all other patients were stage I and II. Although Hao et al. did not find any association between HOMAIR and non invasive markers of fibrosis (APRI score and AAR score)[15], Moucari et al. found that IR is associated with severe fibrosis (Stage III-IV) with OR=2.7[18]. This disconcordance because the later study included more patient with severe fibrosis.

Because IR has a relation to obesity and type 2 DM, we tested the relation between HOMAIR and BMI. We found that there is no significant difference between mean BMI in insulin resistant patients and insulin sensitive patients. However, when we classified BMI categorical to above 25 kg/m and equal to or less than 25kg/m, we found that 13/16 with BMI>25 (81.3%) had IR and 19/34 (55.9%) of patients with BMI≤25 had IR (P=0.08). Elevating the cutoff value for HOMAIR to 4 showing that only 5/34 (14.7%) of patients with BMI ≤25 had IR in comparison to 8/16 (50%) of patients with BMI>25 (P=0.008). Moucari et al. found the same significant association between BMI>25 and HOMAIR[18]. This finding means that patients with CHCV and BMI>25 are 2.1 folds more risky to have IR than patients with BMI ≤25 (OR= 2.1). Whether this IR affects rapid virological response or not is in need to be clarified.

Predictors of rapid virological response: In our study, we found

that BMI, AST, serum albumin, TSH, and IR with HOMAIR>4, and inflammatory activity were associated with RVR in univariate analysis. In logistic regression analysis, only IR (OR=17.8, P=0.04) and BMI (OR=0.04, P=0.02) were significantly negative predictors of RVR and serum albumin (OR=0.03, P=0.02) is a positive predictor of RVR. Because most of the studies were interested in SVR, Moucari et al. found a significant association between insulin sensitivity and SVR in HCV G4 patients(18). Same finding was confirmed by Bakir et al. from HCV Egyptian patients(26). As the RVR is predictor of SVR, we found here same finding of association of IR with RVR. Saad et al. have reported also that BMI and IR are negative predictors for SVR in CHCV Egyptian patients(35).

In conclusion, we found that IR is highly prevalent among CHCV Egyptian patient and associated with elevated AST and correlated with ALT and is a negative predictor for RVR after adjusting for all other variables.

This study has shortcomings of small sample size which makes it sometimes under power and representative only for lean non diabetic without severe fibrosis or cirrhosis patients.

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HIGH INSULIN RESISTANCE IS ASSOCIATED WITH LOW RAPID VIROLOGICAL RESPONSE IN CHRONIC HCV PATIENTS

Mohamed A. Metwally MD, Roshdy M. Khalaf Allah MD and Inas A. Ahmed MD

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BALB/C MICE IMMUNISED WITH CENTRIN-3 VACCINE IS PROTECTED AGAINST LEISHMANIA DONAVANI

Fathiya Asteal Ph.D*, Salem Abdalla Ph.D, Aisha Zaidi Ph.D*** and Selman Ali Ph.D******

**Department of Medical Laboratory, Faculty of Medical Technology, Sert University, Libya*

***Department of Pharmacology, Faculty of Medicine, Tripoli University, Libya*

****Department of Biochemistry, Faculty of veterinary Science, Tripoli University, Libya*

*****College of Arts & Science, School of Science & Technology, Nottingham Trent University*

Abstract

Leishmaniasis is a parasitic protozoal disease affecting humans and animals with phlebotomine sand flies as intermediate vectors. There is no effective vaccine in use against this parasite and production relies on finding potent immunogenic antigens with long lasting memory response. As a part of searching for new Leishmania antigens of a potential vaccine application, the immunogenicity of L. donovani centrin-3 (Ldcen-3) was investigated in a Balb/c model. Ldcen-3 is a calcium binding protein that has been shown to be involved in duplication and segregation of the centrosome in higher and lower eukaryotes. The Ldcen-3 gene was cloned in various vectors and coated on gold particles for gene gun immunisation. Significant protection was induced by immunisation with 1μg DNA of pcDNA3.1-Ldcen-3 or pCRT7/CT-TOPO-Ldcen-3 constructs. Protection against challenge with live parasite was vector dependent where better protection was induced by pCRT7/CT-TOPO-Ldcen-3. Splenocytes from Balb/c mice immunised with pcDNA3.1-Ldcen-3 or pCRT7/CT-TOPO-Ldcen-3 had a potent CTL response against DC targets loaded with SLA or tumour cells transfected with Ldcen-3 plasmid construct.

Key words: *Immunogenicity, Leishmania, Centrin, vaccine*

Introduction

Immunisation with plasmid DNA encoding Leishmania antigens represents a promising approach to vaccination against Leishmaniasis as it induces both humoral and cell mediated immune responses and results in long lasting immunity[1]. Therefore, DNA vaccination could potentially treat and prevent Leishmaniasis. Many vaccine strategies have been pursued, including the use of whole cell lysate, killed, virulent or irradiated parasite[2]. Leishmania DNA vaccines and purified or recombinant parasite antigens have also been tested. Most of the studied antigens have so far shown a limited degree of effectiveness as a potential vaccine in animal models but little or no protection in humans. New antigen discovery strategies are essential to identify new antigens with potential as a novel vaccine candidate.

The immunogenicity of centrin genes have not been previously studied and very little is known of their biology in Leishmania. A DNA-encoding N-terminal domain of the proteophosphoglycan (PPG) gene, which is a surface-bound protein in both promastigotes and amastigotes, was investigated as a vaccine in hamsters against challenge with L. donovani. A protection efficiency of about 80% was observed in vaccinated hamsters with more than 6 months survival after challenge. The efficacy was supported by a surge in inducible nitric oxide synthase, IFN-γ, TNF-α, and IL-12 mRNA levels along with down-regulation of TGF-β, IL-4, and IL-10. The level of Leishmania specific IgG2 was also increased which was indicative of an enhanced cellular immune response. It was concluded that the N-terminal domain of L. donovani PPG is a potential DNA vaccine against visceral Leishmaniasis[3].

Centrins are cytoskeletal calcium binding proteins that are localized in the microtubule organising centre (MTOC) of eukaryotic cells[4]. There are a number of eukaryotic centrin genes that have been identified, including four genes identified in mice, and three in humans[5,6]. The recently completed genome database for two trypanosomatids, i.e., Trypanosoma brucei, the causative agent of

African sleeping sickness and Leishmania, have revealed five centrin genes in this group of organisms[7]. The functions of some of the centrins have been identified, for example, one group of centrins, which includes: C. reinhardtii centrin, Paramecium centrins 2 and 3, mouse centrins 1 and 2 and human centrins 1 and 2, are involved in centrosome and basal body segregation[8,9]. The other group containing Leishmaniacentrin-1, yeast centrin, mouse centrin-3 and human centrin-3, plays a role in centrosome and basal body duplication[10,11]. The N-terminal non-conserved domain of centrins, which is variable in length, is considered to be responsible for the functional diversity of centrins[6,12]. L. donovani centrin 3 (Ldcen-3)has a significantly smaller N-terminal region compared to centrins from other species[13].

The Ldcen3 gene is 100% homologous in L.donovani, L. major and L. mexicana. Therefore, the use of a DNA vaccine to stimulate an immune response against this protein could represent a novel approach to immunise humans against more than one species of the parasite. A DNA vaccine encoding Ldcen3 could offer protection against both visceral and cutaneous Leishmaniasis because of heterogeneity in DNA sequence to that of human centrin-3 (Fig. 1).

Knocking out the centrin-3 (Ldcen-3) gene reduced the growth rate of both promastigotes and amastigotes, and reduced survival in human macrophages in vitro [13,14]. Other studies showed that dominant negative expression of centrin proteins by parasites could result in reduced survival in macrophages in vitro or in reduced virulence in mice in vivo[15].

Methods

In the present study, the immunogenicity of Ldcen-3 was investigated in a Balb/c L. mexicana model, using a gene gun to release a plasmid DNA construct coated on gold particles. The gene gun fires DNA coated gold particles at high velocity directly into epidermal cells, which consist of skin cells, Langerhans cells (LC) and dermal dendritic cells (DC). Inside the cell, plasmid is transported to the nucleus, the encoded gene is transcribed and the protein is

subsequently produced, processed into peptides by host proteases and then presented in the context of MHC class I antigen which then stimulates CD8+ T cells[16,17]. DC directly transfected with DNA vaccine can prime CD8+ cells by presenting the DNA encoded antigen via MHC class I. Immature DC can endocytose soluble proteins and debris from apoptotic transfected cells and express the coded antigen through MHC class I or MHC class II following differentiation into mature DC. Thus, a DNA vaccine can be effective in the stimulation of both CD8+ T cells and CD4+ T cell populations. The ability of DCs to present extracellular antigens into MHC class I and MHC class II is known as cross priming. Accordingly, DCs play an important function in the induction of both humoral and cell mediated immunity following DNA vaccination.

Results

Confirming pCRT7/CT-TOPO as a mammalian vector

Sub cloning of LacZ into pCRT7/CT-TOPO

To provide evidence that pCRT7/CT-TOPO-Ldcen-3 could transfect and express genes of interest in mammalian cells, pCRT7/CT-TOPO-Ldcen-3 was constructed incorporating the lacZgene as a marker and was used to transfect a mammalian cell line (Fig 1). Briefly, the lacZ gene was cut from pcDNA3.1myc-His lacZ (-) vector from both sides using XbaIand Hind III restriction enzymes. The digested fraction was separated by gel electrophoresis (1.5 %). The pCRT7/CT-TOPO vector was also digested using the same restriction enzymes (Fig 2-A, B, C&D). The Ldcen-3 was extracted from the gel and inserted in pcDNA 3.1(-) and then the lacZ gene and the empty pCRT7/CT-TOPO vectors were ligated using a DNA ligase enzyme. The pCRT7/CT-TOPO-lacZ vector was transfected in CT26 cells to establish the expression ability in mammalian cells; a suitable CT26 clone 25 (CT 26-lacZ) mouse tumour cell line stably transfected with lacZ gene, was used as a positive control.

Transfection of CT26 cells withpCRT7/CT-TOPO-lacZ

β-galactosidase, an important reporter gene encoded by the lacZ gene, is commonly used for moni-

toring transfection efficiency in mammalian cells. The β-galactosidase staining kit was used to determine the expression of lacZ following transient or stable transfection of plasmids encoding lacZ. β-galactosidase catalyzes the hydrolysis of X-gal producing a blue colour. Transfected CT26 withpCRT7/CT-TOPO-lacZwas examined under a light microscope for the development of blue stain, which successfully produced a blue colour when compared to control cells CT26-lacZ indicating the ability of pCRT7/ CT-TOPO to express lacZ in mammalian cells (Fig.3).

Subcloning of Leishmania-donovani centrin-3 (Ldcen-3) into pcDNA3.1

Confirmation of the presence of Ldcen-3 by PCR

The pCRT7/CT-TOPO-Ldcen-3 vector was bulked up to obtain sufficient quantities of the plasmid and PCR was used to confirm Ldcen-3 presence using two primers designed for Ldcen-3 (541bp), Ldcen-3F, forward 5`AGA GGC ATT CGT GTT CG-3` and Ldcen-3R, reverse 5`AGG TTG ATC TCG CCA TCT TGA -3` (Fig.4).

To determine the sequence of the Ldcen-3 gene, the DNA sample along with two primers that were used for the PCR amplification were sent to MWG-biotech.com for sequencing. This confirmed the presence of the pCRT7/CT-TOPO-Ldcen-3 gene insert.

Subcloning of Ldcen-3 into pcDNA3.1 (-)

In order to adopt a widely used mammalian vector for DNA immunisation and also to transfect CT26 tumour cells it was decided to sub-clone the Ldcen-3 gene into a pcDNA3.1 (-) vector (Fig.5), which contained a mammalian selectable marker antibiotic gene. Ldcen-3 was cut from both sides by XbaI and Hind III restriction enzymes from the pCRT7/CT-TOPOvector. The digested fractions were separated by gel electrophoresis (Fig.6A). The pcDNA3.1 (-) vector was also cut using the same restriction enzymes. The Ldcen-3 gene and the digested pcDNA 3.1 (-) vectors were then ligated using a DNA ligase enzyme. The presence of the Lcen-3 gene in the pcDNA3.1(-) vector was determined by restriction enzyme digestion (Fig. 6B)

and PCR amplification (Fig. 6C) using forward and reverse primers Ldcen-3F 5`AGA GGC ATT CGT GTT CG-3`andLdcen-3R, reverse5`AGG TTG ATC TCG CCA TCT TGA -3`.

Construction of pCRT7/CT-TOPO empty vector

In order to produce a pCRT7/CT-TOPO empty vector to be used as a negative control in DNA vaccination and protection studies, the Ldcen-3 gene was cut and removed from this vector. The Ldcen-3 gene was cut out from the vector by digestion with XbaI and Hind III restriction enzyme and the product was run into an agarose gel. The band related to the vector was extracted from the gel (Fig. 7). Both free ends of the vector that resulted from digestion were then ligated to each other by the ligase enzyme. The absence of the Ldcen-3 gene in the empty vector was confirmed by PCR using Ldcen-3 primers.

Immunogenicity of Ldcen-3

Protection induced by immunisation with pCRT7/CT-TOPO-Ldcen-3 plasmid construct

To determine the immunogenicity of Ldcen-3 (L. donovanicentrin-3), a pCRT7/CT-TOPO-Ldcen-3was used as a DNA vaccine in a Balb/c mouse model. L. mexicana gp63 construct (VR1012-gp63) was used as a positive control since this gene (L. mexicana gp63) was shown to induce strong immunity by DNA-gene gun immunisation[17]. The results (Fig. 8) clearly demonstrated that mice immunised with Ldcen-3 or gp63 were significantly protected against challenge with live parasites, 5 out of 6 mice were lesion free in Ldcen-3 or gp63 groups.

Protection induced by immunisation with pCRT7/CT-TOPO- Ldcen-3 and pcDNA3.1-Ldcen-3 plasmid construct

The immunogenicity of Ldcen-3 cloned in two different vectors was investigated to confirm the immunogenicity to Ldcen-3. Balb/c mice were immunised by gene gun with 1µg of pcDNA3.1-Ldcen-3. pCRT7/CT-TOPO-Ldcen-3, empty pcDNA3.1, and empty pCRT7/CT-TOPO, PBS was used as a control. The results show that a significant protection was induced by immunisation with 1µg Ldcen-3 constructs which was vector dependent since pCRT7/CT-TOPO-Ldcen-3 (4 out of 6 free of lesion) induced

better protection than pcDNA3.1-Ldcen-3 (3 out of 6 free of lesion) (Fig. 9). The empty pCRT7/CT-TOPO (0/6) vectors did not protect mice from challenge. Although immunisation with empty pcDNA 3.1 vector slowed down lesion development in immunised mice.

CTL activity in Balb/c mice immunised with pcDNA3.1 (-)-Ldcen-3 and pCRT7/CT-TOPO-Ldcen-3 by gene gun

To evaluate the role of cytotoxic T cells in immunity to Leishmania, a standard 4-hour ^{51}Cr-release cytotoxicity assay was used to assess the ability of L. mexicana Ldcen-3construct to generate specific cytotoxic T lymphocytes by immunisation. Balb/c mice were immunised with pcDNA3.1 (-)-Ldcen-3 and pCRT7/CT-TOPO-Ldcen-3. Splenocytes were harvested from immunised mice and cultured in vitro for 5 days together with blasts cells pulsed with LPS and SLA2 (see chapter 2 methods). On day 5, the splenocyte cells were used as effectors in standard 4-hour ^{51}Cr-release cytotoxicity assay against non-adherent DCs loaded with SLAs and DCs alone as target. Splenocytes from Balb/c mice immunised with pcDNA3.1 (-)-Ldcen-3 or pCRT7/CT-TOPO-Ldcen-3 induced significant CTL activity compared with empty vectors (Fig. 10).

Transfection of CT26 cells with pcDNA3.1 (-)-Ldcen-3

In this study, CT26 tumour cells were transfected with pcDNA3.1 (-)-Ldcen-3 DNA using lipofectamine 2000, according to the manufacturer's instructions, to investigate CTL activity against targets expressing Ldcen-3 antigen. The presence of the Ldcen-3 gene was determined in the stable transfected cells by RT-PCR using forward and reverse primers: Ldcen-3F 5`AGA GGC ATT CGT GTT CG-3` andLdcen-3R, reverse5`AGG TTG ATC TCG CCA TCT TGA -3`. The transfected CT26-Ldcen-3 clearly shows a strong band for Ldcen-3. Also, for unknown reasons, non-transfected CT26 cells always showed a faint band when tested with the primers, which is not a specific band compared with transfected CT26 this was also observed when CT26 cells was transfected with L. mexicana gp63 plasmid construct[17] (Fig. 11).

CTL activity in Balb/c mice by immunisation with Ldcen-3construct against tumour targets

Balb/c mice were immunised twice at a two week interval with Ldcen-3 construct coated on gold particles by gene gun. Mice were sacrificed two weeks following the 2nd immunisation and spleens were collected. Splenocytes were harvested and cultured in vitro for 5 days together with blasts cells pulsed with LPS and SLA2 (SLA may contain Ldcen-3 protein). On day 5, the splenocytes cells were used as effectors in a standard 4-hour ^{51}Cr-release cytotoxicity assay against CT26 tumour cells transfected with Ldcen-3 (Fig. 12). The results clearly show that immunisation of mice with Ldcen-3construct induce specific CTL activity against CT26 tumour cells expressing Ldcen-3. The in vitrorestimulation of CTLs by SLA2 loaded blast cells was crucial. It was shown that removing the in vitrorestimulation of the splenocytes prevented the generation of CTL activity in immunised mice and levels were comparable with that of naïve mouse splenocytes re-stimulated in vitro by blast cells loaded with SLA2. Maximum cytotoxity was observed even at the minimum effector to target (E:T) ratio of 6:1 suggesting the need for further testing with different effector to target ratios for unknown reasons.

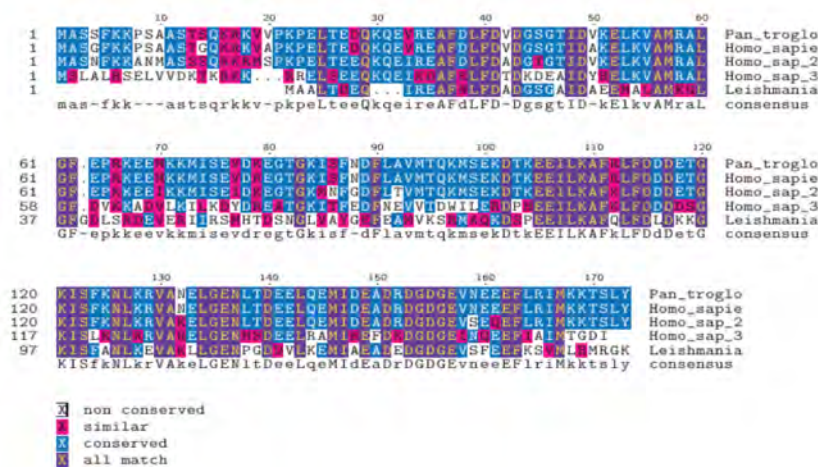

Fig.1: The amino acid sequence of Ldcen-3 compared with human centrins.

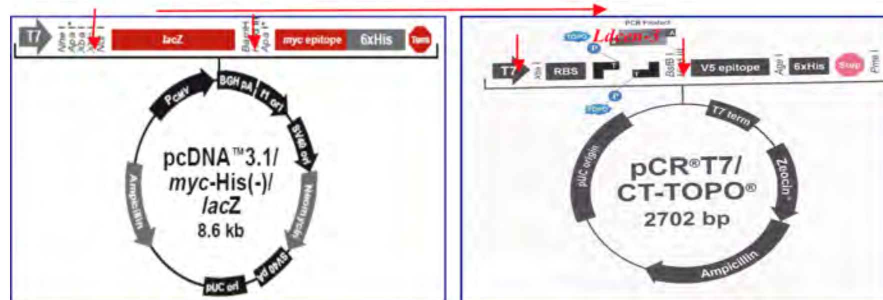

Fig.2A: Map representing pCRT7/CT-TOPO and pcDNA 3.1 myc-His/lacZ: pCRT7/CT-TOPO vector containing -Ldcen-3 gene and pcDNA 3.1 myc-His LacZ (-) to sub cloneLacZ in pCRT7/CT-TOPO.

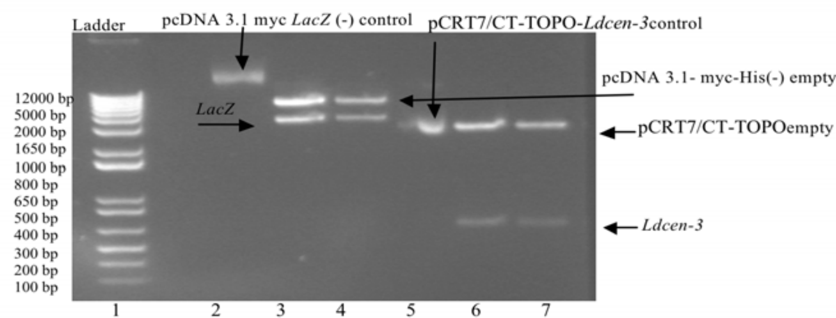

Fig.2B: Digestion of pcDNA 3.1 and pCRT7/CT-TOPO: Both plasmids were cut by XbaI and Hind III **restriction enzymes:** 1- ladder; 2- pcDNA 3.1 myclacZ (-); 3, 4-the above band is the linear - empty pcDNA 3.1 myc-His and lower band islacZ; 5, pCRT7/CT-TOPO-Ldcen-3 control; 6, 7, above band is the linear empty vector pCRT7/CT-TOPO and lower band is Ldcen-3.

Fig.2C: Sub cloning of lacZ into the pCRT7/CT-TOPO vector: The lacZ gene and the digested pCRT7/CT-TOPO vector were collected, purified and ligated using a DNA ligase enzyme, 1- 1kd ladder; 2- empty pCRT7/CT-TOPOand 3- pCRT7/CT-TOPO-lacZ (maps and vectors were obtained from invitrogen).

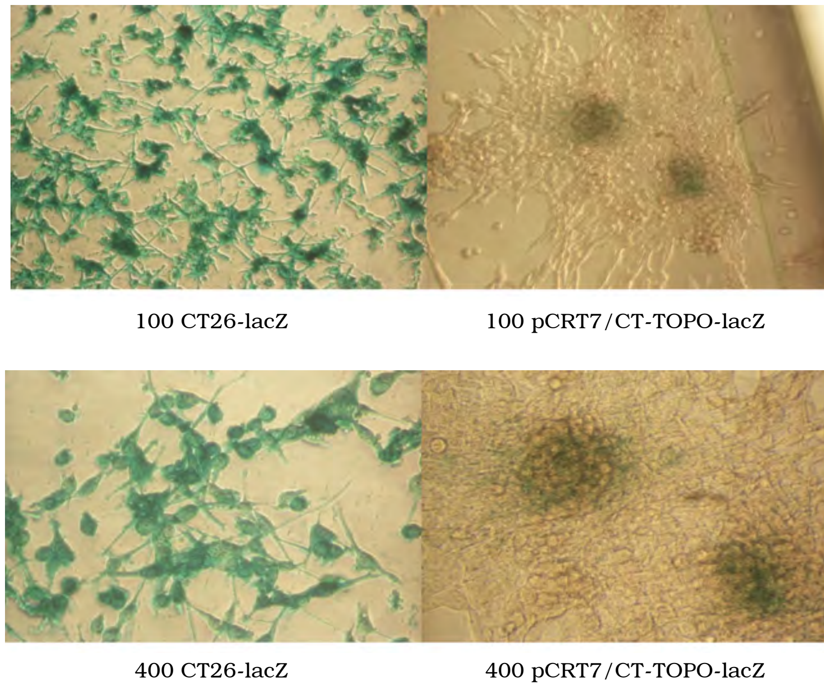

Fig.3: Expression of β-gal in CT26 transfected with pCRT7/CT-TOPO-LacZ CT26 cells transfected with a pCRT7/CT-TOPO-LacZ vector were washed twice with PBS and then fixed for 15 minutes with glutaraldehyde. Cells were then stained with X-gal for overnight to test the expression of pCRT7/CT-TOPO in mammalian cells. Blue colour staining indicates the expression of lacZ; suitable CT26-clone 25, (a stable transfectant with high expression of B-galactosidase) was used as a positive control.

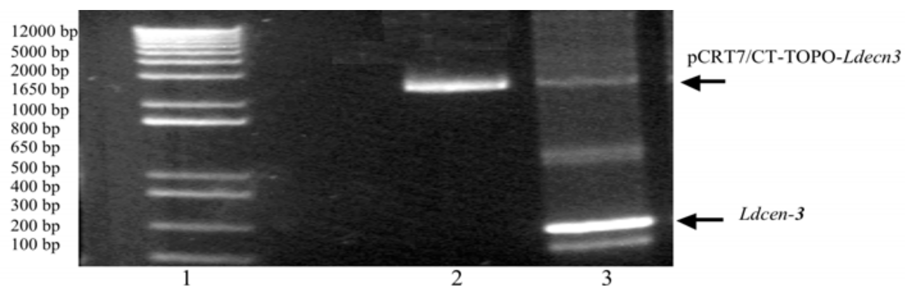

Fig.4: Confirmation of the presence of Ldcen-3 by PCR: The presence of Ldcen-3 was confirmed by PCR amplification using 5`AGA GGC ATT CGT GTT CG-3` forward and 5`AGG TTG ATC TCG CCA TCT TGA -3`reverse primers 1- ladder, 2- pCRT7/CT-TOPO-Ldecn3 as a control and not PCR result, 3- Ldcen-3 (PCR product).`

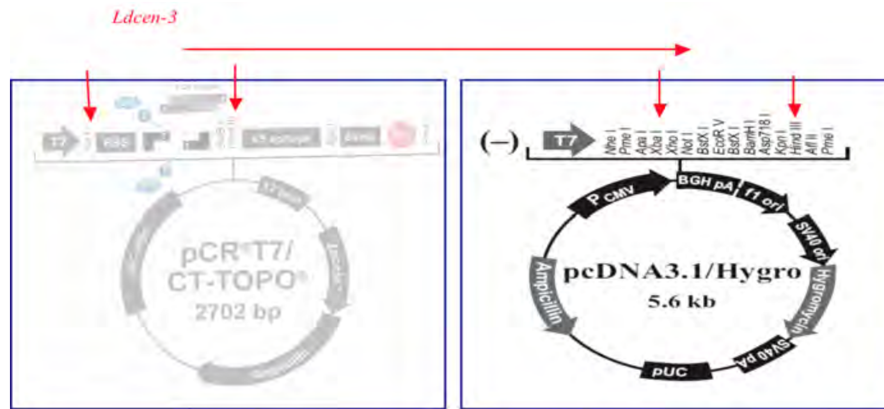

Fig.5: Map representing pCRT7/CT-TOPO-Ldcen-3 and pcDNA 3.1(-) vectors.

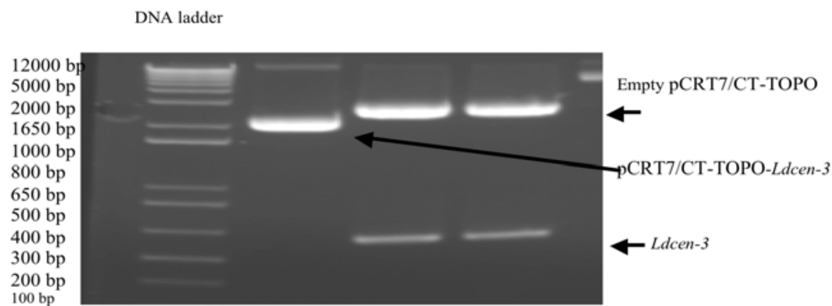

Fig.6A: subcloning of Ldcen-3 into pcDNA 3.1 (-) vectors: pCRT7/CT-TOPO-Ldcen-3 was cut by XbaI and Hind III restriction enzyme 1- ladder; 2- pCRT7/CT-TOPO-Ldcen-3, 3,4-the above band is a linear empty pCRT7/CT-TOPO and lower band is Ldcen-3.

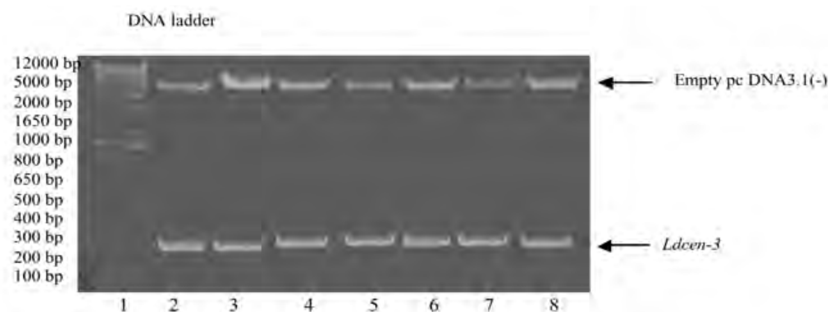

Fig.6B: Confirmation of the presence of Ldcen-3 in pcDNA3.1 (-): Restriction digestion with the same enzymes (Hind III and XbaI restriction enzymes), 1-ladder, 2-8 the upper bands are linear empty pcDNA3.1 (-) and the lower bands are Ldcen-3 after digestion of pcDNA3.1 (-)-Ldcen-3 with Hind III and XbaI.

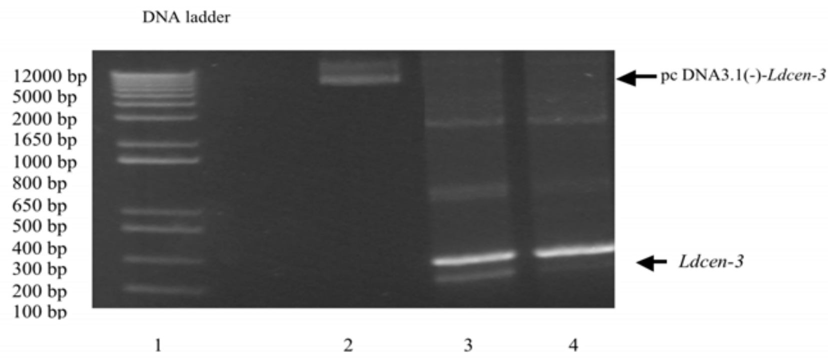

Fig.6C: Confirmation of the presence of Ldcen-3 in pcDNA3.1 (-) by PCR: The presence of Ldcen-3 gene was confirmed by PCR using Ldcen-3 forward primer F 5`AGA GGC ATT CGT GTT CG-3` and the Ldcen-3 reverse primer R, 5`AGG TTG ATC TCG CCA TCT TGA-3`, 1-DNA ladder, 2-empty pcDNA3.1 (-)-Ldcen-3 and 3, 4 Ldcen-3.

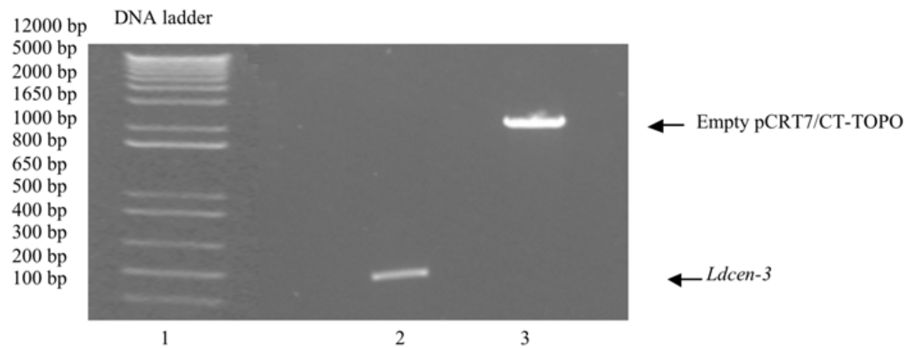

Fig.7: Production of empty pCRT7/CT-TOPO vector.

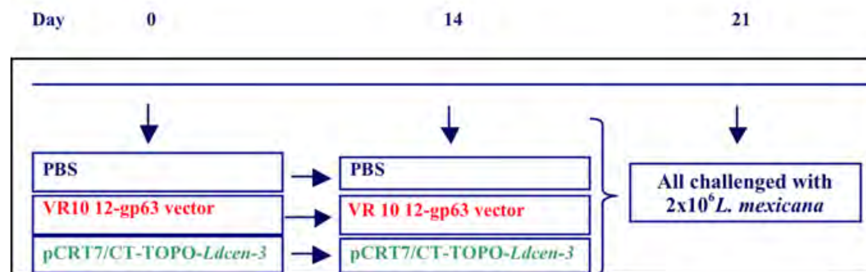

Fig.8A: Immunisation protocol.

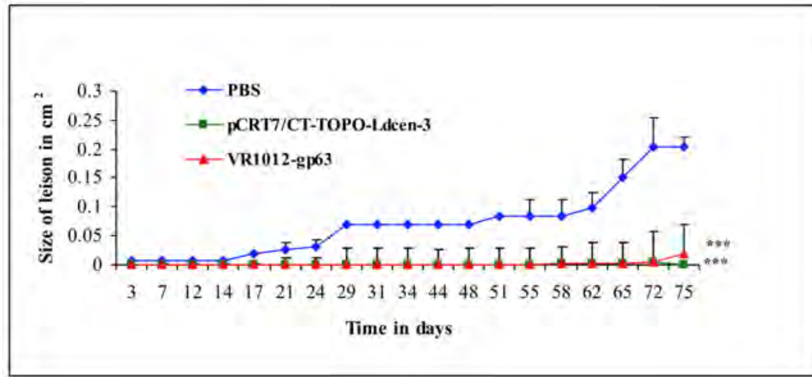

Fig.8B: Protection induced by immunisation with pCRT7/CT-TOPO- Ldcen-3 construct.

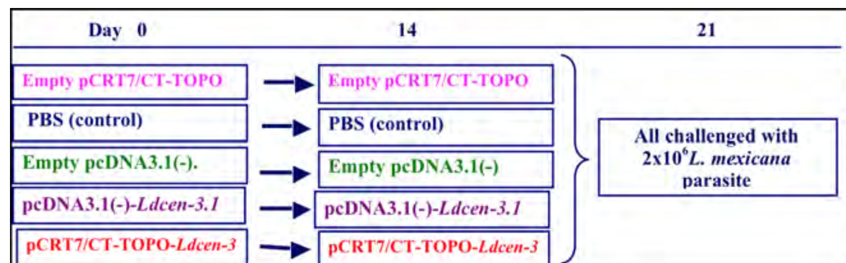

Fig.9A: Immunisation protocol.

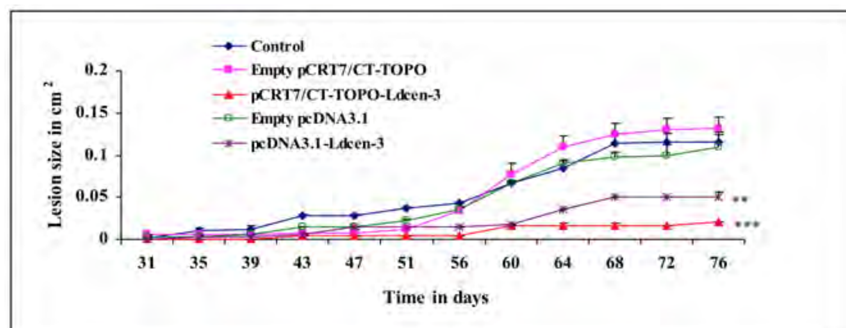

Fig.9B: Protection induced by immunisation with pCRT7/CT-TOPO- Ldcen-3 and pcDNA3.1-Ldcen-3.

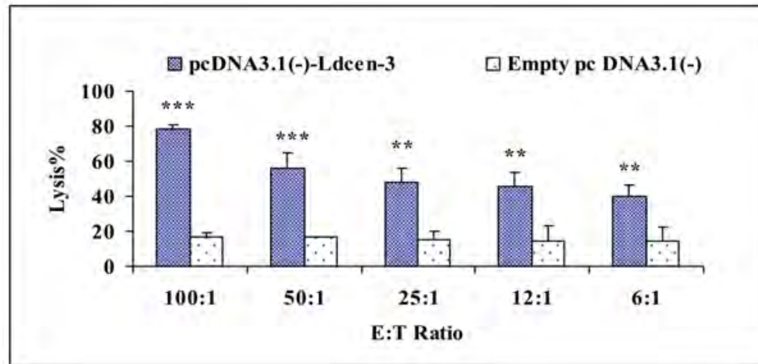

Fig.10A: CTL activity of Balb/c mice immunised with 1μg pcDNA3.1-Ldcen-3 or mice immunised with 1μg empty pcDNA 3.1(-) by gene gun.

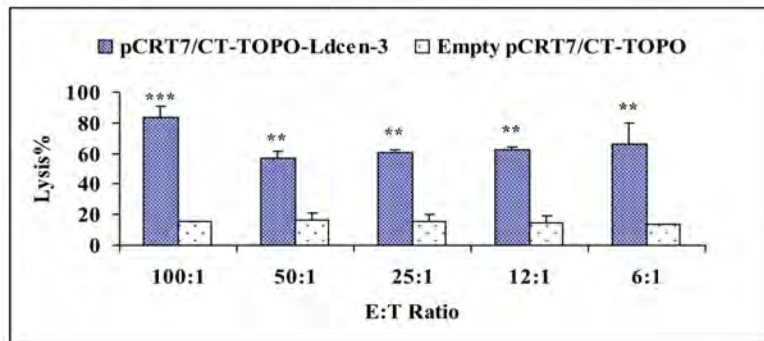

Fig.10B: CTL activity of Balb/c mice immunised with pCRT7/CT-TOPO-Ldcen-3 or immunised with empty pCRT7/CT-TOPO by gene gun. Splenocytes were stimulated with SLA for 5 days and used as effector cells in a standard 4-hour cytotoxicity assay against DCs pulsed with SLA.The graph represents 4 mice in 2 independent experiments P**≤0.01, P***≤0.001 by T test.

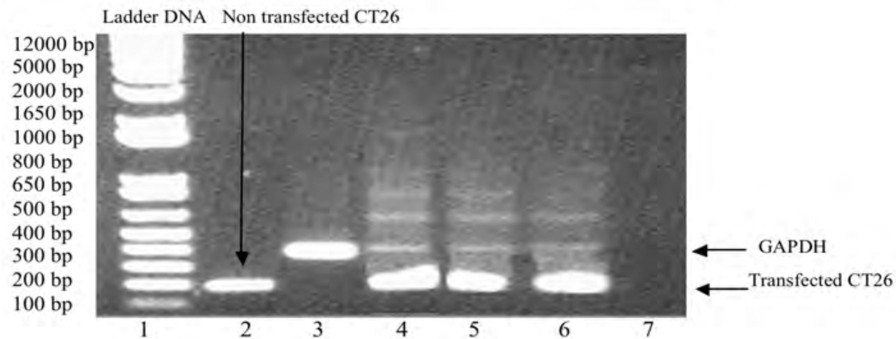

Fig.11: Expression of Ldcen-3 gene in transfected CT26 tumour cells as detected by RT-PCR.

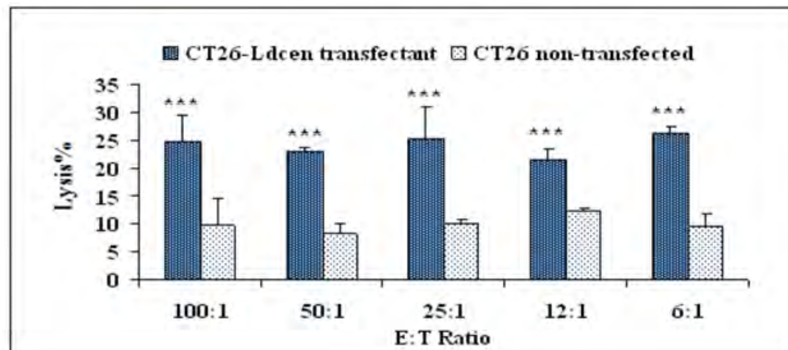

Fig.12: CTL activity of Balb/c mice immunised with 1μg pcDNA-Ldcen-3(-) by gene gun.

Discussion

Immunisation with naked plasmid DNA represents a promising new approach in prevention and treatment of various diseases [18]. DNA vaccines offer a considerable number of advantages over other vaccines and are therefore an appealing approach to vaccination against Leishmaniasis. A number of studies have demonstrated encouraging results with DNA vaccines and have highlighted their potential in both treatment and protection against Leishmaniasis [19-21]. DNA vaccines are usually constructed from bacterial plasmids that are designed to express a gene of interest in the host cells to initiate antigen specific immune responses[22,23]. The plasmid DNA enters the cell and goes to the nucleus where it is transcribed to messenger RNA. The transcribed messenger RNA enters the cytoplasm and is translated on the ribosomes. The expressed antigen is presented to corresponding cells and generates a humoral and cell mediated immune response.

There is a homology in the gene sequence of Ldcen-3 between different species of Leishmania. Ldcen-3 appears to be a suitable candidate for a DNA vaccine, since Ldcen-3 is 100% homologous between L. donovani, L.mexicana and L. major[24]. Selvapandiyan and coworkers[14] have previously shown that immunisation with a live attenuated L. donovanicentrin 1 gene-deleted parasite (LdCen1) could induce significant protection against Leishmaniasis in animals. Balb/c mice immunised with LdCen1

(Leishmania mutant) demonstrated early clearance of virulent parasite challenge compared with mice immunised with killed parasites, which was associated with a significant increase of cytokine (IFN-γ, IL-2, and TNF) producing $CD4^{+}$ T cells. Immunised mice also showed increased IgG2a and NO production in macrophages. Balb/c mice immunised with LdCen1were cross-protected against L. braziliensis suggesting that LdCen1is a safe and effective vaccine candidate against visceral and mucocutaneousLeishmaniasis.

To transfect CT26 tumour cells with Ldcen-3 to be used as targets to assess CTL activities in Balb/c mice immunised with Ldcen-3 plasmid construct, it was decided to sub clone the Ldcen-3 from pCRT7/CT-TOPO into a known mammalian pcDNA 3.1 plasmid. Garmory and coworkers[25] have reported that pcDNA 3.1 is a suitable mammalian vector having the cytomegalovirus (CMV) promoter which is required for optimal expression in mammalian cells. Also, pcDNA3.1/hygro is a suitable vector for a DNA vaccine. pcDNA3, which is very similar to pcDNA3.1/Hygro, has been used in other studies as a back bone for DNA vaccines against Leishmaniasis[26,27]. Therefore, Ldcen-3 was sub cloned from pCRT7/CT-TOPO into pcDNA 3.1 to be used as a vaccine and also to be transfected into CT26 tumour cell to be used as target cells in CTL assays.

CT26 transfected with Leishmania centrin is expected to present centrin-3 antigen on their surface MHC I and would be a suitable target for CTL activity against Leishmania antigens. Stable transfectants expressing Leishmania antigens would provide a suitable alternative target to fresh DCs in cytotoxicity assays. Splenocytes from Balb/c mice immunised with pcDNA3.1-Ldcen-3 or pCRT7/CT-TOPO-Ldcen-3 induced a potent CTL response compared to the control group against either DC targets loaded with SLA2 or CT26 tumour cells expressing Ldcen-3. Tumour cells can act as professional APC that would specially generate CTL if they express a tumor peptide-MHC class I complex and co-stimulatory molecules[28,29]. The immunogenicity of Ldcen-3cDNA

cloned in the pCRT7/CT-TOPO plasmid and pcDNA3.1 was determined via DNA vaccination in a Balb/c mouse model in vivo. Mice immunised with Ldcen-3 in pCRT7/CT-TOPOor in pcDNA3.1 (-) were significantly protected against challenge with live parasites; the known immunogenicity gp63 gene was used as a positive control. A dominant Th1 response was shown to have been correlated with protection in several animal models for Leishmania infection. Immunisation of Balb/c mice with a plasmid DNA vaccine containing gp63 gene from L. major, induced a dominant Th1 response that was protective against challenges with live parasites in vivo [17,30]. Susceptibility of Balb/c mice to Leishmania major infection was correlated to an inability to generate a Th1 response which could be restored by administration of IL-12[31,32]. This Th response would aid the development of $CD8^+$ CTLs capable of killing cells expressing appropriate antigen.

DNA vaccines produce potent CD8 CTL responses in mice against antigens from parasites and tumours. The construction of DNA vaccine-encoded antigens able to produce a CTL response includes whole protein, truncated protein and fusion with another protein[33,34]. Conry et al.[28] and Jacobsen et al.[35] have found that if the tumour cells are tranfected with plasmid DNA containing a tumour antigen gene then a specific CTL may be generated. In this work, CT26 tumour cells transfected with pcDNA3-Ldcen-3 were shown to be susceptible target cells to CTLs derived from Balb/c mice immunised with pcDNA3-Ldcen-3by gene gun. Ali et al.[17] have demonstrated a potent CTL activity in cultured splenocytes from Balb/c mice immunised L. mexicana gp63 DNA plasmid using I.M. injection and gene gun immunisation with against CT26 tumour cells transfected with pcDNA-gp63. Qin et al. [36] have shown a method of DNA immunisation using a prime-boost immunisation strategy (two different vaccines, each encoding the same antigen, given several weeks apart); better protection was obtained by gene gun immunisation. In addition, Gurunathan et al.[37] have reported the presence of long term antigen-specific Th1 activity

in mice immunised with a DNA vaccine containing a gene that coded for a Leishmania antigen. Rodriguez-Cortes et al.[1] found that a multivalent DNA vaccine, encoding TRYP which is a key enzyme of the trypanothione dependent metabolism for removal of oxidative stress in Leishmania, LACK and gp63, did not protect dogs against L. infantum experimental challenge, inspite of the hypothesis that an effective immune response was more likely to be generated following exposure to more than one antigen. Alternatively, Carter et al.[38] established that Balb/c mice immunised intramuscularly by parasite enzyme gamma glutamyl cysteine synthetase DNA vaccine protected them against L. donovani.

In conclusion, this study had shown for the first time that Balb/c mice immunised with pcDNA 3.1-Ldcen-3 or pCRT7/CT-TOPOLdcen-3constructs by gene gun induced potent protection against challenge with L. mexicana which was also correlated with CTL activity.

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BALB/C MICE IMMUNISED WITH CENTRIN-3 VACCINE IS PROTECTED AGAINST LEISHMANIA DONAVANI

Fathiya Asteal Ph.D, Salem Abdalla Ph.D, Aisha Zaidi Ph.D and Selman Ali Ph.D

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EFFECT OF CIRCULATING LEVEL OF INTERLEUKIN-6 (IL-6), INTERLEUKIN-8 (IL-8) AND TUMOR NECROSIS FACTOR-α (TNF-α) ON SURVIVAL IN PATIENTS WITH EARLY STAGE LUNG CANCER

Mona M. Halim MD*, Osama Elbaz MD and Nahla Anber MD

*Department of Clinical Oncology and Nuclear Medicine*and Department of Clinical Pathology, Mansoura University*

Abstract

Background: *Some patients diagnosed with early-stage lung cancer and treated according to standard care survive for only a short period of time, while others survive for years, for reasons that are not well understood. Associations between markers of inflammation and survival from lung cancer have been observed.*

Materials and methods: *Our study investigated whether circulating levels of inflammatory markers are associated with long versus short survival in stage I and II lung cancer. Patients who had survived either <80 weeks (short survivors, SS) or >150 weeks (long survivors, LS) were selected. Logistic regression was used to calculate adjusted odds ratios (ORs) and corresponding 95% confidence intervals (CIs). The false discovery rate was calculated to adjust for multiple testing.*

Results: *A total of 57 LS and 83 SS were included in this analysis from Mansoura University Hospital in the period between January 2007 to January 2008. Markers (TNF-α, IL-6 and IL-8) had adjusted OR on the order of 2- to 5-fold when comparing the upper and lower levels with regard to the odds of short survival versus long. IL-8 and TNF-α were the most significant markers associated with increased odds of short survival (OR=3.05, 95%CI 1.31-7.1 & P=0.002 and OR=2.92, 95%CI 1.25-6.78 & P=0.007 respectively). Smoking and chronic obstructive pulmonary disease were not associated with marker levels.*

Conclusion: *Our results provide some evidence that down regulation of inflammatory responses may play a role in the survival of early-stage*

lung cancer. These findings will require confirmation in future studies.

Keywords: *Interleukins, survival and early lung cancer .*

Introduction

Lung cancer is the leading cause of cancer death worldwide[1]. About 15% of lung cancers are diagnosed at early stages when the disease is more likely to be successfully treated[2]. But even in those patients diagnosed with early-stage lung cancer who receive optimal treatment, survival remains poor[2]. Treatment guidelines and prognosis are strongly based on disease stage at the time of diagnosis. Among those diagnoses with early-stage disease and treated by standard approaches, there is substantial heterogeneity in patient survival outcomes for reasons that are not well understood[3-5]. The identification of markers that reveal the underlying mechanisms related to survival from early-stage disease could aid in the development of new therapies.

Evidence from epidemiological and clinical studies suggests that inflammatory diseases of the lung, such as chronic obstructive pulmonary disease (COPD) or asbestosis, increase the risk of lung cancer mortality[6-8]. Similarly, smoking, also associated with poor survival after lung cancer diagnosis[9-11], is thought to alter the inflammatory microenvironment of the lung[12-14]. Genetic and molecular evidence has added support to the association between inflammation and lung cancer survival. Several studies have reported associations between single nucleotide polymorphisms in immune-related genes and survival in lung cancer[15-19]. Gene expression of 11 cytokines was capable of identifying a group of patients with poor survival in lung adenocarcinoma patients in a different study[20]. Elevated preoperative serum levels of several inflammatory markers such as basic fibroblast growth factor, an angiogenic cytokine, interleukin-8-6 (IL-6, IL-8), a pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) or angiogenic CXC chemokines have been associated with poor outcomes[21-24].

Despite these associations, the

studies included subjects with all disease stages who received varied treatments. In addition, studies frequently do not examine whether markers add information beyond standard clinical and pathological variables. Further, only a limited number of markers and their association with survival have been investigated. Multiplex assay platforms enable simultaneous evaluation of a large number of inflammatory circulating markers in small amounts of plasma[25]. Such an approach allows investigators to perform a comprehensive evaluation of the role of circulating levels of inflammatory markers in epidemiologic studies.

Patients and Methods

140 patients with stage I and II lung cancer were collected from Mansoura University Hospital in the period between January 2007 to January 2008. Lung cancer was diagnosed by standard clinical criteria and confirmed by pathology reports from surgery, biopsy, and imaging[27]. Vital status was determined by means of clinical records. The primary end point for this study was death due to any cause. The follow-up time was calculated as the time from diagnosis to the date of death from any cause or lost follow-up. Patients surviving either <80 weeks (short survivals) or >150 weeks (long survivals) were selected for this analysis (table 1).

Blood samples and laboratory methods:

Blood samples were collected from studied patients after end of standard therapy. Samples were tested for circulating acute phase proteins TNF-α, IL-6 and IL-8. Specimens within the acute phase protein were tested at a 1:2000 dilution. Values below the minimum detectable level were assigned half of the minimum level, while those above the maximum detectable level were given the maximum value.

Statistical analysis:

Marker levels were log-transformed to approximate a normal distribution. A Pearson X2 test was used to compare differences in detectability proportions for each marker between long survivors (LS) and short survivors (SS). Median levels of analytes were compared between LS and

SS using the Mann-Whitney test. Odds ratios (ORs), defined as the relative odds of being a SS versus a LS, and corresponding 95% confidence intervals (CIs) were estimated using adjusted logistic regression models for the levels of the markers. P-value <0.05 is considered statistically significant.

All analyses were done using the R software package (version 2.13.0). Principal component analysis (PCA) was carried out using the prcomp function in R for purposes of data dimension reduction. The weight (of loading) of each marker with each principal component (PC) was the correlation between the PC and the marker.

Results

A total of 140 lung cancer patients, 57 LS and 83 SS, were included in this analysis. The median follow-up time for LS was ~141 weeks (Table 1) and was ~45 weeks for SS. 44 patients (77.2%) of LS were still alive at the end of the study, 10 patients (17.7%) died from lung cancer and 3 patients (1.71%) died from other causes. 69 patients (83.2%) of SS died from lung cancer and 14 patients (11.62%) died from other causes. Other characteristics of the participants are shown in Table 1. Specifically, SS were more likely to be older, be diagnosed with stage IB or II lung cancer, currently smoke, have moderate or severe spirometer-based COPD and not have undergone surgery.

Median follow up time was 141 weeks for LS while was 44.6 weeks for SS with range of 89.4-189.1 weeks for LS and 20.6-61.3 weeks for short survivals. The most common ages of LS group were <65 years and from 65 years to <70 years, while SS were mostly of 70 years to <75 years. Male sex was predominant in both survival groups (35.6 & 63.8%) respectively.

Most common stage of LS group was IA (29.8%) while IIA was the most common stage in SS (%24). Adenocarcinoma represents 70.2% of LS patients and squamous cell carcinoma represents 62.7% of SS. Long survivals were mostly non smokers (38.6%) and short survivals were mostly current smokers (60.2%) (table I).

Self reported COPD was detected in 29.2% of long survivals and 61.4% of short survival patients. 48 patients (57.8%) with short survival was suffering of moderate to severe degree of spirometer based COPD.

Line of treatment vary according to stage of the disease and performance status of our patients. Among long survivals, surgery was the cornerstone therapy in 47 patients (82.5%), while among short survivals surgery was only performed in 33 patients (39.8%) of cases. Only 37 patients received chemotherapy in the form of VP (Vepsid-Cisplatin) protocol for squamous cells carcinoma group. On the other hand, Paclitaxel based chemotherapy was given for patients of adenocarcinoma pathology. 25 patients among long survivals (43.8%) received radiotherapy, while 53 patients (63.8%) among short survivals received radiotherapy.

Characteristics	**LS >150 w (n=57)**		**SS <80 w (n=83)**	
Median follow up time (weeks) IQR	141.0 (89.4-189.1)		44.6 (20.6-61.3)	
Age (years) n%	**N**	**%**	**N**	**%**
<65	20	35	10	12.1
65 to >70	20	35	25	30.1
70 to <75	7	12.2	35	42.1
≥75	10	17.8	13	15.7
Sex n%	**N**	**%**	**N**	**%**
Males	30	52.6	53	63.8
Females	27	47.4	30	36.2
Stage	**N**	**%**	**N**	**%**
IA	17	29.8	18	21.7
IB	15	26.3	10	12.1
IIA	13	22.8	20	24
IIB	12	21.1	35	42.2
Histology n%	**N**	**%**	**N**	**%**
Adenocarcinoma	40	70.2	31	37.3
Squamous cell carcinoma	17	29.8	52	62.7
Smoking status	**N**	**%**	**N**	**%**
Never smoker N%	22	38.6	20	24.1
Former smoker	20	35.1	13	15.7
Current smoker	15	36.3	50	60.2
COPD self reported	**N**	**%**	**N**	**%**
No	40	69.8	32	38.6
Yes	17	29.2	51	61.4
COPD spirometer based	**N**	**%**	**N**	**%**
Mild	32	56.2	35	42.2
Moderate or severe	25	43.8	48	57.8

Table (2): Distribution of treatment lines of lung cancer patients by survival status.

	LS >150 w (n=57)		SS <80 w (n=83)	
Surgery n%	N	%	N	%
No	10	17.5	50	60.2
Yes	47	82.5	33	39.8
Chemotherapy treatment n%	N	%	N	%
No	20	35.1	40	48.2
Yes	37	64.9	43	51.8
Radiation treatment n%	N	%	N	%
No	32	56.2	30	36.2
Yes	25	43.8	53	63.8

Table (3): Adjusted analysis for the associations between inflammatory circulating markers and survival status.

Markers	Median		P-value	95% CI	OR
	LS	SS			
IL-8	7.11	9.77	0.002	1.31-7.1	3.05
TNF-α	8.56	9.44	0.007	1.25-6.78	2.92
IL-6	4.51	5.69	0.48	1.08-7.47	2.84

Markers ordered from the most significant to the least significant according to P-value.
P-value <0.05 is considered significant.

Discussion

Our study evaluated the associations of three circulating markers of immunity and inflammation with long-versus short-term survival in lung cancer patients diagnosed with stage I and II. After applying a correction of multiple comparisons, among the studied 3 markers IL-6, IL-8 and TNF-α, we found that IL-8 and TNF-α significantly increased the odds of short survival by almost 3.05, 2.92 respectively with 95% CI ranged (1.31-7.1) and (1.25-6.78) respectively with statistically significant p-value (0.002 & 0.007 respectively). While, in our study IL-6 increased the odds of short survivals by almost three-fold OR (2.84) and 95% CI (1.08-7.43) but with statistically insignificant p-value (0.48).

A study of 334 lung cancer patients found that cytokine IL-6 was associated with a significant increased risk of mortality in Caucasians (95% CI 1.22-2.40) comparing levels higher to lower than the median value[23]. Although that study included patients staged I-IV with ~50% of them with stage I and II, the analysis

was only broadly adjusted for stage (II-IV versus I)[23]. In our study, IL-6 increased the odds of short survivals by almost 3 folds OR (2.84), 95% CI (1.08-7.43) but the difference did not reach statistical significance after adjusting for multiple testing.

Although the different sampling schemes in the two studies make it difficult to directly compare the magnitude of the risk associated with higher levels of IL-6, both studies found that higher levels of IL-6 were associated with poor survival.

Our results coordinate with Pardigol et al. (1998) which reported that chemokines TNF-α and IL-8 circulating levels coded by special genes expression are expressed in lung leukocytes and macrophages and are associated with recruitment of inflammatory cells in asthmatic patients and has been observed to promote angiogenesis.

There are some limitations that could have affected our results. Additional true associations could exist, but we may have failed to detect them because of insufficient statistical power. Another limitation is that we measured circulating levels of inflammatory markers which might not be a reflection of the tumor microenvironment, but rather of the systemic state of the patient. The strengths of our study were the rich epidemiological and clinical data. In addition we compared early stage lung cancer patients at the extremes of the survival distribution to maximize the chance of detecting an association between marker levels and survival.

Conclusion

In summary, in this comprehensive study of circulating levels of some immune and inflammatory markers in relation to survival in early-stage lung cancer, cytokines (IL-8, TNF-α) was significantly associated with poor survival beyond other demographic, clinical, pathological, and treatment variables. The results provide some evidence that deregulation of inflammatory responses may play a role in the survival of early-stage lung cancer, but these findings will require confirmation in further studies.

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EFFECT OF CIRCULATING LEVEL OF INTERLEUKIN-6 (IL-6), INTERLEUKIN-8 (IL-8) AND TUMOR NECROSIS FACTOR-α (TNF-α) ON SURVIVAL IN PATIENTS WITH EARLY STAGE LUNG CANCER

Mona M. Halim MD, Osama Elbaz MD and Nahla Anber MD

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TISSUE DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF RIGHT VENTRICULAR FUNCTION AND PULMONARY ARTERY PRESSURE BEFORE AND AFTER 6 CYCLES OF PALLIATIVE CHEMOTHERAPY FOR INOPERABLE STAGE III PANCREATIC ADENOCARCINOMA

Mona M. Halim MD and Shaheer K. George MD

Departments of Clinical Oncology and Nuclear Medicine & Internal Medicine (Cardiovascular Unit), Mansoura University

Abstract

Aims: *The aim of this study was to evaluate effect of combined Gemcitabine-Cisplatin chemotherapeutic agents on right ventricular function and pulmonary artery pressure in patients with stage III inoperable pancreatic adenocarcinoma without prior cardiovascular disease nor evident cardiovascular risk factors.*

Subjects and Methods: *40 patients with inoperable stage III pancreatic adenocarcinoma in Mansoura University Hospital in Mansoura, Egypt were planned to receive 6 cycles of combined Gemcitabin 1000 mg/m^2 IV (30 min infusion), Cisplatin 50 mg/m^2 IV (1 hour infusion) chemotherapeutic agents at day 1, 15 four week apart for palliation. The patients were enrolled between January to November 2011. Tissue Doppler echocardiographic examination was performed before the onset of chemotherapeutic regimen (Echo-1) and after completion of the sixth cycle of chemotherapy (Echo-2). Patients who complained from any cardiovascular symptoms during cycles of chemotherapy were excluded from the study.*

Results: *The mean age for participants was 46.8±7.3 years. 60% were males and 40% were female. No significant change regarding heart rate, rhythm nor blood pressure after completion of 6 cycles of chemotherapy. Echocardiographic examination revealed significant increase in RVEDD (cm), RAD (cm) as well as RAV (cm^3) in Echo-2 when compared*

to Echo-1 and the results were statistically significant (P=<0.001, 0.007 and <0.001 respectively). There was also significant decrease in pulmonary velocity (m/sec) and pulmonary acceleration time (msec) as well as increase in SPAP (mmHg) in Echo-2 when compared with Echo-1 and the results were also statistically significant (P=<0.008, <0.008 and 0.007 respectively). Tissue Doppler echocardiographic examination revealed decease in tricuspid annular early diastolic velocity (TAE), tricuspid annular systolic velocity (TAS) as well as tricuspid E/A ratio (TE/A) in Echo-2 when compared to Echo-1 and the results were statistically significant (P=0.002, 0.007 and 0.005 respectively). However, there is increase in tricuspid annular late diastolic velocity in Echo-2 when compared to Echo-1 but the result is statistically insignificant (P=0.09).

Conclusion: *Chemotherapy with combined Gemcitabine and Cisplatin in patients with inoperable stage III pancreatic adenocarcinoma can lead to deterioration of R.V function and elevation of SPAP after repeated cycles as evaluated by tissue Doppler echocardiography. Future studies including larger number of patients, different chemotherapeutic agents and patients from different cancer sites could be target for future multicentre studies.*

Keywords: *Tissue Doppler Echocardiography, right ventricular function and pancreatic adenocarcinoma.*

Introduction

Chemotherapeutic regimens which contain anthracyclines, cyclo-phosphamide, fluorouracil, taxanes, and trastuzumab cause early and late cardiotoxicity[1]. However, combined Gemcitabine-cisplatin combination used for palliation in patients with pancreatic adenocarcinoma was poorly evaluated[1]. Chemotherapy-induced cardiotoxicity includes cardiomyopathy with or without overt congestive heart failure. Impairment in diastolic cardiac function was suggested as an early finding of chemotherapy-induced cardiac damage[2]. Tissue Doppler echocardiography (TDI), which allows measurement of systolic and diastolic velocities of ventricular walls, has been a useful method recently for earlier detection of local abnormalities in cardiac functions before the heart is globally affected[3,4].

Although the left ventricular systolic and diastolic functions after chemotherapy were well studied, there have been scarce investigations about right ventricular functions and pulmonary artery pressure whose prognostic value increases in the presence of left ventricular systolic dysfunction[5].

Echocardiographic assessment of right ventricle is difficult due to it's crescentic anatomical and morphological structure. M-mode echocardiography, two-dimensional echocardiography, conventional Doppler echocardiography, and myocardial Doppler tissue imaging are all used to evaluate right heart and provide accurate prognostic information especially when used in combination[6].

We investigated the effect of combined Gemcitabine-Cisplatin combination regimen that is frequently used in our university hospital for palliative treatment of stage III inoperable pancreatic adenocarcinoma on R.V function and PAP after 6 cycles of therapy.

Methods

Study population:

Forty patients with stage III pancreatic adenocarcinoma in Department of Medical Oncology and Nuclear Medicine, Mansoura University Hospital were included between January and November 2011. We did not involve patients older than 55 years of age, because diastolic function of the heart may be impaired by increasing age. Patients with hypertension, diabetes mellitus, established diagnosis of coronary artery disease, rhythm other than sinus, any organic valvular heart disease, renal impairment (serum creatinine >1.5 mg/dl), hepatic dysfunction (serum ALT and/or AST > 3 times of upper level) were excluded from the study. According to the recommendations of NCC guidelines 2010, those patients were planned to receive 6 cycles of combined Gemitabine + Cisplatin chemotherapy on palliative basis. We evaluated right ventricular function as well as pulmonary artery pressure using tissue Doppler echocardiography at baseline before starting therapy (Echo-1) and after the end of the 6th cycle (Echo-2) provided that no emerged any cardiovascular symptoms during the cycles of

chemotherapy.

Echocardiographic examination:

All participants had undergone echocardiographic evaluation before the start of chemotherapeutic regimen (Echo-1) and at the end of the 6th cycle (Echo-2) .

All echocardiographic examinations were performed by the same cardiologist using a Vivid 5 ultrasound with a 2.5-3.5 MHz transducer in the left lateral decubitus position. Parasternal and apical projections were obtained according to the recommendations of American Society of Echocardiography[7].

Standard two-dimensional echocardiographic evaluation for left and right ventricular size and function was performed. Left and right ventricular diameters were measured from a parasternal long-axis view by M-mode examination recorded at the speed of 50 min/s. Left ventricular ejection fraction (EF) was measured from the apical, four-chamber view using biplane Simpson's rule[7,8].

Pulmonary artery systolic pressure was estimated by calculating the systolic pressure gradient between the right ventricle and the right atrium by the maximum velocity of the tricuspid regurgitant jet using the modified Bernoulli equation and then adding the estimated right atrial pressure based on the size of the inferior vena cava and the change in the caliber of it with respiration to this value [9]. To measure the pulmonary acceleration time, pulsed Doppler was used to record the right ventricular outflow tract systolic spectral signal, and time-to-peak duration of the spectral signal across the pulmonic valve was measured from the short-axis view[10].

TDI values of the right and left ventricles were obtained from the apical four-chamber view using a sample volume placed at the lateral corner of the tricuspid annulus; and anterior, inferior, medial, and lateral sections of the mitral annu lus[11].

Statistical analysis:

Data, analysis was performed by using Statistical Package for Social Sciences (SPSS) version 11.5 software (SPSS Inc., Chicago. IL. USA). For the continuous vari-

ables, parametric test conditions were tested. Descriptive statistics were shown as mean + standard deviation or median (maximum-minimum) where appropriate. While the mean differences between measurement times were compared by repeated measures of ANOVA. Degrees of association between continuous variables were calculated by Spearman's correlation analysis. A P=value <0.05 was considered statistically significant.

Results

Table (1) shows the basal characteristics of the study population. The mean age of participants was 46.8±7.3, 60% were males and 40% were females. No significant changes were observed regarding heart rate, rhythm or blood pressure after completion of 6 cycles of chemotherapy.

Table (2) shows echocardiographic measurements for the right ventricle and the pulmonary artery. There was significant increase in RVEDD (cm), RAD (cm) as well as RAV (cm^3) in Echo-2 when compared to Echo-1 and the results were statistically significant (P=<0.001, 0.007 and <0.001 respectively). There was also significant decrease in pulmonary velocity (m/sec) and pulmonary acceleration time (msec) as well as increase in SPAP (mmHg) in Echo-2 when compared with Echo-1 and the results were also statistically significant (P=<0.008, <0.007 and 0.003 respectively).

Table (3) shows the data of tissue Doppler echocardiographic examination results. There was decrease in tricuspid annular early diastolic velocity (TAE), tricuspid annular systolic velocity (TAS) as well as tricuspid E/A ratio (TE/A) in Echo-2 when compared to Echo-1 and the results were statistically significant (P=0.002, 0.007 and 0.005 respectively). However, there is increase in tricuspid annular late diastolic velocity in Echo-2 when compared to Echo-1 but the result is statistically insignificant (P=0.09).

Table (1): Basal characteristics of the study population.

	Echo-1	Echo-2	P-value
Age (years)	46.8±7.3	-	-
Systolic BP (mmHg)	121.4±11.6	123.5±10.2	0.8
Diastolic BP (mmHg)	78.2±6.1	80.1±5.0	0.09
Heart rate(bpm)	67.4±4.0	69.0±5.0	0.09
Height (m)	1.59±4.8	-	-
Weight (kg)	68.4±5.13	66.2±4.3	0.07
Apical S3	-	-	
Apical S4	5	6	
Pansystolic murmer on apex	-	-	
Pansystolic murmer on tricuspid area	4	6	

Table (2): Right ventricular echocardiographic examination

	Echo-1	Echo-2	P-value
RVEDD (cm)*	2.7 (2.5-3.4)	2.8 (2.5-3.4)	<0.001
RAD (cm)*	3.3 (2.9-4.2)	3.5 (3.0-4.3)	0.007
RAV (cm^2)*	27 (18-44)	30 (19-44)	<0.001
Pulm vel (m/s)**	1.61 + 0.14	0.85 + 0.16	<0.008
Pulm acc time (ms)**	123.1 + 16.01	113.2 + 12.4	<0.007
sPAP (mmHg)*	31 (28-29)	38 (29-44)	0.003

RVEDD, right ventricular end-diastolic diameter, RAD, right atrial diameter; RAV, right atrial volume; Pulm vel, pulmonary velocity; Pulm acc time, pulmonary acceleration time; sPAP, systolic pulmonary artery pressure; Echo-1, before chemotherapy; Echo-2 after the completion of 6 cycles of chemotherapy.
**P < 0.05 considered significant.

Table (3): Tissue Doppler echocardiographic measurements from lateral tricuspid annulus.

	Echo-1	Echo-2	P-value
TAE (cm/s)	17.3 + 1.80	14.44 + 2.71	0.002
TAA (cm/s)	11.4 + 1.53	12.1 + 1.66	0.09
TAS (cm/s)	16.6 + 2.1	11.4 + 1.85	0.007
T E'/A'	1.62 + 0.18	1.21 + 0.32	0.005

TAE tricuspid annular early diastolic velocity; TAA, tricuspid annular late diastolic velocity; TAS, tricuspid annular systolic velocity; T E/A, tricuspid annular E/A ratio; Echo-1, before chemotherapy; Echo-2 after completion of 6 cycles of chemotherapy. P<0.05 considered significant.

Discussion

We have demonstrated that chemotherapeutic drugs cause a decrease in right ventricular systolic and diastolic functions, however, although most echocardiographic indices remain in the normal range and do not cause any clinical signs of right heart failure. Tissue Doppler examination revealed significant subclinical decrease in tricuspid annulus systolic velocity, TE/A ratio, ↓ pulmonary velocity, ↓ in pulmonary acceleration time and ↑ sPAP.

In numerous studies involving mostly anthracyclines, subclinical cardiac damage was demonstrated[12] and especially diastolic impairment in the left ventricle was shown to precede systolic derangement[13,14,15]. TDI has been suggested as the preferred method to detect early cardiac damage caused by cancer chemotherapy in many articles([16,17]. However, myocardial deformation parameters (strain and strain rate) have been recently reported to detect the subclinical myocardial damage earlier than myocardial velocity measurements[18]. Jurcut et al.[19] reported a significant reduction in radial strain after three cycles of pegylated doxorubi-cin treatment given every 3 weeks and a significant reduction in both longitudinal and radial strain and strain rates after six cycles of the same regimen. In a study using TDI, subclinical systolic and diastolic myocardial abnormalities were shown to persist up to 6 years of follow-up[20].

The study by Ganame et al.[21] was similar to our study in terms of the investigation of short-time effects of chemotherapy. After each of the first three doses of low-to-moderate dose anthracycline therapy, cardiac systolic and diastolic functions were acutely impaired as detected by TDI and strain rate imaging. Although some other studies also described subtle myocardial involvement detected by TDI in a short-term interval[22]. Appet et al.[23] did not find a significant impairment in most of the echocardiographic indices, other than a modest reduction in EIA ratio by TDI or conventional echocardiography after three cycles of low-dose Epirubicin infusion. However, the cardiotoxic effect of Epirubicin is about half that of Doxorubicin at same doses[24].

In histological studies, the cardiotoxic damage was more prominent in the subendocardial part of the cardiac walls[25]. The thinner right ventricle should be more sensitive to the-toxic effects of chemotherapy, although data are lacking to support this hypothesis.

In general, the issue of right ventricular involvement during or, after chemotherapy is not adequately studied. Belham et al.[26] reported that low-dose anthracycline administration was associated with an increase in the left ventricular Tei index (myocardial performance index), whereas there was no significant change in the right ventricular Tei index. Cottin et al.[27] evaluated the cardiac functions by radionuclide angiography in 33 women treated with anthracycline therapy and found an impairment in the systolic and diastolic left ventricular radionuclide parameters without any alteration in the right heart functions. Yildirim et al.[16], investigated left ventricular and right ventricular functions using dobutamine stress echocardiography and TDI in asymptomatic pediatric long-term survivors of different types of malignancies who were treated with anthracyclines and detected that right ventricular E/A was impaired[16].

Despite a decrease in right ventricular systolic and diastolic functions, most parameters remain in the normal ranges provided in the recent guideline of American Society of Echocardiography for the echocardiographic assessment of the right heart[28]. The reason for the discrepancy between our results and the results of the above-mentioned studies may be due to the difference in echocardiographic indices evaluated. Also, it may be related to the difference in the chemotherapeutic regimen used and its dose.

We detected a slight significant decrease in pulmonary acceleration time and a slight increase in estimated pulmonary artery pressure, although the echocardiographic threshold for pulmonary hypertension was not reached. It has been known for a long time that a shorter acceleration time reflects an increase in pulmonary artery pressure[29], Lopez-Candales

et al.[30] have shown that right ventricular outflow signal could also be used to assess the presence of right ventricular dysfunction. Although invasive pulmonary vascular resistance calculation was not performed in this study, we think that this finding reflected a decrease in right ventricular performance rather than an impairment in pulmonary resistance because of the absence of severe manifestations of left ventricular failure to induce reactive (out of proportion) post-capillary pulmonary hypertension. Still, it should be kept in mind that chemotherapeutic agents are among the possible causes of idiopathic pulmonary arterial hypertension by increasing pulmonary vascular resistance, although not frequent [31].

In conclusion, we have demonstrated an impairment in right ventricular echocardiographic indices, as well as elevation of sPAP during chemotherapy with Gemcitabine + Cisplatin in patients with inoperable stage III pancreatic adenocarcinoma as palliative therapy, in a relatively short time, although this has not caused any, clinical deterioration. This investigation may give rise to attention on right heart in cancer patients and may lead to larger trials which also test the possible association of short-term changes in right heart echocardiographic indices with the long-term prognosis. After analysing the possible association between the early impairment in each of the abovementioned parameters and the long-term prognosis, the parameters which should be preferred for risk assessment would be more clearly identified. However, at the moment we suggest a combined use of tissue Doppler echocardiography and conventional echocardiography to assess the right heart functions during ongoing chemotherapy starting from the relatively early phases of the treatment.

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TISSUE DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF RIGHT VENTRICULAR FUNCTION AND PULMONARY ARTERY PRESSURE BEFORE AND AFTER 6 CYCLES OF PALLIATIVE CHEMOTHERAPY FOR INOPERABLE STAGE III PANCREATIC ADENOCARCINOMA

Mona M. Halim MD and Shaheer K. George MD

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