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Early Clinical and Echocardiographic Effects of Elective Percutaneous Coronary Intervention

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Abstract: Early effect of elective percutaneous coronary intervention (PCI) is important in clinical practice. Knowledge of different variables for suboptimal effects of PCI may help to refine indications of and to guide strategies aimed at improving outcome. Objectives. To detect the early effects of elective PCI on different clinical and echocardiographic variables in the studied group and in different patient subgroups. Methods. Twenty-four patients with established coronary artery disease who are candidates for elective PCI of one or more of coronary vessels were enrolled in the study after exclusion of pts with acute MI and any contraindication for coronary angiography. After obtaining complete history and full clinical examination; every pt was subjected to *clinical assessment* using Minnesota questionnaire & Canadian Cardiovascular Society Angina Classification (CCSAC), *transthorasic echocardiographic examination* with measuring of the following: Left ventricular end-diastolic and end-systolic volumes (LVEDV & LVEDV) with calculation of LV ejection fraction (LVEF), 17 segment model scoring system, wall motion score index (WMSI) & Myocardial performance index (MPI). *Elective coronary angiography* was performed with implantation of one or more stents. Clinical assessement and echocardiographic parameters were reevaluated 1 month post PCI. Analysis of data was done in the studied population & in pts subgroups according to risk factors, medications type, lesion classifications, type of revascularization, stent type and number of vessel affected. Results. 24 pts; 17 males & 7 females with mean age 55.7±9.3 years were studied at the Critical Care Medicine Department, Cairo University. The commonest risk factors were hypertension, and obesity each in 54.2%, followed by Diabetes Mellitus and family history of CAD each in 41.7%, smoking in 37.5%, and finally dyslipidemia in 33.3%. Both CCSAC and Minnesota questionnaire significantly improved after PCI. Mean LVEDV was 117±26.9 at baseline and 110.9±26.3 after 1 month, mean LVESV was 58.8±22 and 53.1±21.2 at follow up and mean LVEF was 50.9±8.1% and 53.1±7.5 after 1 month, all with significant p value. The Minnesota Score, LVEF, LVESV & MPI significantly improved in pts with impaired baseline LVEF than those with normal LVEF. 408 segments were analysed, 84 segments showed RWMA in the form hypokinesia in 18%, and akinesia in 3%. Analysis of the affected segments revealed hypokinesia in 86.9% and akinesia in 13.1%. At follow up 33 segments (39.3%) showed improvement in the RWMS. According to different risk factors, the presence of HTN, absence of DM, & the absence of dyslipidemia were in favor with more improvement in the LV volumes and EF. None of the other risk factors showed significant effect before & after PCI. According to the type of medications, the use of β-Blockers (βB) or angiotensin converting enzyme inhibitors (ACEIs) or the absence of Ca Channel Blockers (CCB) was in favor of significant improvement in the Minnesota score, LV volumes and function, and regional LV function. According to angiographic criteria; pts with type A lesion (38%) showed improvement in Minnesota score, EF, ESV, MPI, and RWMSI than type B (33%) or C (29%). All patients showed clinical improvement by CCSAC whatever the lesion type. Pts with single vessel disease (58%) showed significant improvement in Minnesota score, EF, EDV, ESV, and MPI than those with 2 or more vessel affection. Pts who underwent total revascularization (62.5%) showed significant improvement in Minnesota score, EF, EDV, ESV, and MPI than those with subtotal revascularization (37.5%). Patients using Bare Metal Stents (75%) showed significant improvement in Minnesota Score, EF, EDV, ESV, MPI, and RWMSI than those using Drug Eluting Stents (25%). Conclusion. Early clinical & echocardiographic improvements after elective PCI. Absence of DM or dyslipidemia or the use of βB or ACEIs was in favor of better PCI effect. Complex lesion and higher number of affected vessels showed less improvement after elective PCI.
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Key words: Elective PCI, early, clinical effect, echocardiography

Introduction

Over the past 30 years, dramatic improvements have been achieved in the safety of percutaneous coronary intervention (PCI) procedures, despite the increasing complexity of clinical and anatomic conditions treated. The need for emergent bypass surgery has declined from 8% in 1990 to far less than 1% in the current era, and the rate of vascular complications has declined dramatically as techniques have improved and procedural experience has increased [(1)].

In patients with significant amounts of viable myocardium, LV function may improve markedly, and even normalize, following successful revascularization.[(2-4)] Given the remarkable current periprocedural safety profile of elective coronary

intervention, it is important to predict the rate of success and study the factors affecting elective PCI outcome.

Aim of the work:

To detect the early effects of elective PCI on different clinical and echocardiographic variables in the whole studied population and in different patient subgroups.

2. Patients & Methods:

The study included twenty-four patients with established coronary artery disease who are candidates for elective PCI of one or more of coronary vessels. The study was conducted in the Critical Care Medicine Department, Cairo University, from the period of September 2009 to May 2010.

Exclusion criteria:

We excluded from the study pts with acute MI and those with any contraindication for coronary angiography.

All patients were subjected to:

Complete history and full clinical examination including 12-lead electrocardiogram (ECG); clinical assessment; echocardiographic examination and elective PCI.

A- Echocardiographic examination

All patients underwent conventional transthoracic echocardiographic examination before and one month after PCI. Each patient was examined in the left lateral decubitus position according to the recommendations of the American Society of Echocardiography using an ATL HDI 5000 colored echocardiographic machine using a 3.5 MHz. transducer.

The following parameters were measured

1- Global systolic function

- **Ejection fraction (EF):**

Left ventricular end-diastolic and end-systolic volumes were measured and LVEF was calculated using Simpson's rule (normal range 55 % - 75 %) [5].

$$EF = \frac{LVEDV - LVESV}{LVEDV} \times 100$$

2- Regional left ventricular function:

- A wall motion score index (WMSI) is derived by dividing the sum of the wall motion scores by the number of visualized segments (17 segments) [5].

3- Myocardial performance index:

Calculated by the following equation:

$$MPI = \frac{IVRT + IVCT}{ET}$$ (Normal: 0.39), Figure (1). [5]

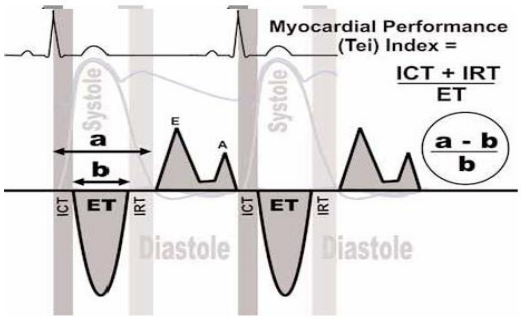

Figure (1) Calculation of MPI

B- Clinical assessment:

- Clinical assessment was done before and 1 month after PCI using:
  - Minnesota questionnaire (Table 1) and,
  - Canadian Cardiovascular Society Angina Classification.

Canadian Cardiovascular Society Angina Classification:

- Class 0: Asymptomatic.
- Class 1: Angina with strenuous exercise.
- Class 2: Angina with moderate exertion.
- Class 3: Angina with mild exertion :
  Walking 1-2 level blocks at normal pace
  Climbing 1 flight of stairs at normal pace
- Class 4: Angina at any level of physical exertion.

B- Revascularization technique (elective PCI):

- The procedures were performed using ***Integris H 300 (Philips NL company)*** catheterization laboratory. Pre-interventional medications included: Intravenous heparin (10000 to 15000 IU), to keep the activated clotting time > 300 sec during the procedure and oral Clopidogrel® 300 mg loading dose 24 hours before the procedure.
- Baseline diagnostic coronary angiography was first performed via femoral artery using Seldingers' technique with 6, 7, or 8 F left Judkins, Q or multipurpose guiding catheters for the detection of severity, and extent of coronary artery disease (CAD), lesion morphology was classified according to the ACC/AHA classification [6].

Table (1): Minnesota questionnaire

The following questions ask how much your heart failure (heart condition) affected your life during the past month (4 weeks). After each question, circle the 0, 1, 2, 3, 4 or 5 to show how much your life was affected. If a question does not apply to you, circle the 0 after that question.

Did your heart failure prevent you from living as you wanted during the past month (4 weeks) by -	No	Very Little				Very Much
1. causing swelling in your ankles or legs?	0	1	2	3	4	5
2. making you sit or lie down to rest during the day?	0	1	2	3	4	5
3. making your walking about or climbing stairs difficult?	0	1	2	3	4	5
4. making your working around the house or yard difficult?	0	1	2	3	4	5
5. making your going places away from home difficult?	0	1	2	3	4	5
6. making your sleeping well at night difficult?	0	1	2	3	4	5
7. making your relating to or doing things with your friends or family difficult?	0	1	2	3	4	5
8. making your working to earn a living difficult?	0	1	2	3	4	5
9. making your recreational pastimes, sports or hobbies difficult?	0	1	2	3	4	5
10. making your sexual activities difficult?	0	1	2	3	4	5
11. making you eat less of the foods you like?	0	1	2	3	4	5
12. making you short of breath?	0	1	2	3	4	5
13. making you tired, fatigued, or low on energy?	0	1	2	3	4	5
14. making you stay in a hospital?	0	1	2	3	4	5
15. costing you money for medical care?	0	1	2	3	4	5
16. giving you side effects from treatments?	0	1	2	3	4	5
17. making you feel you are a burden to your family or friends?	0	1	2	3	4	5
18. making you feel a loss of self-control in your life?	0	1	2	3	4	5
19. making you worry?	0	1	2	3	4	5
20. making it difficult for you to concentrate or remember things?	0	1	2	3	4	5
21. making you feel depressed?	0	1	2	3	4	5

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Follow up

Both clinical assessement and echocardiographic parameters were reevaluated 1 month post PCI

D- Statistical analysis:

Data were collected on special format, verified and then coded when needed prior to analysis. All continuous data were expressed as mean ± SD, categorical data were expressed as frequency in tables. Paired T test and non parametric t test (Mann Whitney test) were used for comparing means of values before and after revascularization. Tools to assess the accuracy of diagnostic test have been calculated. P value < 0.05 considered significant (CI is 95%). All analysis has been performed using SPSS 12 and graphics by MS excel.

3. Results

Our study was conducted on 24 patients who were admitted to the Critical Care Medicine Department, Cairo University, during the period from September 2009 till May 2010. All the studied populations were diagnosed to have CAD and underwent elective PCI to one or more coronary vessel.

Our results will be presented as follow:

A- Descriptive data: (Demographic, clinical, angiographic and echocardiographic data)

B- Comparative data: between baseline and one month post PCI; (For the whole population and in different pts subgroups)

A- Descriptive data

I. Demographic & clinical data

The mean age of the studied population was 55.7±9.3 years ranging from 40 to 71 years. Seventeen pts (70.2%) were males and 7 pts (29.8%) were females. Nine pts (37.5%) were smokers, Thirteen pts (54.2%) were hypertensive, 10 pts (41.7%) were diabetic, 8 pts (33.3%), were dyslipidemic, 10 pts (41.7%) had family history of CAD and 13 pts (54.2%) were obese (as measured by BMI).

II. Baseline angiographic data

1-Vessels affected:

Fifteen pts (62.5%) had LAD lesion, 11 pts (45.8%) had LCX lesion, 12 pts (50%) had RCA lesion

2-Number of vessels affected:

Ten pts (41.7%) had more than one vessel disease.

3-Type of lesion:

Nine Pts (38%) had type A lesion, while 8 pts (33%) had type B lesion and those with type C lesion were 7 pts (29%).

4- Type of revascularization:

Fifteen pts (62.5%) underwent total (complete) revascularization, while 9 pts (37.5%) had subtotal revascularization.

5-Number and type of stent:

A total number of 33 stents were deployed. Eighteen pts (75%) received 25 bare metal stent and six pts (25%) had 8 DES.

III. Baseline Echocardiographic data:

1-LV volumes and EF:

Mean LVEDV was 117±26.9, mean LVESV was 58.8±22 and mean LVEF was 50.9±8.1%. Ten pts (42%) had normal EF (≥50%) while 14 pts (58%) had reduced EF (< 50%)

408 segments were studied, 84 segments (21%) showed regional wall motion abnormalities in the form

hypokinesia in seventy three (18%), and akinesia in 11 pts (3%). Analysis of the affected segments revealed hypokinesia in 86.9% and akinesia in 13.1%. At follow up 33 segments (39.3%) showed improvement in the RWMS.

B) Comparison between before and one month post PCI data:

I) Whole population:

Clinical assessment

Both Canadian Cardiovascular Society Angina Classification and Minnesota questionnaire significantly improved after PCI (Table 2).

Table (2): Clinical and echocardiographic data before and after PCI:

	Before	After	P value
CCSAC	3.3±0.8	1.4±0.7	0.0001
MINNESOTA score	65.1±13.3	46.6±17.4	0.001
LVEDV (ml)	117±26.9	110.9±26.3	0.034
LVESV (ml)	58.8±22	53.1±21.2	0.014
EF (%)	50.9±8.1	53.1±7.5	0.004
MPI	0.66±0.12	0.6±0.097	0.008

Echocardiography

EDV and ESV showed a significant decline one month after revascularization. Patients showed a highly significant improvement in myocardial performance index one month after revascularization (Table 2) .

II) Patient Subgroups:

The effect of elective PCI on the measured variables was evaluation in different pts subgroups before and 1 month after PCI.

1) Diabetes Mellitus:

Both groups showed significant improvement in CCASC, Minnesota score and RWMAs index. The **non diabetic** group showed additional improvement in EF, EDV, ESV and MPI one month after PCI (Table 3)

Table (3): The effect of revascularization on diabetic pts

Diabetes	**Non diabetic (No=14)**			**Diabetic group (No=10)**		
	Before	**After**	**P val.**	**Before**	**After**	**P val.**
CCSAC	3.3±0.8	1.35±0.6	0.001	3.3±0.67	1.6±0.69	0.001
Minnesota	67.16±14.2	45.3±19.6	0.033	63.6±13.5	47.6±16.7	0.013
EF	53.3±7.5	56.2±6.4	0.003	47.6±8.1	48.9±7.2	NS
EDV	111±25.2	100.9±18.4	0.020	125.4±28.1	124.8±30.2	NS
ESV	53.1±19.9	44.5±13.4	0.010	66.9±23.2	65.3±24.6	NS
MPI	0.63±0.12	0.57±0.06	0.032	0.68±0.11	0.65±0.12	NS
RWMSI	1.19±0.12	1.12±0.07	0.001	1.29±0.1	1.23±0.1	0.025

2) Hypertension:

Both groups showed significant improvement in CCASC, ESV, MPI, and RWMSI. The **hypertensive** group showed additional significant improvement in Minnesota score, EF, and EDV. (Table 4).

Table (4): The effect of revascularization on hypertensive pts

Hypertension	**Non hypertensive (No= 11)**			**Hypertensive (No= 13)**		
	Before	**After**	**P- val.**	**Before**	**After**	**P- val.**
CCSAC	3±0.6	1.2±0.6	0.001	3.6±0.7	1.6±0.6	0.001
Minnesota	57.5±16.2	41.2±22	NS	70.8±7.3	46.8±14	0.002
EF	51.4±7	53.4±6.6	NS	50.5±9.2	52.9±8.5	0.039
EDV	107.3±23	105.4±27	NS	125±27.8	115.2±26	0.034
ESV	53.1±18.2	50.2±20	0.027	63.6±24.3	55.6±22.4	0.03
MPI	0.61±0.09	0.58±0.08	0.014	0.69±0.13	0.62±0.1	0.029
RWMSI	1.19±0.09	1.14±0.1	0.004	1.26±0.14	1.19±0.1	0.004

3) Obesity:

Both groups showed significant improvement in CCASC, Minnesota score, and RWMSI. The **non obese** group showed additional improvement in EF, and ESV (Table 5) .

Table (5): The effect of revascularization on obese pts

Obesity	Non Obese (No= 11)			Obese (No= 13)		
	Before	**After**	**p val.**	**Before**	**After**	**p val.**
CCSAC	3.5±0.7	1.3±0.5	0.001	3.1±0.8	1.5±0.8	0.001
Minnesota	58±12.3	37.8±15	0.011	69.8±12	53.2±16	0.032
EF	53.2±8.3	56±7.3	0.021	49±7.2	50.6±7.1	NS
EDV	111.6±22	101.9±10	NS	121.5±30.4	118.4±33	NS
ESV	53.2±19.	44.7±10.1	0.038	63.6±23.9	60.3±25.5	NS
MPI	0.63±0.11	0.56±0.06	NS	0.68±0.12	0.63±0.11	NS
RWMSI	1.18±0.09	1.11±0.06	0.001	1.27±0.13	1.21±0.1	0.014

4) Effect of revascularization on different baseline treatment groups:

i) Beta blockers

Eighteen patients (75%) were using beta blockers. Both groups showed significant improvement in CCSAC. The group **using βB** showed additional significant improvement in Minnesota score, EF, EDV, ESV, MPI, and RWMSI (Table 6) .

Table (6): The effect of revascularization on pts using beta blockers

B- blockers	No B-blockers (No= 6)			B-blockers (No= 18)		
	Before	**After**	**p val.**	**Before**	**After**	**p val.**
CCSAC	3.0±0.6	2.0±0.89	0.041	3.4±0.7	1.2±0.4	0.001
Minnesota	73±2.9	63±13.5	NS	62.2±14.3	42.1±15.9	0.001
EF	50.1±9.1	50.1±9.9	NS	51.2±8	54.1±6.6	0.002
EDV	118.6±37.6	119±44	NS	116±23.7	108±18.4	0.023
ESV	61.5±29.8	62.5±34.5	NS	58±19.7	50.0±14.6	0.006
MPI	0.69±0.14	0.67±0.14	NS	0.64±0.11	0.57±0.06	0.008
RWMSI	1.26±0.14	1.24±0.16	NS	1.22±0.11	1.14±0.08	0.001

ii) ACE Inhibitors

Fourteen patients (58%) were using ACE Inhibitors. Both groups showed significant improvement in CCSAC, while the group **using ACE Inhibitors** showed additional improvement in Minnesota score, EF, EDV, ESV, and RWMSI (Table 7).

Table (7): The effect of revascularization on pts using ACEI

ACEI	No ACEI (No= 10)			ACEI (No-= 14)		
	Before	**After**	**p val.**	**Before**	**After**	**p val.**
CCSAC	3.1±0.73	1.5±0.7	0.001	3.5±0.75	1.4±0.6	0.001
Minnesota	72.5±3.5	63.5±19	NS	63.5±14.12	43±16.2	0.01
EF	56.4±7.6	56.55±8.2	NS	47±6.1	50.7±6.3	0.001
EDV	103.8±19	103.9±19	NS	126.4±28.3	115.9±27.5	0.023
ESV	46±15.7	46.2±15.7	NS	68±21.6	58.2±21.3	0.007
MPI	0.58±0.09	0.57±0.08	NS	0.71±0.11	0.62±0.09	0.013
RWMSI	1.17±0.1	1.14±0.11	NS	1.27±0.12	1.18±0.24	0.0001

iii) Calcium channel blockers

Seven patients (29.1%) were using CCB. Both groups showed significant improvement in CCSAC. The group **not using CCB** showed additional significant improvement in Minnesota score, EF, EDV, ESV, MPI, and RWMSI (Table 8) .

Table (8): The effect of revascularization on pts using CCB

CCB	No CCB (No= 17)			CCB (No= 7)		
	Before	After	p val.	Before	After	p val.
CCSAC	3.4±0.7	1.2±0.4	0.0001	3±0.5	1.8±0.8	0.015
Minnesota	62.9±14.3	42.1±15.9	0.001	73.3±2.8	63±13.5	NS
EF	50.8±8.1	54.1±6.8	0.001	51.2±8.7	50.7±9.1	NS
EDV	117.2±24	108±18.8	0.026	116.2±34	116±40.8	NS
ESV	58.8±20	50.3±15	0.005	59±28	60±32.4	NS
MPI	0.65±0.11	0.58±0.05	0.013	0.67±0.13	0.65±0.15	NS
RWMSI	1.22±0.12	1.14±0.08	0.001	1.24±0.14	1.22±0.15	NS

5) Effect of revascularization on patients with different angiographic criteria.

A- Type of lesion

Patients were divided into three groups: patients with type A lesion (9 patients, 38%), type B lesion (8 patients, 33%) and type C lesion (7 patients, 29%). All groups showed significant improvement in CCSAC. In pts with **type A lesion,** there was additional improvement in Minnesota score, EF, ESV, MPI, and RWMSI. While in pts with **type B lesion**, there was significant improvement in Minnesota score, EF, FM%, and RWMSI (Table 9).

Table (9): The effect of revascularization on pts with different lesion types:

Lesion type	Lesion type A (No= 9)			Lesion type B (No-=8)			Lesion type C (No-=7)		
	Before	After	P-val	Before	After	P-val	Before	After	P-val
CCSAC	3.4±0.8	1.44±0.7	0.001	3.3±0.9	1.25±0.4	0.0001	3.1±0.3	1.7±0.77	0.003
Minnesota	69±14.7	43±18.7	0.028	56.2±14.3	35.2±5.1	0.021	71.5±2.3	65.5±9.3	NS
EF	50.8±8.6	54.6±7.9	0.001	53.1±8.1	56.2±4.9	0.047	48.5±8	47.7±7.6	NS
EDV	112.7±22.8	104.3±21	NS	112±23.9	104±9.2	NS	128.1±34.7	127±39.2	NS
ESV	56.3±19.2	47.3±15.5	0.025	53.7±19.4	45.8±8.7	NS	68±27.9	68.2±30.4	NS
MPI	0.66±0.13	0.58±0.06	0.042	0.63±0.11	0.57±0.04	NS	0.67±0.13	0.66±0.15	NS
RWMSI	1.21±0.14	1.13±0.09	0.004	1.22±0.1	1.13±0.06	0.009	1.27±0.11	1.24±0.14	NS

B- Number of affected vessels

Fourteen pts (58%) had single vessel disease, while 10 pts (42%) had multivessel affection. Both groups showed significant improvement in CCSAC, and RWMSI. The group with **single vessel disease** showed additional improvement in Minnesota score, EF, EDV, ESV, and MPI (Table 10) .

Table (10): The effect of revascularization in relation to the number of vessels affected:

No of vessels	*Single vessel disease (No= 14)*			*Multi vessel disease (No= 10)*		
	Before	*After*	*p val.*	*Before*	*After*	*P val.*
CCSAC	3.4±0.85	1.35±0.49	0.001	3.2±0.6	1.6±0.8	0.001
Minnesota	64.3±12.9	41.5±±15.6	0.002	66.6±15.5	55.8±18.13	NS
EF	49.7±8.8	52.6±8.08	0.009	52.6±7.1	53.9±7.2	NS
EDV	123.7±28	112.5±26.4	0.013	107.6±23.4	108.6±27.4	NS
ESV	63.7±23.7	54.2±21.8	0.009	52.1±18.3	51.6±21.3	NS
MPI	0.68±0.13	0.61±0.1	0.026	0.62±0.101	0.59±0.09	NS
RWMSI	1.24±0.13	1.16±0.12	0.001	1.22±0.11	1.16±0.1	0.034

C. Type of revasualrization

Fifteen patients (62%) underwent total revascularization and 9 patients (38%) underwent incomplete revascularization. Both groups showed significant improvement CCSAC, and RWMSI, while only the group that underwent **total revascularization** showed significant improvement in Minnesota score, EF, EDV, ESV, and MPI post PCI (Table 11) .

Table (11): The effect of total and subtotal revascularization

Revascul. type	*Total (No=15)*			*Subtotal (No= 9)*		
	Before	**After**	**P-val.**	**Before**	**After**	**P-val.**
CCSAC	3.3±0.8	1.2±0.41	0.001	3.3±0.5	1.8±0.7	0.001
Minnesota	60.4±14.6	38.6±12	0.002	73.6±4.1	61±16.9	NS
EF	50.8±8.9	54±7.9	0.002	51.1±7	51.7±7.1	NS
EDV	118.8±29	110.3±26.6	0.041	114±23	111.7±27.4	NS
ESV	60±24.5	52±21.8	0.014	56.8±18.8	55.1±21.2	NS
MPI	0.66±0.03	0.59±0.1	0.026	0.65±0.11	0.61±0.09	NS
RWMSI	1.22±0.13	1.15±0.12	0.0001	1.24±0.1	1.18±0.1	0.040

C- Type of the stent:

Eighteen patients (75%) implanted Bare Metal Stents (BMS) while 6 pts (25%) used Drug Eluting Stents (DES). Both groups showed significant improvement in CCSAC. Patients using **BMS** showed significant improvement in Minnesota Score, EF, EDV, ESV, MPI, and RWMSI. (Table 12)

6) Effect of revascularization on pts with normal and impaired baseline EF.

Ten pts (42%) had normal baseline LVEF (≥50%), while 14 pts (58%) had reduced EF. Both groups showed significant improvement in CCSAC, and RWMSI. The group with **impaired baseline EF** showed additional improvement in Minnesota score, EF, ESV, and MPI. (Table 13)

Table (12): The effect of revascularization on pts using BMS and DES.

Type of stent	*BMS (75%)*			*DES (25%)*		
	Before	***After***	***p val.***	***Before***	***After***	***p val.***
CCSAC	3.33±0.8	1.3±0.6	0.001	3.33±0.51	1.66±0.8	0.011
Minnesota	67.1±13.8	46.1±16.2	0.003	60.2±12.5	48±22.7	NS
EF	51.3±7.9	54.1±7.6	0.001	49.8±9.2	50.16±7.3	NS
EDV	112.7±25	106.5±25	0.018	129.6±29.5	124±28.4	NS
ESV	56.4±20.9	49.9±19.9	0.005	66.8±23.7	62.2±23.7	NS
MPI	0.65±0.12	0.59±0.1	0.024	0.67±0.107	0.62±0.09	NS
RWMSI	1.22±0.13	1.14±0.1	0.001	1.27±0.07	1.22±0.11	NS

Table (13): Effect of PCI on pts with normal and impaired baseline EF

Baseline EF	**Normal EF (No= 10)**			**Reduced baseline EF (No= 14)**		
	Before	**After**	**p val.**	**Before**	**After**	**p val.**
CCSAC	3.1±0.7	1.4±0.6	0.001	3.5±0.7	1.5±0.6	0.001
EF	59.5±3.7	60.1±4.2	NS	44.8±3.3	48.2±5.1	0.002
EDV	95.5±10.6	93.1±10.2	NS	132.2±24	123.5±27.2	NS
ESV	38.7±5.4	36.7±4.3	NS	73.2±17.3	64.9±20.5	0.030
MPI	0.55±0.04	0.53±0.04	NS	0.73±0.1	0.65±0.09	0.015
RWMSI	1.13±0.05	1.09±0.05	0.011	1.3±-0.1	1.22±0.11	0.001

4. Discussion:

There is a relation between myocardial blood flow and systolic function, the so-called "flow function" relation [(7)] . As blood flow is reduced, there is a corresponding reduction in contractile performance ("perfusion-contraction matching"). There may no ischemic symptoms or necrosis when this occurs slowly since blood flow and function are once again in equilibrium [(8)] .

In the past, severe left ventricular (LV) dysfunction was considered an irreversible condition, as regional akinesis was thought to represent infarcted myocardial tissue. It is now understood that, among patients with ischemic cardiomyopathy, LV systolic dysfunction can result from myocardial necrosis, myocardial hibernation, or repetitive myocardial stunning. While myocardial necrosis is irreversible, systolic dysfunction resulting from hibernation and stunning are potentially reversible states of ventricular dysfunction [(9)] .

Myocardial revascularization using PCI is widely used and improves clinical outcome particularly in post infarction patients with markedly reduced LVEF [(10)] .

Echocardiography has become an established and powerful tool for diagnosing the presence of coronary artery disease and defining its consequences in patients with acute ischemic syndromes and those with chronic coronary atherosclerosis. Transthoracic imaging and Doppler techniques are generally sufficient for

evaluating patients with suspected or documented ischemic heart disease. [11]

Echocardiographic studies may help in planning revascularization procedures by demonstrating the functional significance of a given coronary stenosis. Moreover, because restenosis is a common complication, reassessment roughly 1 month after angioplasty is a reasonable time frame within which to assess the functional results of angioplasty [12] .

In our study, we found that PCI is associated with a significant improvement in heart failure and angina symptoms as shown by improvement in Minnesota Score and Canadian Cardiovascular Society Angina Classification.

Our results agree with those of ***Zellweger et al.,*** [13] who documented the beneficial effect of PCI on symptoms and extent of ischemia and ***Buszman et al.,*** [10] who found a significant improvement in heart failure symptoms as shown by improvement in NYHA class. This also agrees with ***Momtahen et al.,*** who conducted his study on 110 patients and found that PCI was associated with a significant improvement in clinical outcome as shown by functional improvement in NYHA class and angina severity [14] .

Our results suggest that PCI is associated with a significant improvement in global functions as shown by the significant improvement in LVEF and MPI. This improvement of the global LV functions was significant one month post-PCI.

Our results agree with those of ***Dzavik and colleagues,*** who studied 244 patients and showed that the restoration of coronary patency of non-acute occluded coronary arteries is associated with a small but significant improvement in global LV function [15] . Also with results of ***Zellweger et al.,*** who found a significant decrease in ESV and EDV after PCI as compared to pre-PCI findings which points to a positive effect on left ventricular remodeling even in the absence of significant changes in EF [13] .

And again in concordance to ***Momtahen et al.,*** who conducted his study on 110 patients with CAD and assessed LV function by echocardiography before and after revascularization. He found that PCI was associated with a significant improvement in global LV function (as shown by improvement in LVEF) and this improvement of LV contractility was significant one month post-PCI. [14]

In our study, we found a significant improvement in regional LV function post revascularization as shown by the significant improvement in RWMSI.

Our result agrees with ***Dzavik and colleagues,*** who showed that the restoration of coronary patency of occluded coronary arteries is associated with a significant improvement in regional function [15] .

This was in concordance to ***Momtahen et al.,*** who stated that PCI was associated with a significant improvement in regional LV function as assessed by RWMSI [14] .

In our study patients with baseline impaired Ejection Fraction (<50%) showed significant improvement in global and regional LV functions and clinical assessment post revascularization.

This agrees with ***Dzavik et al,*** who stated that the improvement in LVEF after revascularization was more significant in patients with base line impaired EF. [15] It also matches the results of ***Buszman et al.,*** and ***Dudek et al.,*** who showed a significant improvement in LV function in patients with baseline depressed LV function after PCI [10,16] .

This again was in concordant to ***Momtahen et al.,*** who stated that The LVEF improvement was nonetheless more pronounced in patients with baseline impaired EF (LVEF ≤ 40%). Therefore, it appears that patients with more severe LV dysfunction will achieve more benefits from PCI [14] .

In our study 62% of patients underwent total (complete) revascularization. These patients showed significant improvement of global LV function represented by significant improvement in EF and MPI, while 38% of patients underwent subtotal (incomplete) revascularization and showed non-significant change in EF and MPI after revascularization.

This was in concordance to ***Kirschbaum et al.,*** who conducted his study at 2010 on 61 patients and assessed LVEF before and 6 months after revascularization. The EF improved significantly after complete revascularization (46% to 51%; $p< 0.0001$) but did not change after incomplete (49% to 49%; with $p=0.88$) or unsuccessful revascularization (49% to 47%; $p=0.11$). [17]

In our study, 14 patients (58.3%) were using ACE inhibitors. This group showed significant clinical improvement in heart failure and angina symptoms as well as global and regional LV functional improvement and a significant reduction in EDV and ESV 1month after revascularization while the group not using ACE inhibitors showed non significant change in heart failure symptoms nor global or regional LV function and didn't show significant reduction in EDV and ESV after revascularization.

This partially agrees with ***Kjøller-Hanse et al.,*** in the APRES study who conducted his study on 159 patients In this study, they found that in patients with asymptomatic and moderate left ventricular dysfunction undergoing invasive revascularization for chronic stable angina pectoris, long-term treatment with ramipril®, initiated shortly after invasive revascularization, reduced the incidence of cardiac death, AMI and development of clinical heart failure but regarding the recurrent angina pectoris, the study showed no significant benefit with ramipril® therapy [18, 19] .

In our study the group of patients (75%) using beta blockers showed a significant clinical improvement and a significant improvement in the global and the regional LV functions 1 month after revascularization while the group not using beta blockers showed only significant improvement in the angina symptoms and non significant change in heart failure symptoms nor global nor regional LV functions.

Our results matched that of the study conducted by ***Chan et al.,*** who showed that beta-blocker use was associated with a marked survival benefit among patients undergoing successful elective percutaneous coronary revascularization [(20)] .

In our study diabetic patients (41%) showed non-significant improvement in global LV systolic functions(LVEF and MPI)and in LV volumes, the non diabetic group showed a significant improvement in both global and regional LV functions ,both groups(diabetics and non diabetic patients) showed a significant improvement in clinical symptoms after revascularization.

Knuesel et al., and Schinkel et al., has designed a study to assess the prediction of improvement of LV function and heart failure symptoms after coronary revascularization in patients with diabetes mellitus and ischemic LV dysfunction, using 18F-FDG imaging. Twenty six percent were diabetics who showed less functional improvement as assessed by echocardiography and when assesses by 18F-FDG SPECT after oral administration of acipimox they concluded that is practical for routine assessment of myocardial viability in patients with ischemic LV dysfunction with or without diabetes mellitus [(, 20)] .

So that one of our limitations that diabetic patients specifically should be assesed by 18F-FDG SPECT rather than echocardiography after coronary revascularization.

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Bone Specific Alkaline Phosphatase and Cardiovascular Morbidity among Patients on Maintenance Hemodialysis

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Abstract: Background: Vascular calcification is common in individuals with chronic kidney disease (CKD) and significantly correlated to the high cardiovascular death risk. In advanced CKD, stages 3 through 5, secondary hyperparathyroidism (SHPT), along with renal osteodystrophy, are common and may be associated with abnormal mineral metabolism and / or abnormal serum or tissue mineral levels, vascular calcification, and poor survival, especially among those who undergo maintenance dialysis treatment. Serum alkaline phosphatase (ALP) is a biochemical marker of bone turnover and is used to monitor metabolic bone disease associated with renal insufficiency. Higher levels of serum ALP were associated with vascular calcification in maitenance hemodialysis patients MHD. Bone-specific ALP (bALP) is a byproduct of osteoblasts and is a more specific measure of bone formation as well as bone turnover and is increased in MHD patients, probably as a result of high turnover bone disease. Atherosclerosis, in addition to being a disease of lipid accumulation, also represents a chronic inflammatory process. Inflammatory markers such as high-sensitivity C-reactive protein (hsCRP) may provide an adjunctive method for global assessment of cardiovascular risk. **Objectives of this work:** (1) Estimate the clinical utility of serum biomarkers of bone metabolism like ALP, bALP, intact parathyroid hormone, calcium, and phosphorus as potential markers and indicators in diagnosis of renal osteodystrophy in MHD patients aiming to improve their clinical outcomes. (2) Evaluate the association between renal osteodystrophy and progression of vascular calcification detected by echocardiography and carotid Duplex in MHD patients. (3) Testing the role of CRP and hsCRP in mediating the increased cardiovascular risk in MHD patients. **Patients and methods:** Seventy MHD patients and 15 healthy volunteers were enrolled in the study. All patients and controls were subjected to echocardiography, carotid duplex and predialysis blood sampling for estimation of routine blood chemistry (Calcium, Phosphorus, urea, creatinine, glucose, albumin, ALT, AST, ALP, cholesterol, triglyceride, HDLc), intact parathormone (iPTH) and hsCRP. Bone specific alkaline phosphatase (bALP) was also measured. **Results**: Plasma levels of ALP, bALP, iPTH, CRP, hs-CRP, urea, creatinine, glucose, phosphorus, were significantly higher in MHD group compared to control group. Statistical analysis revealed highly significance statistical difference in EDD, ESD, EF, IVS, PWT, IMT in MHD group compared to the control group. Mitral valve and aortic valve calcification was found in 27.4%, 71.4% respectively in hemodialyzed patients. b-ALP sensitivity, specificity and positive predictive value of the test at a cut off > 10 IU/L were found to be 89%, 67% and 79% respectively. ***Conclusion:*** Plasma bALP can be measured with a reliable immunoassay in hemodialysis patients represents a highly sensitive and specific biochemical marker of skeletal remodeling in these patients, even better when associated with plasma iPTH levels. Abnormal mineral metabolism and inflammation are pivotal factors for the increased cardiovascular risk in CKD patients.

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Keywords: Bone; Alkaline Phosphatase; Cardiovascular; Morbidity; Patients; Hemodialysis

1. Introduction

Vascular calcification is common in individuals with CKD and significantly correlated to the high cardiovascular death risk (1). Both intimal and medial calcification is observed frequently in patients with CKD (2,3). Several mechanisms have been implicated for the high prevalence of vascular calcification in CKD, inducing the high burden of conventional cardiovascular risks such as diabetes, hypertension, and dyslipidemia; bone and mineral disorders such as calcium load and secondary hyperparathyroidism and chronic inflammation (4).

In advanced CKD, stages 3 through 5, secondary hyperparathyroidism (SHPT), along with renal osteodystrophy, is common and may be associated with abnormal mineral metabolism and / or abnormal serum or tissue mineral levels, vascular calcification, and poor survival, especially among those who undergo maintenance dialysis treatment (5).

Serum ALP is a biochemical marker of bone turnover and is used to monitor the metabolic bone disease associated with renal insufficiency. Higher serum levels of minerals and higher ALP levels, were associated with increased all- cause death risk in a 2-year cohort of 58,000 MHD patients (5).

Experimental studies suggested that alkaline phosphatase might promote vascular calcification (6). Indeed, higher levels of serum alkaline phosphatase were independently associated with progressive arterial calcification in a longitudinal study of stage IV and V (CKD) patients (7).

Clinical studies have found serum ALP to be associated with coronary artery calcification and all-cause mortality in patients with CKD and on hemodialysis (8, 9).

Bone-specific ALP is a by-product of osteoblasts and is a more specific measure of bone formation as well as bone turnover (8). Bone-specific alkaline phosphatase (bALP) is increased in MHD patients, probably as a result of high turnover bone disease. Indeed, a statistical association between bALP level and the presence of aortic calcification are present in patients with osteoporosis (10). Similarly, in vivo study showed an increased level of circulating bALP in patients with CKD in presence of aortic calcification (11).

Apart from vascular calcification, inflammation is another potential mechanism for the association between higher serum alkaline phosphatase levels and increased mortality. Laboratory and experimental evidence indicate that atherosclerosis, in addition to being a disease of lipid accumulation, also represents a chronic inflammatory process (12). Thus, researchers have hypothesized those inflammatory markers such as high-sensitivity C-reactive protein (hsCRP) may provide an adjunctive method for global assessment of cardiovascular risk (13). Plasma levels of hsCRP have been associated with increased vascular event rates (14). Mendall *et al*., 2000 (15) demonstrated the association between hsCRP and all-cause mortality. Several large-scale prospective studies demonstrated that hsCRP is a strong independent predictor of future myocardial infarction and stroke among apparently healthy men and women. Pasceri et *al*., 2000 (16) described CRP within atheromatous plaque, as a correlate of endothelial dysfunction, and as having a direct role in cell adhesion molecular expression that raised the possibility that CRP may also be a potential target for therapy. Highly sensitive hsCRP has the potential to play an important role as an adjunct for global risk assessment in primary prevention of cardiovascular disease (14).

Aim of the Work:

1. Estimate the clinical utility of serum biomarkers of bone metabolism like ALP, bALP, intact parathyroid hormone, calcium, and phosphorus as potential markers and indicators in diagnosis of renal osteodystrophy in MHD patients aiming to improve their clinical outcomes.
2. Evaluate the association between renal osteodystrophy and progression of vascular calcification detected by echocardiography and carotid Duplex in MHD patients.
3. Testing the role of CRP and hsCRP in mediating the increased cardiovascular risk in MHD patients.

2. Methods:

Seventy adult uremic patients from dialysis unit of Theodor Bilharz Research Institute, (23 females and 47 males), were included in this study. Their mean (± SD) age was 56 ±14 years. All patients were treated by conventional hemodialysis, 3 sessions weekly, 4 hours each, for a period ranging from 7 to 10 years.

Exclusion criteria included liver diseases, ethanol or drug abuse, active malignancy and pregnancy.

Fifteen (age and sex matched) healthy control subjects, selected from medical and paramedical staff are included in this study.

All patients and controls in this study were subjected to the following:

- Full clinical examination and routine laboratory investigation.
- Electrocardiography.
- Echocardiography: Standard transthoracic M. mode, two dimensional, continuous and pulsed wave Doppler echocardiograms using 2.5 MHz transducer. All echocardiographic measurements were performed according to the recommendations of the American Society of Echocardiography (Sahn *et al.,* 1978).
- Carotid Duplex: Ultrasonographic studies on common carotid arteries were performed by using a 7.5 MHz high resolution probe. IMT was defined as a low-level echo gray band that does not project into the arterial lumen (Berglund, 1994) and was measured during end-diastole as the distance from the leading edge of the second echogenic line of the far walls of the distal segment of the common carotid artery, the carotid bifurcation, and the initial tract of internal carotid artery on both sides.
- Biochemistry: Predialysis blood sampling was performed after a 12-h fast. Routine chemistry (calcium, phosphorus, urea, creatinine, glucose,

albumin, ALT, AST, cholesterol, triglyceride, HDL and ALP were performed using autoanalyzer Beckman CX3 Delta analyzer. Plasma iPTH and hsCRP were measured by a solid-phase chemiluminescent enzyme-labeled immunometric assay using Immulite/ Immulite 1000. Bone ALP was measured by using a radioimmunometric assay provided by Hybritech Europe S.A. Belgium.

Statistical Analysis:

All results are given as the means ± SEM. Statistical analysis was performed comparison between two groups, parametric (*t*-test) or non-parametric (Mann–Whitney test) unpaired *t*-tests were used. The significance of the magnitude of correlation coefficients of biochemical compared with echocardiographic and duplex data was assessed by linear regression analysis. The P value was regarded significant if <0.05 at confidence interval 95%. The statistical significances 0f differences in frequencies of variants between the groups (expressed as percent) were tested using the chi-square (χ^2). Post-test probability parameters (sensitivity, specificity and predictive value positive and negative), receiver operator characteristic (ROC) curves and areas under the curves were obtained using Analyze Software for SPSS version 18.0 (Analyze software, LTD, Leeds, UK). ROC curves are a plot of the true positive rate (sensitivity) against false positive rate (1- specificity) and the area under the curve is a measure of test accuracy.

3. Results:

The demographic data of patients and controls revealed mean ages 56.1 ± 14 years and 51.6 ± 10.1 years respectively. Twenty three were females (33%) and forty seven were males (67%). Duration of hemodialysis ranged from 7-10 years with a mean 6.4±4.9 years. Systolic and diastolic blood pressure measurements ranged 80-190 mmHg with a mean 129.01 ± 19.25 and 50-110 mmHg with a mean 80.14 ±11.58 respectively in MHD group.

Plasma levels of ALP, bALP, iPTH, CRP, hsCRP, urea, creatinine, glucose, phosphorus, were significantly higher in MHD group compared to control group (P<0.001). However, Ca and albumin levels were significantly lower in MHD group when comparing the two studied group (P<0.001). No significant difference was observed for plasma level of AST, ALT, triglycerides and HDLc (Table 1 and figure 1).

Statistical analysis revealed highly statistical significant difference in echocardiographic data particularly EDD, ESD, EF, IVS, PWT, IMT in MHD group compared to the control group (Table 2).

The calcification percentage of the cardiac valves and pericardium in the studied groups are shown in (Table 3), which were significantly higher in MHD group compared to control group.

Positive correlations were found between bALP versus ALP (Figure 2) and iPTH (Figure 3) (r = 0.99), (P< 0.001), (r = 0.77) (P<0.001) respectively. However negative correlation was found between bALP and albumin (Figure 4) (r = - 0.448) (P < 0.001).

Similarly positive correlation was found between ALP and iPTH (r=0.4) (P<0.001) but ALP showed negative correlation with albumin (r= -0.35) (P <0.01).

Intact PTH showed a positive correlation with creatinine (Figure 5) (r = 0.289) (P < 0.01), and a negative correlation with albumin (r = -0.352) (P < 0.01).

Positive correlations were found between phosphorus and CRP, hsCRP and creatinine (r = 0.301) (P < 0.01), (r = 0.357) (P < 0.01) and (r = 0.478) (P < 0.001) respectively.

Posterior wall thickness showed positive correlation with ALP, bALP, iPTH and IMT (r = 0.245) (P < 0.02), (r = 0.249) (P < 0.02), (r = 0.246) (P < 0.02) and (r = 0.3) (P < 0.01) respectively.

IMT showed positive correlation with ALP, bALP, iPTH and hsCRP (r= 0.212) (p<0.05) , (r = 0.207) (P < 0.05) (r = 0.270) (P < 0.01) and (r = 0.22) (P < 0.04) respectively.

b-ALP revealed sensitivity 89%, specificity 67% and positive predictive value of the test 79% at a cut off > 10 IU/L as shown in figure (6).

However the test result variables of b–ALP and cholesterol, EDD, urea, creatinine and EF revealed area under curve 0.792,0.742 and 0.256 and significance 0.011, 0.035 and 0.033 concerning urea, creatinine and EF (Figure 7).

Table 1: Comparison Between Plasma Biochemical Parameters In The Studied Groups.

	Control group (n = 15)	MHD group (n = 70)	t-test	P value
ALP (IU/L) Range Mean±SEM	 18 – 65 45.07 ± 3.69	 20 – 330 144.20 ± 12.48	7.617	P<0.001
b-ALP (IU/L) Range Mean±SEM	 3.37 -12.85 8.84 ± 0.73	 3.85 – 110 43.73 ± 4.55	7.576	P<0.001
Ratio b-ALP/ALP Range Mean±SEM	 0.19 – 0.20 0.20 ± 0.004	 0.17 -0.34 0.27 ± 0.07	8.30	P<0.001
i PTH (pq/ml) Range Mean±SEM	 7.3 -82.7 35.60 ± 4.66	 14.80 -1746.70 437.82 ± 51.38	7.802	P<0.001
CRP (mg/L) Range Mean±SEM	 0.20 – 0.80 0.57 ± 0.033	 0.60 -4.80 0.97 ± 0.0.10	3.806	P<0.001
hsCRP (mg/L) Range Mean±SEM	 0.06 – 0.98 0.26 ± 0.06	 0 -7.20 1.07 ± 0.16	4.719	P<0.001
Urea (mg/dl) Range Mean±SEM	 15 – 42 29.50 ± 1.13	 19 – 272 117.51 ± 5.15	16.638	P<0.001
Creatinine (mg/dl) Range Mean±SEM	 0.40 – 1.20 1.23 ± 0.047	 1.7 -13.40 7.34 ± 0.31	19.34	P<0.001
Bl Sugar (mg/dl) Range Mean±SEM	 70 – 103 87.80 ± 2.6	 17 – 410 125.14 ± 9.68	3.719	P<0.001
Ca++ (mg/dl) Range Mean±SEM	 8.3 – 10.20 9.70 ± 0.18	 2.80 – 15.80 8.69 ± 0.24	3.482	P <0.001
Phosphorus (mg/dl) Range Mean±SEM	 2.50 – 4.50 3.28 ± 0.93	 1.30 – 11.50 5.36 ± 0.23	6.158	P<0.001
AST (IU/L) Range Mean±SEM	 12 – 32 21.93 ± 1.73	 5 – 51 18.50 ± 1.04	1.697	NS
ALT (IU/L) Range Mean±SEM	 18 – 35 24.80 ± 1.35	 5 – 48 21.93 ± 1.14	1.624	NS
Albumin (g/L) Range Mean±SEM	 3.60 – 4.30 2.20 ± 0.13	 1.2-4.3 2.86 ± 0.09	6.128	P <0.001
Cholesterol (mg/dl) Range Mean±SEM	 95 – 155 129.13 ± 4.18	 85 – 250 147.63 ± 5.16	2.784	P <0.001
TG (mg/dl) Range Mean±SEM	 77 – 127 101.33 ± 3.65	 15 – 928 117.73 ± 14.90	1.157	NS
HDLc (mg/dl) Range Mean±SEM	 35 – 43 38.73 ± 3.10	 30 – 49 37.56 ± 4.37	1.197	NS

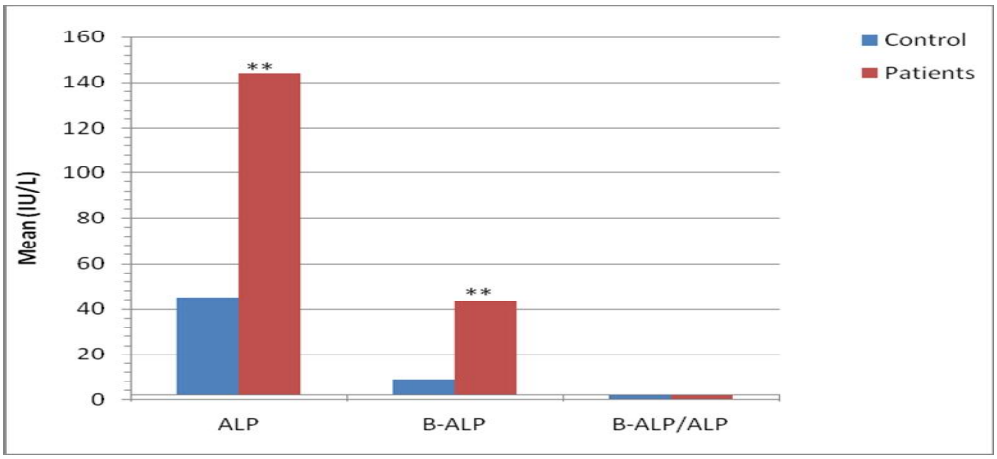


Figure (1) Comparison between ALP, b-ALP and b-ALP/ALP in the studied groups.

Table (2): Descriptive Statistics of Echocardiographic and Duplex Data of Studied Groups.

	Controls (n=15) Mean±SEM	Patients (n=70) Mean±SEM	U-test	P value
EDD	42.1 ± 1.29	49.8 ±1.04	4.65	P<0.001
ESD	26.6 ± 1.3	33.4 ± 0.63	4.99	P<0.001
IVS	8.9 ± 0.3	11.6 ± 0.27	6.83	P<0.001
PWT	8.9 ± 0.28	11.1 ± 0.11	6.68	P<0.001
EF	68.2 ± 1.4	63.1 ± 1.2	2.69	P<0.013
IMT	0.79 ± 0.03	1.11 ± 0.08	3.59	P<0.001

EDD: End diastolic diameter ESD: End systolic diameter
IVS: Interventricular septum PWT:Posterior wall thickness
EF: Ejection fraction IMT: Intima media thickness

Table (3): The calcification of cardiac valves and pericardium of the studied groups

	Patients (n=70)	Controls (n=15)	χ^2	P value
Aortic valve	50 (71.4%)	2 (10%)	15.1	0.05
Mitral valve	15 (27.4%)	1 (5%)	0.93	NS
Pericardium	60 (85.7%)	2 (10%)	29.2	0.01

Table (4):The correlation between different parmeters

	PWT	iPTH	IMT	Albumin	bALB	hsCRP	CRP	Creatinine
Phosphorus						r=0.357 P<0.002	r=0.3 P0.01	r=0.478 P<0.001
iPTH	r= 0.246 P<0.02			r=-0.35 P<0.01				r= 0.289 P<0.01
IMT	R=0.3 P<0.01	r = 270 P<0.01				r=0.22 P<0.04		
bALB	r=0.249 P<0.02	r=0.77 P<0.001	r=0.207 P<0.05	r=-0.448 P<0.001				
ALP	r= 0.245 P<0.02	r=0.4 P<0.001	R=0.212 P<0.05	r = -0.35 P<0.003	r=-0.99 P<0.001			

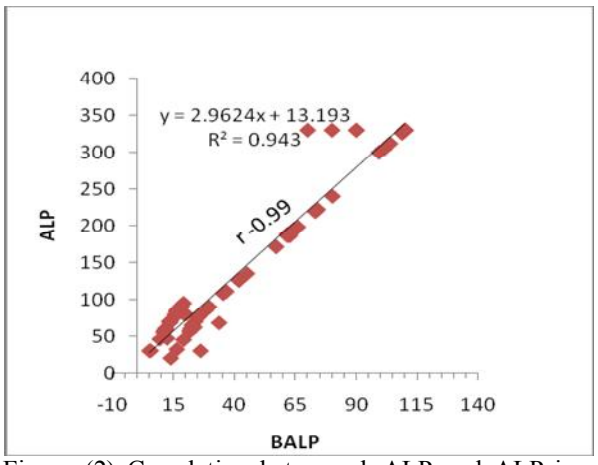


Figure (2) Correlation between b-ALP and ALP in MHD patients

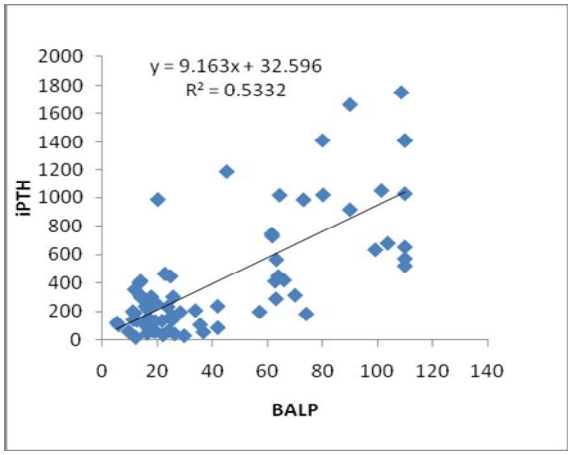


Figure (3) Correlation between b-ALP and i-PTH in MHD patients.

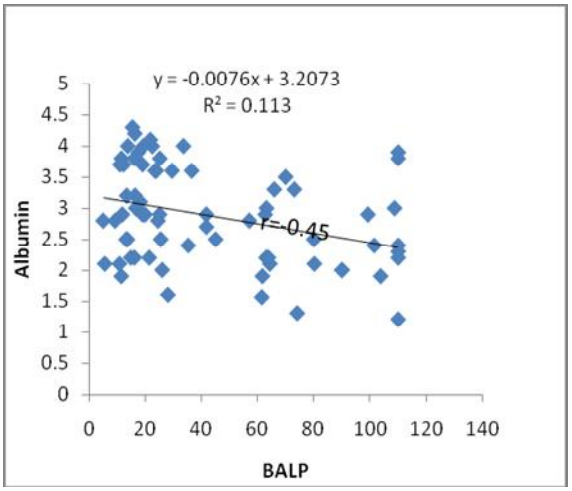


Figure (4) Correltion between b-ALP and albumin in MHD group.

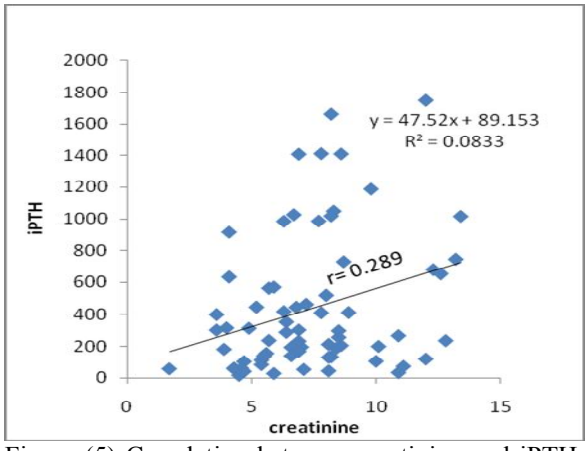


Figure (5) Correlation between creatinine and iPTH in MDH group.

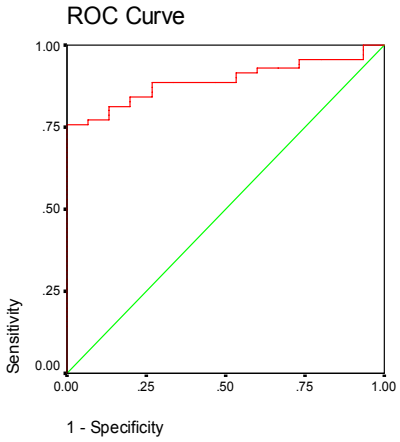
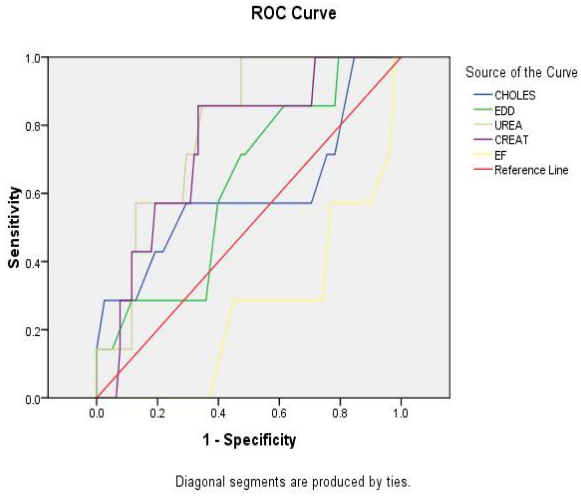


Figure (6) ROC curve of b-ALP



Figure(7) ROC curve b-ALP and cholesterol, EDD, urea, creatinine and EF.

4. Discussion:

In advanced chronic kidney disease (CKD; stage 3 through 5), secondary hyperparathyroidism (SHPT), along with renal osteodystrophy is common and may be associated with abnormal mineral metabolism and / or abnormal serum or tissue mineral levels, vascular calcifications and poor survival especially among those who undergo maintenance dialysis treatment (17). Progression of vascular calcification closely parallels bone loss, (18).

In the current study plasma levels of ALP, bALP and iPTH are significantly higher in MHD group compared to control group (P<0.001). This coincides with study of (19) who reported that: Serum ALP concentration is usually increased in renal osteodystrophy, especially in high-turnover bone disease (19). The KDOQI guidelines state that the deleterious effects of high serum PTH levels may be manifested by elevated b-ALP activity as a result of associated bone resorption (20).

The magnitude of bALP enzyme elevation may indeed be a more reliable marker of severity of the high-turnover osteodystrophy than increased PTH levels, especially because the circulating serum bALP originates directly from the pathologic bone system. Consistent with the foregoing motion, a meta-analysis showed that the treatment of renal osteodystrophy by means of vitamin D analogs can effectively decrease bALP, even through such a treatment may not decrease serum PTH consistently (21), hence the reported link between vitamin D analogs and improved survival in CKD may be via the bALP pathway (22).

Bone-specific ALP (bALP) is secreted by osteoblast cells, and it is thought that bALP plays a major role in bone formation and skeletal mineralization .It is increased in MHD patients, probably as a result of high-turnover bone disease (11); indeed, a statistical association between bALP level and the presence of aortic calcification is present in patients with osteoporosis (10). Similarly, an in vivo study showed an increased level of circulating bALP in patients with CKD in the presence of aortic calcification (11).

In the study presented by Urena and his colleagues in 1996 (23) demonstrated that bone formation and bone resorption parameters correlated better with plasma bALP levels, rather than with ALP or iPTH levels. Accordingly, plasma bALP levels were the best predictors bind with plasma iPTH levels, the predictive value of this marker was even increased. Previous studies suggested that plasma bALP could be a sensitive marker in the assessment of bone turnover in uremic patients with secondary hyperparathyroidism. They correlated with both bone formation and resorption parameters better than iPTH and ALP did (24).

Elevated levels of bone alkaline phosphatase virtually exclude an adynamic renal bone disease (25); however, elevations of bALP along with total ALP may be seen in cases of severe osteomalacia. Combinations of biochemical markers hold promise at least for the differentiation for high-turnover versus adynamic forms (26).

It may be considered that high-turnover state of bone is driven by secondary hyperparathyroidism, which is characterized by poorly differentiated osteoblast precursors manifesting a fibroblastic phenotype and by increased osteoclastic activity. This results in net bone resorption, fibrosis of the bone marrow space and release of calcium and phosphate into the extracellular fluid (27). Both ions could function as promoters of vascular smooth-muscle cell phenotypic differentiation into osteoblast-like cells and initiate the vascular calcification process (18). Concerning the role of secondary hyperparathyroidism on vascular calcification, Neves and his colleagues in 2007(28) showed that normal and uremic rats submitted to parathyroidectomy that subsequently received PTH replacement in supraphysiological doses developed intense aortic medial calcification, with some animals showing coronary calcification. These findings suggested that high PTH levels induced high bone turnover and medial calcification independent of uremia (18).

In this study left ventricular diameters include interventricular septal thickness at end diastole (IVS), posterior wall thickness at end diastole (PWT) and left ventricular internal diameter at end diastole (EDD) and systole(ESD) were highly significant increased in patients with chronic hemodialysis than control group (P<0.001). Also left ventricular Ejection fraction (EF) was highly significant decreased in patients with chronic hemodialysis than control group. Resic and his colleague in 2009 in their study found increase in left ventricular thickness and hypertrophy in 55.8% and left ventricular dysfunction in 60% of patients with chronic hemodialysis than control group.

The present study, revealed that intima-media thickness was highly significant increased in patients with chronic hemodialysis than control group (P<0.001). Common carotid artery intima-media thickness as a measure of subclinical vascular disease was found to be increased in patients with chronic hemodialysis than control group. (30).

In this study it was found that mitral valve calcification was found in 27.4% and aortic valve calcification was found in 71.4% of hemodialyzed patients. Vascular calcification is highly correlated

with cardiovascular disease mortality, especially in patients with ESRD. In addition to the devastating effects of inappropriate biomineralization seen in cardiac valvulopathies, calciphylaxis, and idiopathic arterial calcification, valvular calcification is common in patients with end-stage renal disease, and is associated with an unfavorable prognosis. Raggi and his colleagues in 2004(31) found that mitral valve calcification was seen in 46% of subjects and aortic valve calcification in 33% in hemodialysis patients. Also valvular calcifications, predominant in aortic and mitral positions, were found in 30-50% of hemodialyzed patients (32).

Beddhu and his colleagues in 2009(7) observed that independent of liver function tests and serum calcium and phosphorus, serum alkaline phosphatase might be a risk factor for death in African-American patients in CKD stages III and IV. The potential mechanisms for this observation remain unclear. ALP has been shown in histologic sections of vessels obtained from patients with CKD-associated calcific uremic arteriopathy (33 &34), ALP seems to play a mediating and instrumental role (6). It is likely that the ALP-mortality association in CKD, including the observed link with cardiovascular death, is related to vascular calcification through its pyrophosphate link (4),

However, there is mounting evidence that alkaline phosphatase can promote vascular calcification by hydrolyzing pyrophosphate in the arterial wall (3). For instance, in calcified diabetic arteries, alkaline phosphatase is upregulated (35). In the aorta of uremic rats, the hydrolysis rate of pyrophosphate was increased compared with controls (3). This increase was reduced by levamisole, a nonspecific inhibitor of alkaline phosphatase. These experimental data suggested a role for alkaline phosphatase in the development of uremic calcification. Indeed, in a longitudinal study of 134 stage IV and V CKD patients, higher levels of serum alkaline phosphatase were associated with progressive arterial calcification (36).

The protective effects of low bALP and ALP levels include the mechanisms via pyrophosphate. Experimental data link high ALP to the development of coronary artery calcification with their ability to hydrolyze inorganic pyrophosphate (37). Pyrophosphate is a potent inhibitor of vascular calcification, and its biologic action is reduced by phosphatases. High levels of bALP were associated with mortality and specific fatal events (38). They concluded that high levels of bALP were strongly associated with short term mortality in dialysis patients, pointing out the important impact of bone turnover. Longitudinal assessments of bALP may be useful for the treatment monitoring in clinical practice in dialysis patients.

Indeed, genetic ablation of tissue-nonspecific ALP leads to amelioration of soft tissue calcification in animal studies. Some novel inhibitors of the physiologic pyrophosphatase activity of ALP are capable of reducing vascular calcification in animal models (6), however, the ALP-death link may have additional causes, such as its relationship with inflammation or malignancies (8). The current study shows that CRP and hsCRP are significantly higher in MHD group compared to the control group ($P<0.001$). In addition to vascular calcification, there are other potential mechanisms that may mediate the associations of serum alkaline phosphatase with increased mortality. One of the potential explanations is that higher serum alkaline phosphatase might be associated with inflammation. Damera *et al.*, 2011 (39) found that serum alkaline phosphatase level has been associated with elevated CRP level which is a marker of inflammation. Atherosclerosis has been well established to be an inflammatory process, (40), in another study of Chinese adults, higher serum alkaline phosphatase levels were also associated with elevated CRP levels. (41).

Several studies have documented the importance of abnormal mineral metabolism (42) and inflammation (43) as pivotal factors for the increased cardiovascular risk in CKD patients. Two previous studies in hemodialysis, with a limited number of patients, showed that a high Ca x P was associated with high CRP concentrations (44). In summary elevated serum alkaline phosphatase levels might reflect not only altered bone mineral metabolism but also an atherogenic milieu. these data may have major clinical and public health implications given the high burden of vascular calcification in patients with CKD (9). Because of the significant association of osteodystrophy with cardiovascular calcification, cardiovascular disease and death, diligent treatment of high-turnover bone disease may be an effective measure to improve survival in CKD. Close monitoring of ALP levels may be useful when considering initiation or changes of the therapy. To better understand the natural course of renal osteodystrophy and its complications in CKD; and to evaluate the effectiveness of current and future treatments including vitamin D analogs, calcimimetics and other medications such as ALP inhibitors in improving osteodystrophy and clinical outcome in CKD population.

Conclusion:

Plasma bALP should be measured with a reliable immunoassay in hemodialysis patients. It represents a highly sensitive and specific biochemical marker of skeletal remodeling in these

patients, even better when associated with plasma iPTH levels. Abnormal mineral metabolism and inflammation are pivotal factors for the increased cardiovascular risk in CKD patients.

These findings may have major clinical and public health implications given the high burden of vascular calcification in patients with CKD and potential therapeutic strategies e.g. ALP inhibitors to modulate this pathway to mitigate or prevent risk for vascular calcification and improve the poor survival of patients with CKD which can be evaluated and detected by echocardiography and carotid Duplex .

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Liver Transplantation: An Experience in Post-Operative Follow Up Of 80 Patients

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Abstract: Background: Orthotopic liver transplantation (OLT) is regarded as the only choice for treatment of patients with end stage liver disease (ESLD) and liver failure. Outcome for those patients is much better after liver transplantation resulting in reasonably good quality of life, provided complications are detected and treated promptly. Evaluation of needle liver graft biopsies and extensive clinicopathological correlation play an important role in the determination of liver allograft dysfunction after transplantation. **Objective:** To evaluate possible post-transplant outcome of patients underwent OLT. **Methods:** We analyzed 80 patients with liver cirrhosis who underwent deceased donor liver transplantation (DDLT) over a 10–year period in a cohort and observational study. The study was performed from June 2000 to June 2010 and included 75 men and 5 women. **Results:** Among all patients; origin of cirrhosis was post-viral in 76 patients, brimary biliary cirrhosis (PBC) in 2 cases, autoimmune hepatitis (AIH) in one case and cryptogenic cirrhosis in another case. The cases of post-viral cirrhosis were all of viral C etiology with 20 cases associated with hepatocellular carcinoma and 2 associated with hepatitis B viral infection. The patients are followed up for at least 18 months after enrolling in the study. They all had routine tests at the start of the study used as baseline for each patient. These tests are repeated according to the requirements of the individual patient. All patients had Tacrolimus (FK 506) as an immunosuppressive agent. Patients with hepatitis B viral infection had hepatitis B immunoglobulin, along with Lamivudine for relapse prophylaxis. Out of 80 patients, postoperative liver biopsy was performed, at least once, for 73 patients. The results of the biopsies revealed that recurrent HCV was detected in 46 (63.01%) cases, acute rejection in 14 (19.18%) cases, chronic rejection in 4 (5.48%) cases, cirrhosis in 2 (2.74%) cases, fibrosing cholestatic hepatitis in 2 (2.74%) cases, chronic active hepatitis with cholangitis and bile duct obstruction in 2 (2.74%) cases, recurrent primary biliary cirrhosis in 2 (2.74%) cases and one case (1.37%) with acquired schistosoma japonicum. A total of 24 (30%) patients died during follow up. **Conclusion:** The results of liver graft biopsies revealed that recurrent HCV is the prominent cause of organ dysfunction. Meanwhile; organ rejection was less frequently encountered. The complications of liver transplantation can be controlled and managed if diagnosed promptly and treated early.

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Keywords: Orthotopic liver transplantation, Post operative follow up, Experience.

Abbreviations: AIH: Autoimmune hepatitis, CMV: Cytomegalovirus, DDLT: Deceased donor liver transplantation, ESLD: End stage liver disease, HCV: Hepatitis C virus, OLT: Orthotopic liver transplantation, PBC: Primary biliary cirrhosis

1. Introduction

Once regarded as a last resort therapy for patients with end stage liver disease, liver transplantation has become a viable therapeutic option because of improvement in surgical techniques and the development of more powerful immunosuppressive agents. Orthotopic liver transplantation (OLT) is now the therapy of choice for liver failure that results from all types of acute and chronic liver disease as well as hepatic neoplasms. The pathological evaluation of liver allograft biopsies plays an integral role in the management of patients following liver transplantation. In certain cases it is regarded as the "Gold Standard" because no other reliable diagnostic marker exist. The clinicians relies on the pathological interpretation of liver biopsy report to diagnose or rule out graft rejection, drug toxicitiy, recurrence of transplant disease (e.g. viral hepatitis) or development of a new disease (e.g. Cytomegalovirus infection or de novo autoimmune hepatitis) (1). Since the early 1980`s, there has been a consistent improvement in the results of liver transplantation and current one year survival is almost 80%. (2).

Liver transplantation is potentially applicable to any acute or chronic conditions, in both children and adults, resulting in irreversible liver dysfunction provided that the recipient does not have other conditions that will preclude a successful transplant.

The major indications in children are biliary atresia and inherited or genetic disorders of metabolism associated with liver failure (3).

Since the start of hepatic transplantation as a treatment for end-stage hepatic diseases, various factors, such as the surgical techniques, postoperative treatments, and immunosuppressant drugs have improved exponentially. These improvements have ultimately decreased morbidity and mortality, as well as increased global survival of both the graft and the patient. Currently, this procedure offers a 5-year survival rate of 70%. However, it is not free from complications (4).

After liver transplantation, there are three types of graft rejection that may occur. Hyperacute rejection (which is humoral B cell mediated) is a rare event that happens within minutes to hours after transplantation. The presence of dual blood supply protect the organ from ischemia. Kupffer cell binding of performed antibodies and removal of immune complexes reduces the susceptibility of the liver to hyperacute humoral rejection. Acute rejection is T cells mediated reaction that involves direct cytotoxicity and cytokine mediated pathways. It occurs within days, weeks, or months after transplantation and it is the most common form of rejection. Chronic rejection is the presence of any symptom or sign of rejection after 1 year. The cause of chronic rejection is still unknown but an acute rejection is a strong predictor of chronic rejection (5).

The aim of this study is to evaluate possible post-transplant outcome of patients underwent OLT in other centers and presented to Police Hospital, Giza, 3 months after liver transplantation for following up .

2. Patients and Methods

This Study conducted on 80 patients with cirrhosis underwent liver transplantation and presented to Police Hospital, Giza (three months after transplantation) during the period from June 2000 to June 2010; (As liver transplantation is performed only in highly specialized tertiary care centers, a significant proportion of cases are followed elsewhere according to the residence, health insurance or other factors). It was a cohort non randomized and observational study. Liver transplantation was performed in England (n=69), China (n=10) or Germany (n=1), and it was cadaveric liver in all cases. All patients had certain routine laboratory, radiologic and clinical studies at the start of the follow up used as baseline for follow up. These were repeated according to the requirement of the individual patient. The investigations included complete blood count, coagulation profile, liver function tests, kidney function tests, antimitichondrial and antinuclear antibodies, electrolytes, blood sugar and serum immunosuppressive level. All patients also had baseline ultrasound and CT or MRI images. Cytomegalovirus PCR, IgM and IgG are also performed. If liver transplantation was due to hepatitis B viral (HBV) infection, then HBs Ag and anti -HBV were investigated. If transplantation was due to HCV, then PCR for HCV RNA was tested. All patients receive immunosuppressive treatment according to indicated regimen. Patients having transplantation secondary to hepatitis B receive immunoglobulins every month (according to anti HBs Ag level) in addition to Lamividine.

Percutaneous Liver biopsy was performed under ultrasound guidance at least once for 73 patients. Adequate core including at least 6 small portal tracts was obtained. The samples were fixed with 10 % formaldehyde, embedded in paraffin. Three to four micrometers sections were cut and routinely stained with haematoxylin-eosin, PAS and Masson's trichrome stains. Immunostain for CK 7 was performed whenever needed. The liver biopsies were evaluated for hepatitis activity and fibrosis according to the modified Knodell`s as well as METAVIR scoring systems · For allograft rejection; we used Banff schema and template for allograft liver biopsies (6).

Patients who enrolled early in the study were followed up for 10 years whereas the last patient enrolled was followed up for 18 months, thus the majority of the patients were followed up for more than 5 years .

3. Results:

Results are shown in Tables 1-5 and Figures 1-12.

Among 80 patients; 75 were males and 5 were females. Their ages ranged from 38 to 60 years apart from one girl aged 6 years with a mean age of 51.1 ±6.4 years**.** The causes of liver failure and indication for liver transplantation of all studied patients are shown in table (1). The main indication for liver transplantation was predominantly HCV infection 76 (95%); with 20 (26.31%) patients associated with hepatocellular carcinoma (HCC) and 2 (2.63%) accompanied with HBV infection. Other indications included primary biliary cirrhosis (PBC) in 2 (2.5%) autoimmune hepatitis (AIH) in 1(1.25%), and cryptogenic cirrhosis in 1(1.25%).

Out of 76 patients with HCV infection; 61(80.3%) showed preoperative associated diseases as featured in table (2). Also preoperative interferon therapy performed for 32 cases, and sclero-therapy in 2 cases for oesophageal varices.

During the post-operative follow up period, operative complications or newly acquired diseases are demonstrated in table (3).

Out of 80 patients, postoperative liver biopsy was performed, at least once for 73 patients. It was indicated by elevated liver enzymes or sonographic findings. For the remaining 7 cases, no biopsy was obtained, at least during our follow up. The results of

the histopathological examination of liver graft biopsies revealed that recurrent HCV is the prominent cause of organ dysfunction. Meanwhile; organ rejection was less frequently encountered. These results are detailed in table (4) and figures (1-12).

During our follow up; 24 (30%) patients died for various causes and at various post operative periods of survive as mentioned in table (5). To summarize the causes of 24 deaths; HCV represented the major cause (14 cases, 58.3%) , Graft rejection (4 cases,16.6%), RI (1 case,4.2%), HBV (1 case, 4.2%), Recurrent PBC (1 case, 4.2%), operative complications (1 case, 4.2%) and acquired infection such as tuberculosis and fungal infection (2 cases, 8.3%).

Table (1): Indications for liver transplantation, age and sex distribution of 80 cases

Indications for liver transplantation	No of patients	Age (range) at time of transplantation	Sex	
			M (no)	F (no)
-HCV:	76 (95%)	38-60 Y	73	3
HCV+HCC	20/76(26.31%)	45-59 Y	20	0
HCV+HBV	2/76 (2.63%)	45-60 Y	2	0
-PBC	2 (2.5%)	6-38Y	1	1
-AIH	1(1.25%)	48 Y	0	1
-Cryptogenic cirrhosis	1(1.25%)	40 Y	1	0

AIH: Autoimmune hepatitis, F: Female, M: Male, PBC: Primary biliary cirrhosis

Table (2): Preoperative associated diseases in 61 HCV patients:

Associated diseases	No. of patients
• Hepatitis B virus (HBV)	2
• Hepatocellular carcinoma (HCC)	20
• Bilharziasis	3
• Hypertension (HTN)	16
• Renal impairment (RI)	5
• Diabetes mellitus	11
• Portal vein thrombosis (PVT)	3
• Tuberculosis (TB)	1
Total	61

Table (3): Post-operative complications or acquired diseases in all studied patients (no=80):

Disease	No. of patients
• Recurrent HCV	76 (all HCV cases)
• Diabetes Mellitus (DM)	19
• Renal impairment (RI)	1
• Acquired HBV (donor)	2
• Cytomegalovirus (CMV)	6
• Pulmonary fungal infection	1
• Tuberculosis (TB)	1
• Biliary stricture	6
• Acquired (donor) schistosomiasis	1

Table (4): Results of postoperative liver graft biopsy examination in 73 patients:

Diagnosis of liver biopsy	No. of patients
• Chronic active hepatitis (CAH)	46 (63.01%)
• CAH associated with cholangitis and biliary obstruction	2 (2.74%)
• Cirrhosis	2 (2.74%)
• Fibrosing cholestatic hepatitis	2 (2.74%)
• Acute rejection	14 (19.18%)
• Chronic rejection	4 (5.48%)
• Recurrent primary biliary cirrhosis	2 (2.74%)
• Acquired schistosoma japonicum in transplanted liver	1 (1.37%)
Total	73

Table (5): Duration of post-operative survival and cause of death in 24 transplanted patient.

Surviving period (Y)	No. of cases	Cause of death	Original disease
‹ 1 year	2	T.B.& Pulmonary fungal infection (1) Operative complication (1)	AIH (1) HCV (1)
2 years	2	HCV relapse	HCV
3 years	8	HCV relapse (7) RI (1)	HCV (4) HCV+HCC (3) HCV+HCC+PVT (1)
4 years	3	HCV relapse	HCV (2) HCV+HCC (1)
5 years	1	HCV relapse+ RI	HCV+HCC+ Grade II nephropathy
6 years	1	Acquired HBV+ Gilbert syndrome	HCV
7 years	1	Recurrent PBC	PBC
8 years	5	CR (3) CR +PVT (1) TB peritonitis (1)	HCV
9 years	1	HCV relapse+ Cirrhosis	HCV
Total	24		

AIH: autoimmune hepatitis, CR: chronic rejection, HBV: hepatitis B virus, HCC: hepatocellular carcinoma, HCV: hepatitis C virus, RI: renal impairment, PVT: portal vein thrombosis, T.B: tuberculosis.

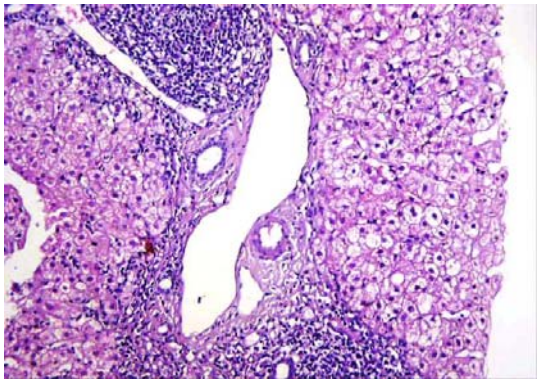

Figure (1): Recurrent viral C hepatitis with portal expansion by lymphocytic aggregates, fibrosis, interface hepatitis, dilated portal vein and fibrosis. Bile ducts are near normal, (Hx&E x200).

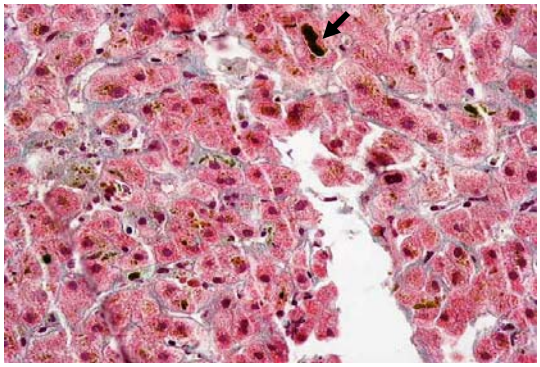

Figure (2): Fibrosing cholestatic variant of recurrent viral hepatitis C showing diffuse swelling of hepatocytes with intracellular cholestasis and bile thrombi (arrow). No significant lobular inflammation noticed (Masson Trichrome stain x400).

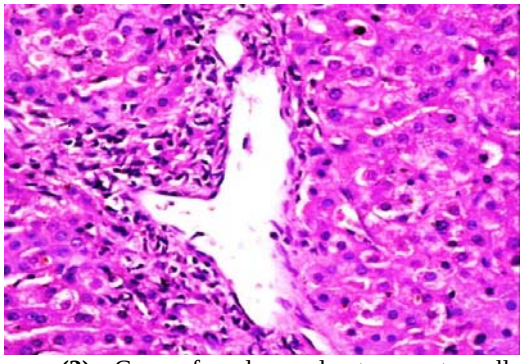

Figure (3): Case of early moderate acute cellular rejection showing portal venulitis .Few lymphocytes adhere to the luminal surface of endothelium , subendothelial lymphocytic infiltrate, lifting of the endothelial lining and perivenular parenchymal inflammation, (Hx&E x400)

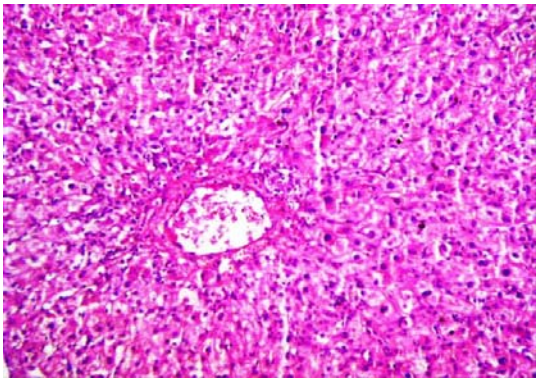

Figure (4): Acute cellular rejection showing perivenular central zonal necrosis, hemorrhage and inflammation with drop out of hepatocytes. No evidences of steatosis. (Hx&E x200).

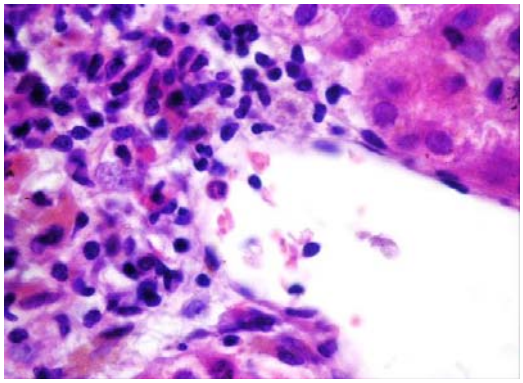

Figure (5): Case of acute cellular rejection with portal mixed inflammatory cell infiltrate including neutrophils, eosinophils, blast cells and lymphocytes with focal venulitis . The interface is mostly respected in this examined field, (Hx&E xOil).

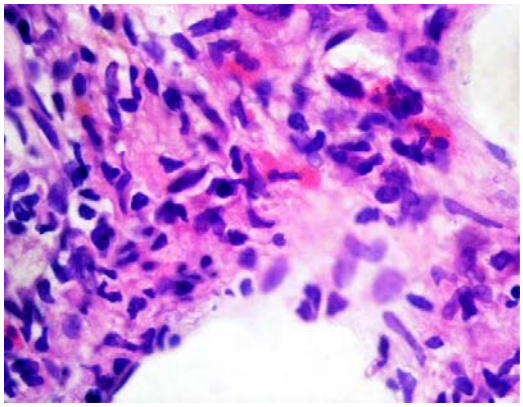

Figure (6): Aportal tract showing many eosinophils, lymphocytes and blast cells, (Hx&E xOil).

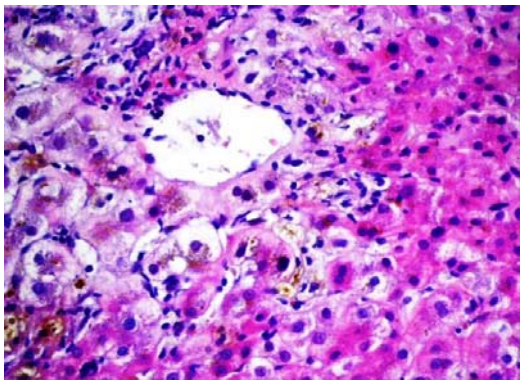

Figure (7): Evolving chronic rejection. Pericentral ballooning degeneration of hepatocytes, hepatocyte drop out and sinusoidal inflammation with cholestasis, (Hx&E x400).

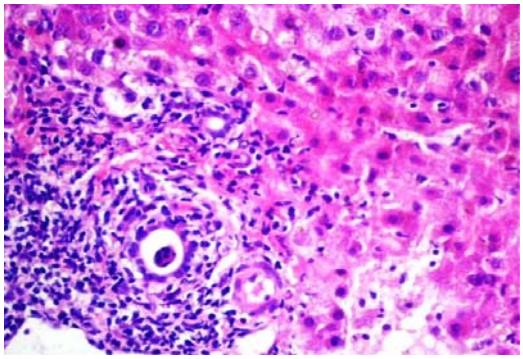

Figure (8): Case of acute cellular rejection showing portal mixed inflammatory infiltrate and periductal cuffing by inflammatory cells with luminal exfoliation of epithelial lining .No evidences of hepatocellular steatosis detected. Adjacent portal artery is present (Hx&E x200)

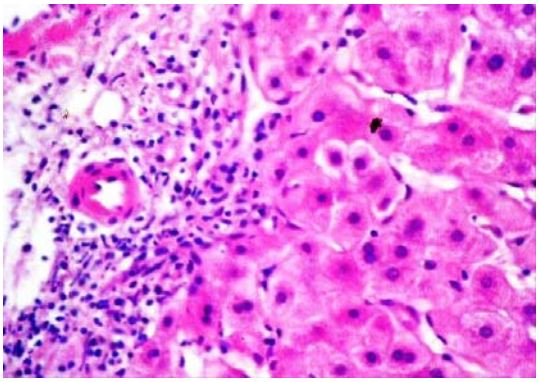

Figure (9): Case of chronic rejection showing portal tract devoid of bile ducts and widow artery with reduction in the overall degree of cellular infiltrate of portal tract and minimal interface inflammation (Hx&E x400).

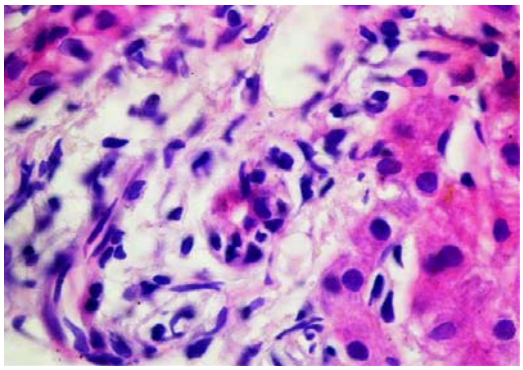

Figure (10): Evolving chronic rejection .An interlobular bile duct shows lymphocytic infiltration ,nuclear pleomorphism, hyperchromasia and disordered polarity . Scanty portal inflammatory cells with no prominent interface reaction noticed (Hx&E x Oil).

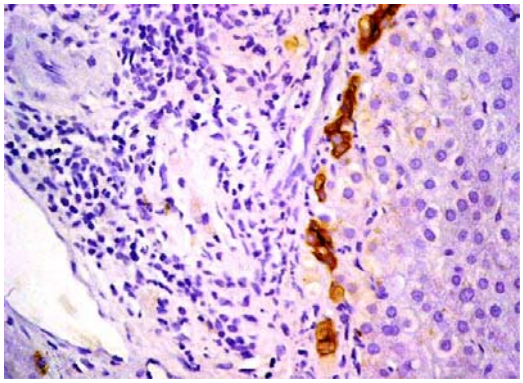

Figure (11) : Case of chronic rejection showing paucity of bile ducts in the portal tract, with preservation of the normal marginal bile ductules, (immunostain for CK7 x400).

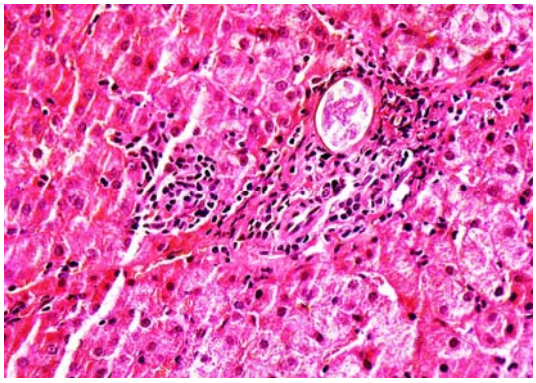

Figure (12): Case of postoperative bilharziasis of the liver showing ova of acquired schistosoma japonicum, (Hx&E x200).

4. Discussion:

It could be seen by reviewing data of our study that outcome for end stage liver disease patients is much better after liver transplantation resulting in reasonably good quality of life, provided complications are detected and treated promptly. Our results reveal survival rate about 70 %. Post transplant 3 and 5 year survival figures in excess of 70% are also achieved in many other places (7). Evaluation of needle biopsies and extensive clinicopathological correlation play an important role in the determination of liver allograft dysfunction after transplantation. Interpretation of these biopsies can be quite difficult because of the high incidence of recurrent diseases that show histopathological, clinical and serological features that overlap with each other and with rejection (3). The main challenge for biopsy examination was to differentiate recurrent viral infection from graft rejection. Accurate diagnosis is important for reduction of immunosuppression as immunodeficiency is likely to be the most important predisposing factor leading to unchecked viral replication. Some of the features may be helpful in distinguishing recurrent viral hepatitis from acute cellular rejection (8).

This study involved 80 patients with liver cirrhosis who underwent orthotopic liver transplantation (OLT) over a 10–year period in a cohort and observational study. The study is performed from June 2000 to June 2010. The patients are followed up for at least 18 months after enrolling in the study. They all had routine tests at the start of the study used as baseline for each patient. Our study included 75 men and 5 women.

Out of 80 patients, postoperative liver biopsy was performed, at least once, for 73 patients. The results of the liver graft biopsies revealed that recurrent HCV is the prominent cause of organ dysfunction 46 (63.01%) cases. Meanwhile organ rejection was less frequently encountered (acute rejection in 14 (19.18%) cases, chronic rejection in 4 (5.48%) cases, cirrhosis in 2 (2.74%) cases, fibrosing cholestatic hepatitis in 2 (2.74%) cases, chronic active hepatitis with cholangitis and bile duct obstruction in 2 (2.74%) cases, recurrent primary biliary cirrhosis in 2 (2.74%) cases and one case (1.37%) with acquired schistosoma japonicum. Moreover; there was no recurrence for HCC encountered in this study (20 cases).

Hepatitis C recurrence is nearly universal after transplantation. It leads to chronic hepatitis and liver cirrhosis in a significant proportion of patients and lowers graft and patient survival. The most useful feature pointing to the diagnosis of acute rejection and differentiating it from recurrent viral hepatitis is the presence of mixed population of inflammatory cells in portal tract infiltrate. These lesions show considerable variation in intensity and it is therefore recommended that a minimum of 5 portal tracts are available for examination (9).

Occasional patients with recurrent HCV after liver transplantation will have an aggressive course characterized histologically by pericellular /sinusoidal fibrosis and cholestasis, known as fibrosing cholestatic hepatitis (FCH). The presence of cholestasis and fibrosis with mild to moderate recurrent HCV should raise the suspicion of FCH. When studying the evolution of these cases, the first abnormality to appear is recurrent HCV and cholestasis, fibrosis develops soon after, and both continue to worsen until the point of allograft failure (10). Schluger et al, 1996 suggests that a minority of patients with recurrent hepatitis C after undergoing liver transplantation develop a severe progressive to cholestatic hepatitis and liver failure (7).

In this study ; there were 2 cases (2.74%) diagnosed as fibrosing cholestatic hepatitis . It is an aggressive and unusual fatal form of viral hepatitis in immunosuppressed patients. It is characterized by progressive cholestasis leading to hepatic failure, and characteristic histopathological features including: periportal fibrosis,

ballooning degeneration of hepatocytes, cholestasis, with minimal inflammation. FCH has only been described in immunosuppressed patients with chronic hepatitis B or C . In contrast to the pathogenesis of chronic hepatitis in immunocompetent patients, attributed to the cellular immune-mediated hepatocytolysis, FCH has been postulated to result from unimpeded viral replication within hepatocytes, culminating in a direct cytopathic effect, in the setting of immunosuppression (11, 12).

Hepatitis recurrence may be difficult to distinguish from cellular rejection. At an early stage following recurrence, acidophilic bodies, minimal portal inflammation and inconstant fatty infiltration favor hepatitis C, whereas portal inflammation with activated lymphocyts including eosinophils suggests associated rejection. Later, the changes of hepatitis recapitulate those seen in the non – transplanted setting. They may show an intimate association with both small bile ducts, which are infiltrated, but not destroyed, and portal venules, which may mimic endotheliitis (12). The associated mild lobular hepatitis and the presence of fatty infiltration do favors HCV infection, but acidophil bodies are similarly found in the rejection and not as useful as in the early stage to discriminate hepatitis C from rejection. In most cases where doubt subsists, the changes are generally mild and HCV RNA results and level of immuno-suppression may be of assistance. It is important to remember that untreated mild rejection is often innocuous, whereas high dose steroids may have a deleterious effect on hepatitis C (13, 14).

The histological diagnostic triad of acute rejection included portal inflammation, bile duct damage and venular endotheliitis. At least two of these features are required for diagnosis of acute rejection. It is suggested that perivenular necrosis may be important as an early diagnostic feature of evolving chronic rejection (12).

All of these inflammatory cell types are implicated in mediating damage of the bile ducts and endothelial cells in rejection. The presence of large number of eosinophils appears to correlate with the more severe degree of rejection and may have a prognostic significance in predicting poor response to additional immunosuppressive therapy (15). A system devised at the Royal Free Hospital, London incorporate portal tract eosinophilia as a fourth feature resulting in overall score ranging from 0 to 12 (16). Eosinophils, for some author a defining feature of acute rejection, are not constant; when present in large number, they may correlate with a more severe degree of rejection and predict a poor response to steroids. Perivenular injury with cholestasis, apoptotic bodies, cell ballooning and confluent cell drop out may reflect a more severe rejection (generally asscociated with hepatic venulitis), a harvesting / reperfusion injury or an ischaemic element (17).

Acute cellular rejection reveals various histologic combinations of the classic triad of mixed portal tract inflammation, bile duct injury and venular endotheliitis (3). The universal incidence of graft failure from chronic rejection was 2.4%. The diagnostic features of chronic rejection are loss of bile ducts and obliterative arteriopathy affecting large or medium sized arteries. The fact that these arteries are not usually biopsied, and that bile duct loss is not always apparent, makes it somewhat difficult in diagnosis (2).

Histological changes of liver allograft rejection have been well characterized, but features which somewhat depart from classical description and newly recognized post transplant complications, especially at a later stage after surgery, may raise diagnostic problems. Important is to evaluate the histological changes in conjunction with clinical information, in particular the primary liver disease and the timing after surgery which are essential (15).

In patients presenting with progressive jaundice, the following histological findings should make early chronic rejection a serious consideration: [1] dysplastic or atrophic bile ducts, [2] pericentral ballooning degeneration of hepatocytes, hepatocyte drop out and intimal or sinusoidal inflammation, [3] cholestasis in the absence of ductular proliferation. An early diagnosis of chronic rejection is imperative so that salvage therapy can be initiated. It is easier to diagnose advanced chronic rejection but the clinical value of this is limited (9).

In USA, pathologists predict features of poor response to immunosuppressive drugs and progression to chronic irreversible rejection. These included bile duct paucity, arteritis, perivenular ballooning and dropout, interstitial hemorrhage and moderate to severe lobular inflammation. These features are then used to define severe acute rejection (13).

Chronic rejection can be defined as immune mediated damage to the liver allograft which is characterized histologically by two main features; loss of bile ducts in more than 50% of portal tracts (chronic ductopenic rejection) and obliterative vasculopathy (chronic vascular rejection) affecting large and medium sized arteries. The vascular lesions do not generally affect small arterial branches and thus seldom detected in needle biopsy. So, the diagnosis of chronic vascular rejection is only made when the whole liver is available. Absence of ductular proliferation or periportal fibrosis distinguishes chronic rejection from other bile duct losing diseases (14).

Chronic rejection remains a diagnostic problem due to inconsistency, insidious development and uneven distribution of the histologic changes and overlapping features with ischaemic cholangitis. Morphologically, chronic rejection is characterized by a progressive loss of the intrahepatic bile ducts (Ductopenic Rejection), an obliterative foam –cell

arteriopathy (Vascular Rejection) and perivenular cell dropout and fibrosis. Most cases will present between 2 and 12 months after transplantation following episodes of acute, or more insidiously over period of months, without previously recognized acute rejection; protracted cases up to 10 years after transplantation are now well recognized and generally follow inadequate immunosuppresion (16).

Persistent perivenular parenchymal cell drop out, supposedly an ischaemic lesion, when associated with early ductopenia, is highly suspicious of evolving chronic rejection, despite of the absence of foam-cell arteriopathy, which is rarely sampled by biopsy needles. Perivenular fibrous tissue deposition is observed later and may reach the stage of extensive centro-central bridging septa with an apparent severe lobulation. Peripheral ductular reaction remains minimal or absent, but it may be conspicuous and bile-duct loss may be delayed when a major bile duct stricture is associated (15).

Early histological changes may show a transitional period when acute cellular rejection persists but the cellular infiltration of morphologically abnormal bile ducts gradually lessens as cholangio-destruction progresses. Light portal inflammation and oedema with degenerative changes affecting the small interlobular bile ducts, perivenular hepatocyte ballooning and drop-out with canalicular cholestasis and a distinctive absence of periportal ductular reaction characterized the early stage. Subsequent biopsy specimens will show a progressive disappearance of the interlobular bile ducts, 50% or more of portal tracts devoid of ducts having been arbitrarily considered minimal diagnostic criteria (17).

Six cases with cytomegalovirus infection were reported in this cohort study. Cytomegalovirus is the most commonly encountered opportunistic viral infection of the liver allograft. The incidence of the disease increases with the overall potency of non-specific immunosuppression. The infection may be the result of recrudescence in a carrier, transmission through blood products or the donor organ, or as acquired disease. It is usually characterized by mild lobular disarray and microabscesses or microgranulomas which are scattered randomly throughout the lobules. The infected cells show nuclear or cytoplasmic inclusions (1).

During this cohort study 24 patients died for several reasons mainly HCV represented the major cause in 14 (58.3%) cases , graft rejection in 4 (16.6%) cases , RI in 1(4.2%) case , HBV in 1(4.2%) case, Recurrent PBC in 1 (4.2%) case , operative complications in 1(4.2%) case and acquired infection such as tuberculosis and fungal infection in 2 (8.3%) cases. Current 1-year survival rates for liver transplantation recipients in the United States are 85% to 90%, and 5-year survival rates are 70% to 75%. Hepatic replacement has gradually shifted from a risky, mostly unsuccessful operation to a routine procedure. One of the most important risk factors for an increased risk of severe post-transplant hepatitis C is treated acute cellular rejection. Another well recognized complication of solid-organ transplant is hyperglycemia and transplant diabetes mellitus. As a result; much attention has been focused on the diabetogenic effect of immunosuppression (17).

By analogy with other solid organ transplantation, rejection may in theory be hyperacute, acute, cellular (the most common form) and chronic (uncommon and seemingly on the decline). Unique to the liver graft is that small bile ducts and vascular endothelia, unlike hepatocytes, normally bear MHC antigens, which make them main targets of the immune attack; a dual blood supply with the portal vein endothelium being first met by allo-reactive T cells (endotheliitis) and a most efficient scavenger system - the kupffer cells –ready to moped up immune complex being formed (15).

In conclusion, the results of graft liver biopsies in the present study revealed that recurrent HCV is the prominent cause of organ dysfunction. Meanwhile organ rejection was less frequently encountered. The complications of liver transplantation can be controlled and managed if diagnosed promptly and treated early and that can be achieved through direct communication between hepatologist and pathologist to avoid insufficient clinical informations and to obtain a correct diagnosis. Moreover; familiarity with the uncommon histological findings, a careful search for subtle morphologic changes and the use of standard terminology could improve the quality of liver transplant biopsy interpretation.

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Assessment of Liver Fibrosis in HCV Infection in Egyptian Patients

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Abstract: One trial to replace liver biopsy with a simple blood test(s) (whose levels can reflect the severity of liver disease) is the aim of this work. The present investigation was carried out on 72 cases (62 fibrotics and 10 hepatocellular carcinomas; HCCs) who referred to the Early Cancer Detection Unit belonging to the Faculty of Medicine, Zagazig University, Egypt for liver biopsy assessment. Their sera were tested for liver enzymes (alanine aminotrasferase [ALT], aspartate aminotransferase [AST] and AST/ALT ratio), HCV viraemia and type, matrix metalloproteinase-9 (MMP-9) and alpha fetoprotein (AFP). The relationships between the values of these serum tests and the stages of liver fibrosis or the presence of HCC were studied in this work. The results indicated that, the serum ALT level at 60 U/L was indicative of significant fibrosis in 81%. Serum AST level at 130 U/L was indicative of significant fibrosis in 88%. However, the transaminases levels can't differentiate, at any level between cancerous and non-cancerous lesions. The transaminases ratio (AST/ALT) at a cut off value 1.0 reflected significant fibrosis in 93% of patients but can't differentiate between cancerous and non-cancerous lesions. Similarly, the serum level of MMP-9 was diagnostic at a level of 160 mg/dl or less for severe fibrosis in 87% of patients but not for HCC. On the other hand, the level of AFP at 1000 ng/ml or more was diagnostic for cancerous lesions in 90% of patients but cannot differentiate at any level between mild and significant fibrosis. Unfortunately, the HCV level of viraemia and type did not affect the severity of liver disease. The age of patient at the biopsy was found to correlate positively with liver disease. The significant fibrosis was found in 81% of patients aged 45 years or more. While, at a cut off value of 55 years, age at the biopsy was diagnostic for HCCs in 88% of patients with a specificity of 71%. On the other hand, the sex of the patient had no effect on severity of liver disease. In conclusion, there is no single blood test whose value can predict the severity of liver diseases in HCV infection with 100% accuracy, but the use of the above significant serum parameters together with age of the patient can help to exclude the need for liver biopsy in many patients, at least those with contraindications for liver biopsy or those refuting this investigation.
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Key words: HCV infection, Egyptian patients, Liver fibrosis, Histopathology, Serum markers.

1. Introduction:

Hepatitis C virus has been encountered worldwide with WHO estimates of 170 million infected patients **(Booth *et al.*, 2001)**. In the United States and Europe, HCV infection has been detected in 1% to 2% of the general population and fewer than 1% of volunteer blood donors **(Alter, 1997)**. In most developed countries, HCV infection is associated with percutaneous blood exposures, such as blood transfusion and injection drug use **(Yoshiba *et al.*, 1996)**. In contrast, a high prevalence of anti-HCV has been found among apparently healthy Egyptian population, such as military recruits (22%-33%) **(Farghaly & Barakat, 1993)**, expatriate workers in the Gulf Region (31%) **(Mohamed *et al.*, 1996)**, and blood donors (28%) **(Arthur *et al.*, 1997)**. Blood transfusion and illicit drug use, as risk factors are rare in Egypt. However, Schistosomiasis infection and the parenteral therapy of this infection have been proposed as risk factors for both hepatitis B virus (HBV) and HCV infection in Egypt **(Frank *et al.*, 2000 and El-Sadawy *et al.*, 2004)**. It is believed that, up to 85% of infected individuals will develop chronic HCV infection. One of the main problems with chronic HCV is that, it generally leads to hepatic fibrosis, cirrhosis and ultimately hepatocellular carcinoma **(El-Serag, 2002)**.

In patients infected with HCV 20% to 30% will progress to cirrhosis in over two to three decades and the development of histologic cirrhosis is silent in most patients **(Wiley *et al.*, 1998)**. The risk of developing cirrhosis appears to be related to the degree of inflammation and fibrosis present on liver biopsy at any given time, and varies from less than 2% risk per year in those with mild disease to over 10% in patients with severe inflammation **(Yano *et al.*, 1996)**. Hepatocellular carcinoma is a significant complication of HCV infection, although it rarely occurs in the absence of cirrhosis **(El-Serag, 2002)**. The degrees of hepatic fibrosis and cirrhosis are undoubtedly associated with patient prognosis and survival. Hepatic fibrosis may occur at varying rates.

However, male sex, higher alcohol use, older age at the time of infection, duration of infection and higher necroinflammatory score at the initial liver biopsy are probable predictors of increasing fibrosis **(Cacciola *et al.*, 1999)**. It is, therefore, important to evaluate the degree of hepatic histopathological changes when diagnosing and treating HCV-infected patients. The evaluation of these changes is difficult without liver biopsy **(Scheuer *et al.*, 1992)**. Nevertheless, the technique of taking liver biopsy have many contraindications and limitations **(Booth *et al.*, 2001).**

The present study is a trial to identify simple tests that might reflect the severity of hepatic fibrosis and the occurrence of liver cancer.

2. Patients and Methods

In the present study, all available cases (82) with liver disease referred to the Early Cancer Detection Unit belonging to the Faculty of Medicine, Zagazig University, Egypt, between April 2001 to April 2004 for liver biopsy assessment were included. Preserved serum samples of these patients were used for the following manifestations:

Anti-HCV antibody testing was done for all serum samples applying the micro particle enzyme immunoassay (MEIA) using the I_{mx} automated system (Abbott Diagnostics, USA) and following the manufacturer's instructions. Negative cases for anti-HCV and positive cases for HbsAg were excluded from this study. Positive samples for anti-HCV and negative for HBsAg were subjected to reverse transcription polymerase chain reaction (RT-PCR) testing to differentiate samples containing HCV-RNA from those with eradicated HCV viraemia. Negative RT-PCR samples were excluded.

To all HCV-infected patients, as confirmed by RT-PCR, the following tests were done:

I- Paraffin bocks of the RT-PCR positive cases were cut and mounted for histopathological, and histochemical assessment:

1- For histopathological studies, the paraffin sections were routinely stained with haematoxylin and eosin according to **Culling (1974)** and examined microscopically by an experienced pathologist blinded to the biochemical results to evaluate the fibrosis stage of each sample using the METAVIR system **(Metavir, 1994)**.

2- The distribution of collagen and reticulin was demonstrated by staining the paraffin sections with Masson's trichrome stain **(Luna, 1972)** and with Gordon and Sweets reticulin stain **(Gordon and Sweets, 1936)**.

II- The serum samples were used for the following assays:

1- Quantitation of the level of viraemia using the Rel Time Detection polymerase chain reaction (RTD-PCR):

A single-tube RT-PCR was optimized for the quantitation of the 5` NCR (non-coding region) of HCV by using the Taqman technology (Roche Molecular Diagnostics), which exploits the 5`-3` nucleolytic activity of AmpliTaq DNA polymerase first described by **Holland et al** (**1991**). The use of a sequence detector (ABI Prism 5700; Applied Biosystems, Foster City, California) allowed measurement of the amplified product in direct proportion to the increase in fluorescence emission continuously during the PCR amplification. The amplification plot was examined early in the reaction at a point that represents the logarithmic phase of product accumulation. The point representing the detection threshold of the increase in the fluorescent signal associated with the exponential growth of the PCR product for the sequence detector is defined as the cycle threshold (C_T). C_T values are predictive of the quantity of the input target **(Heid *et al.*, 1996)**; that is, when the conditions of the PCR are the same, the larger the starting concentration of a template, the lower the C_T.

The standard curve was created automatically by the ABI Prism 5700 detection system (Foster City, CA, USA) by plotting the C_T against each standard dilution of known concentration.

2-HCV serotyping:

The viral serotypes were determined by using the commercial HCV serotyping 1- **6 assay** (Murex diagnostics corporation, UK), and the tests were performed according to the manufacture's instructions.

3- **Liver function tests**, included alanin aminotransferase (ALT), aspartate aminotransferase (AST) and AST/ALT ratio. These biochemical studies were carried out using an automated system (Dimension, DuPont Medical Products Wilmington, Delaware, USA) according to the manufacturer's instructions.

4- **Matrix metaloproteinase-9 (MMP9):** this assay employs the quantitative sandwich immunoassay technique using the ELISA quantikin Kit (R & D, UK) according to the manufacturer's instructions.

5- **Alpha fetoprotein (AFP):** The LIA-mat AFP is a two-site immunoluminometric assay (sandwich principle) using two highly specific monoclonal antibodies. Antibody-coated polystyrene tubes serve as the solid phase. The tracer antibody and the coated antibody react simultaneously with the AFP present in patient samples or standards. Unbound material is removed by a washing step. The AFP values were measured in ng/ml.

Statistical analysis:

All results were expressed in means ± SD. Differences in means of the studied parameters between stages of fibrosis and/or cancerous and noncancerous lesions were tested using one-way analysis of variance (ANOVA). The influence of gender and viral genotype on stage of fibrosis and/or cancerous and noncancerous lesions was tested using a Pearson's chi-square test. The data were entered, checked and analyzed using the SPSS software program (SPSS Inc, hicago,IL). *P* values less than 0.05 were considered to be significant.

The clinical usefulness of the studied serum markers of fibrosis and/or hepatocellular carcinoma (HCC) were assessed, according to **Galen & Gambino (1977)**, by the determination of: sensitivity, specificity, positive and negative predictive values and ROC (receiver operating characteristic) curve.

III- Tabulation, photographing and interpretation of the results were done.

3. Results

Only 72 cases (out of 82), who met the proposed criteria of the study, were used in this work (62 fibrotic cases and 10 cancerous ones). These cases were the subject of the following studies.

I- Histopathological results:

The distribution of fibrosis stages in the studied cases (62 fibrotics) is shown in table (1). From this table, it is clear that, all cases have at least a fibrosis stage 1 or more, none was found in stage 0.

Stage 1 fibrosis (Plate I, A) comprised 7 (11.3%) cases. The Masson's trichrome and reticulin stained sections from this stage showed minimal amounts of collagen and reticulin fibers distributed within the portal tract and pericellular in the hepatic lobules (Plate I, B and C).

Most cases 28 (45.2%) were found in stage 2 fibrosis. At this stage, in addition to portal tract enlargement, there was septa formation between portal areas (Plate II, A). Examination of the trichrome and reticulin stained sections revealed a relative increase in collagen deposition within the portal tracts and pericellular in the hepatic lobules (Plate II, B), with a slight decrease in reticulin staining (Plate II, C).

Table (1): The distribution and frequencies of histopathological changes seen in hepatic tissue sections of hepatitis C virus infected patients.

Pathological changes observed in liver biopsy	No. of cases	%
1- Fibrosis Stages (METAVIR):	**62/72**	**86.1%**
a- Portal tract expansion (stage 1)	7/62	11.3%
b-Portal tract enlargement with rare septa formation (stage 2).	28/62	45.2%
c-Numerous septa formation (stage 3)	16/62	25.8%
d- Cirrhosis (stage 4)	11/62	17.7%
2- Hepatocellular Carcinoma	**10/72**	**13.9%**

Sixteen (25.8%) cases had stage 3 fibrosis. At this stage, there was fibrosotic bridging between portal areas (Plate III, A) and between portal areas and central veins. Masson's trichrome and reticulin stained sections of this stage revealed increased collagen and reduced reticulin fibers within portal tracts and pericellular in hepatic lobules (Plate III, B and C).

In cirrhotics (Stage 4 fibrosis), all pathological changes of stage 3 were present besides pseudolobule formation (Plate IV, A). Eleven cases (17.7%) were present in this stage. Masson's trichrome stained sections revealed thick collagen fibrous bands (Plate IV, B) connecting the portal tracts together and with central veins. These connections were confirmed by reticulin stain, which revealed bridging necrosis and progressive fibrosis (Plate IV, C).

Hepatocellular carcinoma was observed in only 10 cases out of the 72 studied ones (Plate V, A). This figure revealed that, this hepatocellular carcinoma is composed of liver cords which are much wider than the normal liver plate which is two cells thick. There was no discernable normal lobular architecture, though vascular structures were present. Also, the collagenous fibers were increased (Plate V, B) and reticulin fibers were obviously decreased (Plate V, C).

II- Non-Histological Study:

The non-histological study included the historical and the immunochemical features of the studied cases. These features are listed in table 2.

Historical features:

1- Age at biopsy:

The mean age of the studied population was 46.4 ± 10.7 years. It was 43.9 (± 9.2) (range 26-64, median 45 ± 1.17) in fibrotic cases. As a preliminary test, the ages of this population were classified into two groups; group 1 (< 40 years old) and group 2 (>= 40 years old) and the correspondant mean fibrosis stage was calculated. The mean fibrosis stage in the first group was 1.7 while that in the second group was 3.3 (*P= 0.001*) (table, 2). So, one can expect a positive correlation between age at liver biopsy and stage of fibrosis. Also, the distribution of the studied cases was higher in the second group than in the first one

(41 versus 21), indicating an increase in HCV infection in the latter group.

Plate (I)

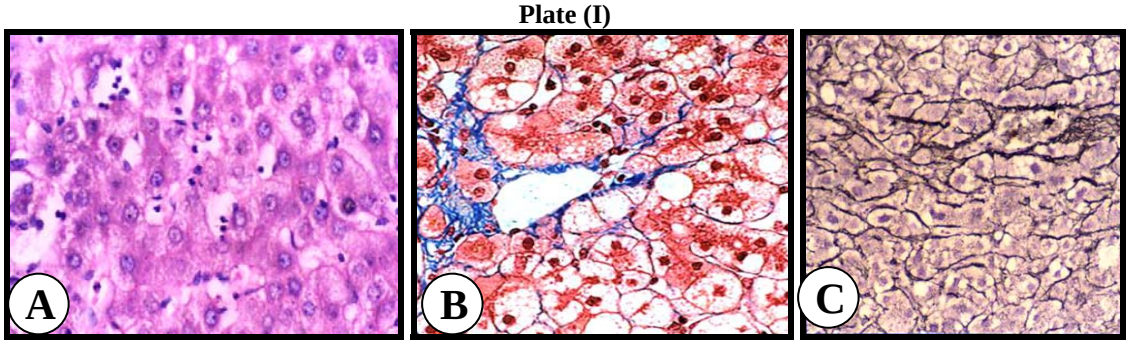

Plate (I): Liver sections from a female patient aged 28 years and infected with HCV showing mild fibrosis: A- Haematoxylin and Eosin stain (x 250), B- Masson's Trichrome stain (x 450) and C- Reticulin stain (x 250).

Plate (II)

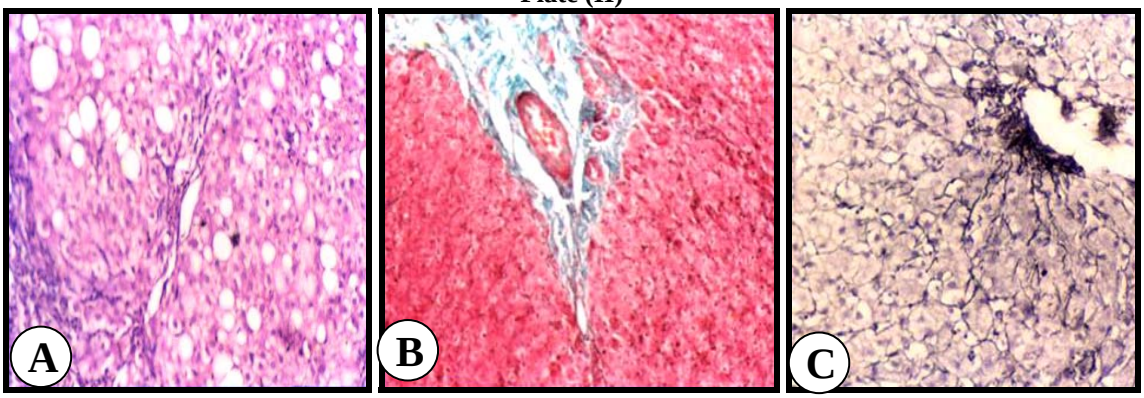

Plate (II): Liver sections from HCV-infected male patient aged 35 years showing short fibrous septa formation (METAVIR stage 2): A- Haematoxylin and Eosin stain (x 250), B- Masson's Trichrome stain (x 250) and C- Reticulin stain (x 250).

Plate (III)

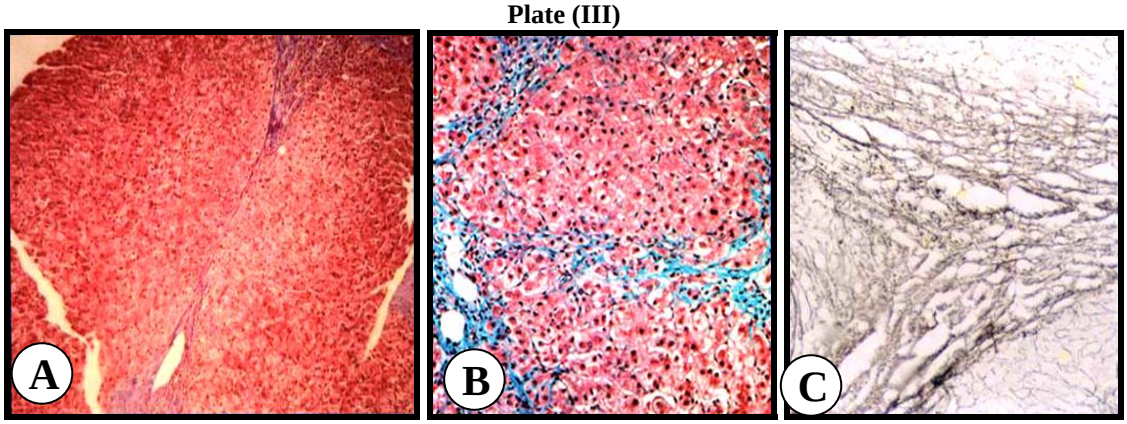

Plate (III): Liver sections showing bridging fibrosis (METAVIR stage 3) from HCV-infected male patient aged 46 years: A- Haematoxylin and Eosin stain (x 250), B- Masson's Trichrome stain (x 250) and C- Reticulin stain (x 250).

Plate (IV)

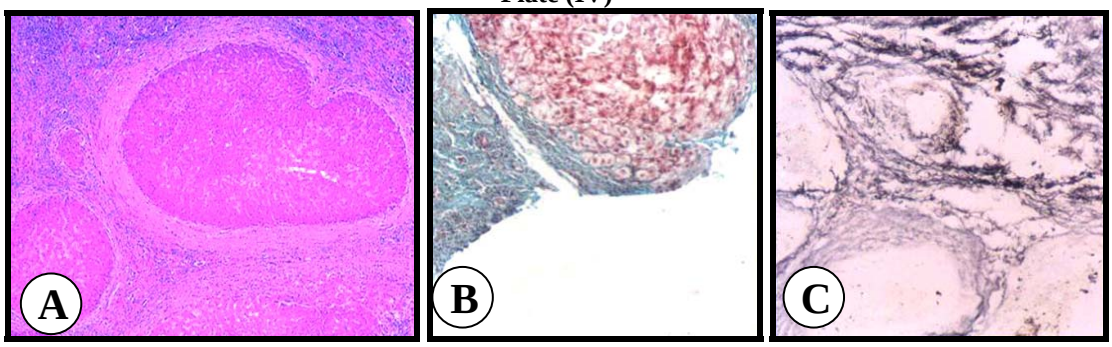

Plate (IV): Liver sections from a male patient aged 52 years and infected with HCV revealing cirrhotic changes (METAVIR stage 4): A- Haematoxylin and Eosin stain (x 150), B- Masson's Trichrome stain (x 150) and C- Reticulin stain (x 150).

Plate (V)

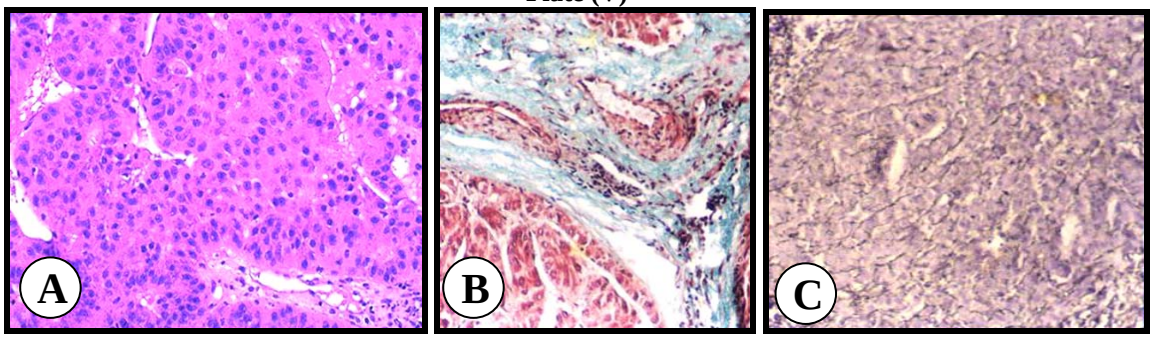

Plate (V): Liver sections from a 63 years old male patient infected with HCV and showing hepatocellular carcinoma: A- Haematoxylin and Eosin stain (x 250), B- Masson's Trichrome stain (x 250) and C- Reticulin stain (x 250).

Table (2): Historical and immunochemical characteristics of fibrotic cases in HCV infected patients.

Character	Number	%	Mean Fibrosis stage (METAVIR)	*P* value
Age (years):				
< 40	21	33.9	1.7	*P* = 0.001
≥ 40	41	66.1	3.3	
Gender:				
Male	52	83.9	2.5	*P* = 0.84
Female	10	16.1	2.3	
ALT (U/L):				
< 40	11	17.7	1.7	*P* = 0.049
≥ 40	51	82.3	2.7	
AST (U/L):				
< 40	6	9.7	1.4	*P* = 0.053
≥ 40	56	90.3	2.7	
AST/ALT:				
< 1.0	35	56.5	1.8	P = 0.001
≥ 1.0	27	43.5	3.3	
Viraemia (copy/ml)				
< 8800	31	50	2.4	*P* = 0.967
≥ 8800	31	50	2.5	
Type:				
1	5	8.1	3.4	*P* = 0.026
4	53	85.5	2.5	
6	4	6.4	2.0	
MMP-9 (mg/dl):				
≤ 160	27	43.6	3.3	*P* < 0.009
> 160	35	56.4	1.8	
AFP (ng/ml):				
< 400	49	79	2.3	*P* = 0.155
≥ 400	13	21	3.4	

Figure (1) is a diagrammatic representaion of fibrosis stages and their correspondant mean ages at biopsy and shows a parallel relationship between age at biopsy and stage of fibrosis. Using a cutoff value of 45 years, age at biopsy was found to have 81% sensitivity, 63 % specificity, 83% positive predictive value and 58% negative predictive value when differentiating between severe and non-severe fibrosis (Table 3). Age at liver biopsy was found to have a fair discriminating power in differentiating severe and non-severe fibrosis (area under the ROC curve= 0.721) (Chart, 1).

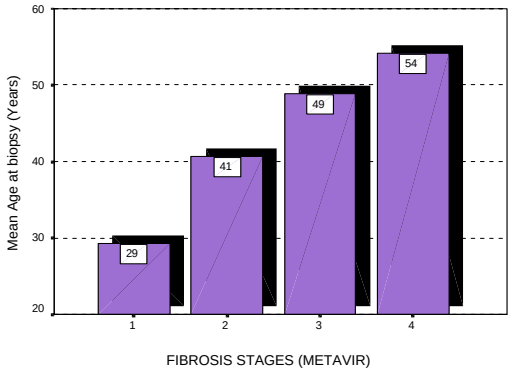

Figure (1): Diagrammatic representation showing the relationship between age at biopsy and stages of liver fibrosis in HCV infected patients.

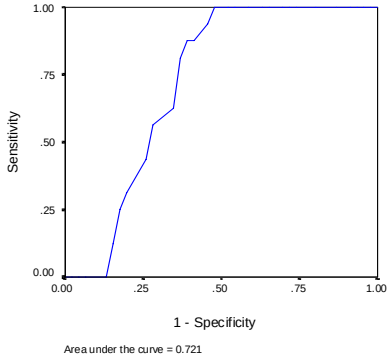

Chart (1): The ROC curve of age at biopsy when used to diagnose significant fibrosis showing a fair discriminating power (Area under the curve = 721).

In HCC cases, the mean age at biopsy was 62 ± 4.2 (median 63 ± 3.1, range 56- 67). When using a cutoff value of 55 years for age to diagnose hepatocellular carcinoma, age at biopsy was found to have 88% sensitivity, 71% specificity, 77% positive predictive value and 68% negative predictive value (Table, 4). The ROC curve of age at biopsy in HCC cases revealed a good discreminating power (area under the curve = 0.78) for differentiating cancerous from non-cancerous lesions (Chart, 2).

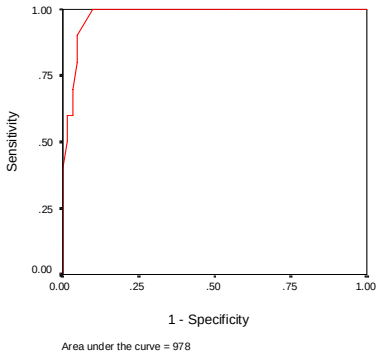

Chart (2): The ROC curve of age at biopsy when used to diagnose hepatocellular carcinoma showing an improvement in its discriminating power (area under the curve = 0.78).

2- Gender:

The population of this study has a high frequency of male sex over females (52 males versus 10 females in fibrotic cases and 9 versus 1 in HCCs) (Table 2). In other words, males have a higher prevalence of HCV infection than females. The mean fibrosis stage of the two sex groups did not differ significantly (2.5 in males versus 2.3 in females, *P*= 0.84, Fig., 2). Therefore, the sex of the patient has no effect on fibrosis progression.

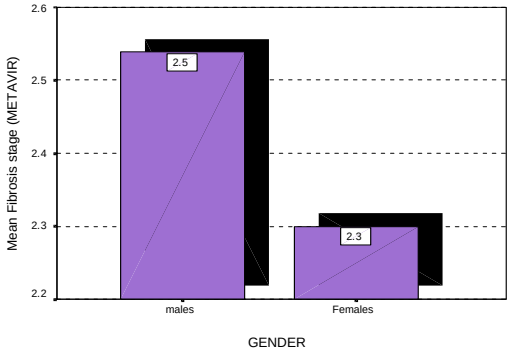

Figure (2): Diagrammatic representation showing no significant difference between males and females in the mean fibrosis stage in HCV-infected patients.

Immunochemical features:

Serum alanine aminotransferase (ALT):

The mean values of serum ALT levels in the studied cases was 82.2 ± 53.3 U/L. In fibrotics it was 70.3 ± 32.4 U/L (range 15-144, median 66 ± 4.1). When the latter cases were divided into two groups according to the serum level of ALT; group (1) with normal levels and group (2) with elevated levels

(Tabe, 2) and the mean fibrosis stage of each group was calculated, a statistical difference in mean fibrosis stages (1.7 versus 2.7) was found between both groups ($P < 0.049$). Figure (3) shows the relationship between mean values of serum ALT levels and the stages of liver fibrosis. In severe fibrotics the mean ALT value was 81.3 ± 14.3, while in mild fibrosis it was 27.4 ± 10.0 U/L. Using a cutoff value of 60 U/L, serum ALT was found to differentiate between severe and non-severe fibrosis by 81% sensitivity, 57% specificity and 80% positive predective value (Table, 3). When it was used for diagnosing severe fibrosis, the level of ALT was found to have a fair discreminating power as the area under the ROC curve = 0.695 (Chart, 3).

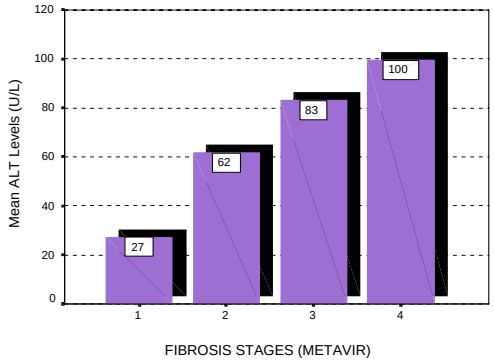

Figure (3): Diagrammatic representation showing an increament in the mean ALT serum levels with progression of liver fibrosis in HCV infection.

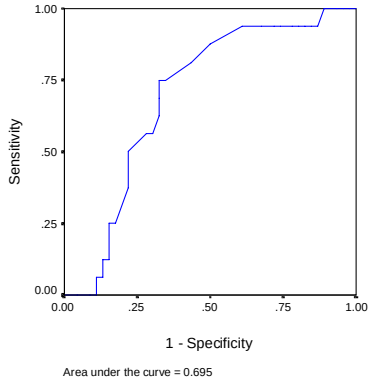

Chart (3): Receiver operating characteristic curve of serum ALT levels for diagnosing significant fibrosis showing a fair diagnostic power (area under the curve = 0.695).

In HCC cases, the mean serum ALT level was 115.7 ± 91.5 U/L (median 112 ± 28.9, range 66 -336), while it was 70.3 ± 32.4 U/L (range 15-144, median 66 ± 4.1) in non-cancerous cases. Nontheless, the serum ALT level was shown not to have any diagnostic ability when differentiating cancerous from non-cancerous lesions (area under the ROC curve = 0.310, Chart, 4).

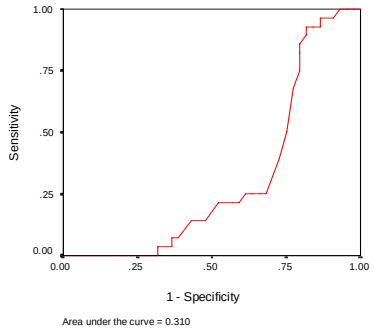

Chart (4): Receiver operating characteristic curve of serum ALT level when used to diagnose hepatocellular carcinoma in HCV-infected patients revealing a bad discriminating ability (area under the curve = 0.310).

Aspartate aminotransferase (AST):

The mean AST level in the studied cases was 149.2 ± 107.7 U/L. It was 125.6 ±74.5 U/L (range 20-322, median 101.5 ± 9.5) in fibrotic cases. When the latter cases were divided into two groups (AST normal group and AST elevated group), the mean fibrosis score of the second group was found to be significantly higher than that of the normal group (2.7 versus 1.4, *P= 0.053*, Tablee, 2). The activities of AST and their relationships with the histologic characters are shown in Fig. (4).

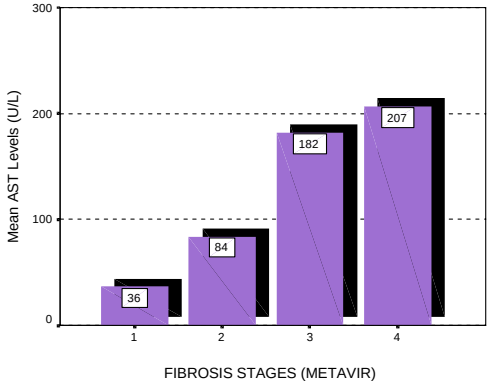

Fig. (4): The relationship between serum AST levels and stages of liver fibrosis in HCV-infection was shown to be parallel.

A cutoff value of 130 U/L for AST concentration was found to have 88% sensitivity, 72% specificity, 84% positive predective value and 71% negative predective value when it was used to differentiate between severe and non-severe fibrosis. The area under the ROC curve when diagnosing severe fibrosis was 0.815 indicating a good discreminating power (Chart, 5).

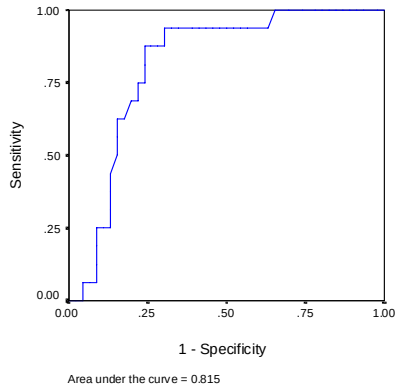

Chart (5):ROC Curve of AST when it was used to diagnose significant fibrosis revealing a good discriminating power (AUC = 0.815).

On the other hand, the mean value of serum AST level in the 10 cancerous cases was 295.7 ± 161.9 U/L (median 232 ± 51.2, range 142 - 621), while in non-cancerous cases it was 125.6 ±74.5 U/L (range 20-322, median 101.5 ± 9.5), indicating a positive relationship. However, the serum level of AST appeared not to have any diagnostic power when diagnosing hepatocellular carcinoma, because the area under the ROC curve was 0.194 (Chart, 6).

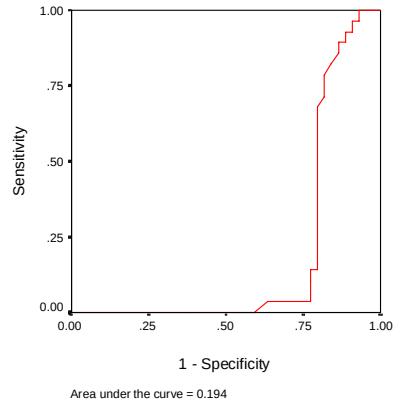

Chart (6):ROC curve of AST level to differentiate between cancerous and non-cancerous lesions in HCV infection revealing a bad diagnostic power (AUC = 0.194).

AST/ALT ratio:

The mean AST/ALT ratio in the studied cases was 1.08 ± 0.35 and it was 1.06 ± 0.37 (range 0.33-2.2, median 0.89 ± 0.05) in fibrotic cases. The fibrotic cases were divided into two groups according to the value of AST/ALT ratio (group 1 < 1, group 2 ≥ 1). The mean fibrosis scores of the two groups were significantly differerent (1.8 versus 3.3, p<0.01).

The mean AST/ALT ratio in severe fibrotics (n: 55) was higher than that in non-severe fibrotics (n: 7) (1.2 ± 0.06 versus 0.7 ± 0.09; P< 0.001). A ratio ≥ 1 had 77% specificity and 89 % positive predictive value in distinguishing severe from non-severe fibrotics, with a 93% sensitivity and 80.7% negative predictive value. The ratio correlated positively with the stage of fibrosis (Fig., 5).

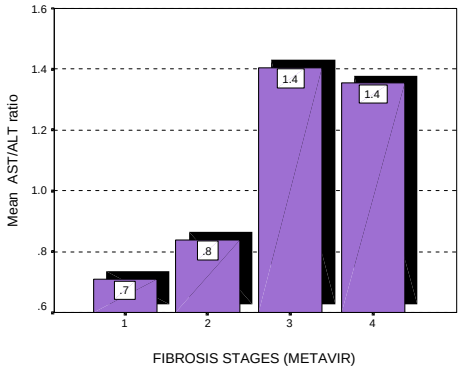

Figure (5): Diagrammatic representation of mean transaminases ratio in the different hepatic fibrosis stages due to HCV infection.

The ROC curve of the transaminases ratio showed a good diagnostic power (AUC = 0.872) when it was used to differentiate between fibrosis stage 1 and higher stages (Chart, 7).

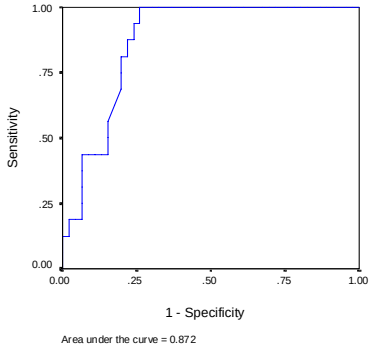

Chart (7): ROC curve of transaminases ratio revealing a good diagnostic power (area under the curve = 0.872) when differentiating severe from non-severe fibrosis.

In HCC cases, the mean AST/ALT ratio was 1.17 ± 0.102 (range 1.02 – 1.32, median 1.2 ± 0.03). While in noncancerous cases it was 1.06 ± 0.37 (range 0.33 – 2.17). Therefore, there was no statistical difference (P = 0.78) between cancerous and non-cancerous cases in terms of AST/ALT ratio. The ROC curve of AST/ALT (Chart 8) revealed a bad diagnostic power for differentiating between cancerous and non-cancerous lesions (AUC: 0.293).

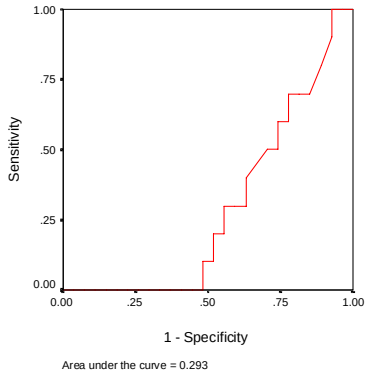

Chart (8): ROC curve of AST/ALT ratio showing a bad discriminating power (area under the curve = 0.293) when diagnosing HCC.

Serum Matrix Metaloproteinase 9 (MMP9):

The mean level of serum MMP9 in the studied population was 217.1 ± 121.7 mg/dl (range 77-654, median 193.5 ± 15.5). When the studied cases were divided into two groups (group 1 with MMP-9 ≥ 169 and group 2 with reduced levels) and the correspondent mean values of fibrosis stages were calculated. The mean fibrosis stage of the first group (≥ 169) was found to be 1.88, while that of the group with reduced levels was 3.25 (*P< 0.009*). The mean serum levels of MMP9 measured in the different histological changes are shown in figure (6). From this figure the mean values of serum MMP9 concentrations decrease, with the severity of the liver fibrosis. In other words, there is a negative correlation between the stage of liver fibrosis and the level of serum MMP9 (*P< 0.0001*).

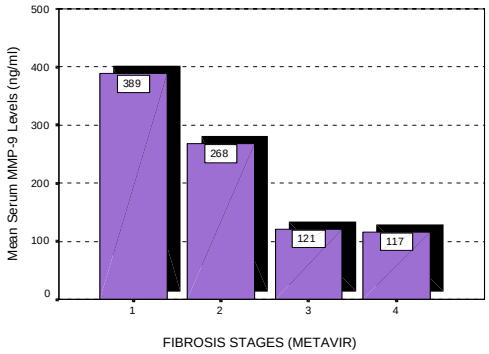

Fig. (6): An inverse relationship between stages of liver fibrosis and the level of serum MMP-9 in HCV infected patients.

Thirty five patients (56.4%) had normal levels of serum MMP9 while 27 patients (43.6%) had reduced levels (Table 2). Moreover, the number of patients having reduced levels increases with fibrosis stage (1 versus 6 in stage 1; 2 versus 26 in stage 2; 14 versus 2 in stage 3 and 11 versus 0 in stage 4).

The test has, at a cutoff value of 160 mg/dl, a 87 % sensitivity and 72% specificity in differentiating severe fibrotics.

The discreminating power of this serum test in diagnosing severe from non-severe fibrosis was good (area under the ROC curve = 0.846, Chart, 9).

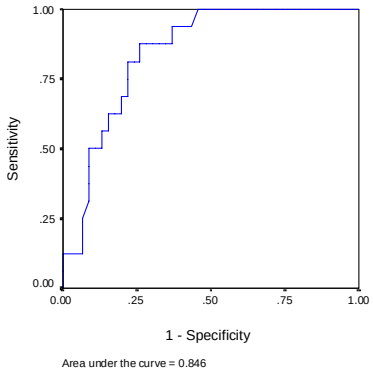

Chart (9):A good discriminating power of serum MMP-9 level when diagnosing severe fibrosis showed by the large area (0.846) under the ROC curve.

The mean level of MMP9 in cancerous cases was 91.2 ± 46.5 mg/dl while in non-cancerous cases it was 119.4 ± 32.7 mg/dl indicating no statistically significant difference (*P* = 0.82) between both groups. Hence, the serum MMP-9 is useful in diagnosing severe fibrosis but not HCC. Moreovere, the area under the ROC curve is 0.196 indicating a bad discriminating power (Chart, 10).

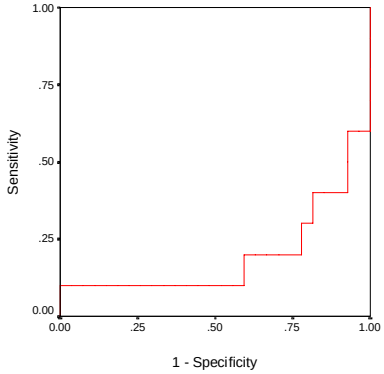

Chart (10): A bad diagnostic power (AUC = 0.196) of serum MMP-9 level when differentiating cancerous from non-cancerous lesions seen in liver sections of HCV infected patients.

HCV-Viraemia:

Qualitatively, HCV infection has been proved by using RT-PCR technique. According to this technique, 72 cases out of 78 were positive (92.3%), indicating that, HCV infection is a major etiological factor for liver disease in this population.

The level of viraemia in the sera of the 72 RT-PCR positive cases was measured by the real-time PCR using ABI prism 5700. Figure (7) shows the amplification curve of some samples. The amount of circulating HCV-RNA in the sera of the studied cases ranged from 140 to 92000000 copies/ml (mean 1836235 ± 10893436.2, median 8750 ± 1283804). In fibrotic cases, the mean level of viraemia is 231657 ± 1172639.4 (median 8800 ± 1489412, range 140 – 92000000).

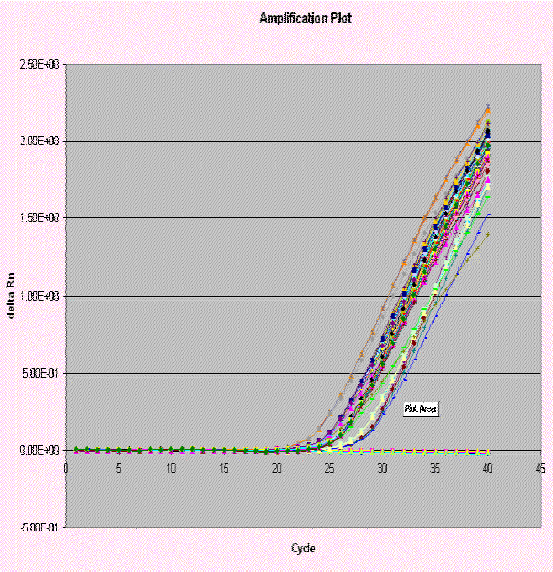

Fig. (7): The amplification curves of some samples using the real time-PCR technique for HCV-RNA quantification.

When the studied cases were divided into two groups according to the level of viraemia (< 8800 and ≥ 8800), the mean fibrosis stage of the first group did not differ significantly from that of the second group (2.25 versus 2.28, *P= 0.967*). Moreover, figure (8) represents the mean values of HCV viraemia calculated in the four fibrosis stages and shows no correlation between stage of fibrosis and level of HCV viraemia. In addition, the area under the ROC curve revealed a bad diagnostic power (Chart, 11, area under the curve = 0.382). Therefore, these data demonstrate that, the amount of HCV-RNA did not correlate with liver histology.

In HCC cases the mean level of viraemia was 191560 ± 231633 while in non-cancerous cases the mean level of viraemia was 231657 ± 1172639.4 (median 8800 ± 1489412, range 140 – 92000000), indicating no significant difference (*P* = 0.334) although the mean level of viraemia is reduced in HCC cases. Also, the ROC curve revealed a bad diagnostic power (Chart 12).

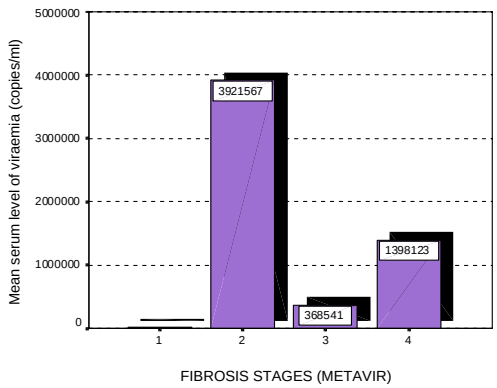

Fig. (8): A fluctuating level of HCV viraemia in the different fibrosis stages indicating no relationship.

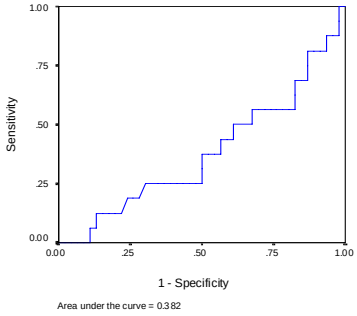

Chart (11): The receiver operating charactaristic curve of serum level of viraemia in fibrotic cases showing a bad discriminating power when differentiating between severe and non-severe fibrosis (AUC = 0.382).

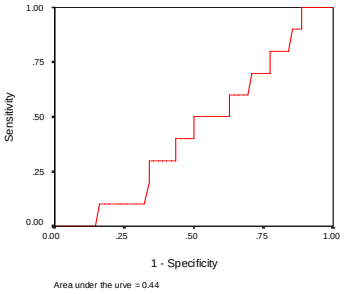

Chart (12):A bad diagnostic power of serum level of HCV viraemia in discriminating between severe and non-severe fibrotics and between cancerous and noncancerous lesions in HCV infected patients.

HCV serotype:

The frequency of the presence of HCV serotypes in the present population was as follows: In fibrotics, the HCV serotypes were represented by only types 1 (5 cases), 4 (53 cases) and 6 (4 cases).

The mean fibrosis stage in patients with HCV type 1 was 2.0, type 4 was 1.9 and type 6 was 1.8; Fig. 9). Thus, the fibrosis stage did not differ significantly by the type of HCV. Therefore, the type of HCV appears to have no effect on fibrosis staging. In HCC cases only types 4 (8/10) and type 1(2/10) were present, while type 6 was absent from HCC cases.

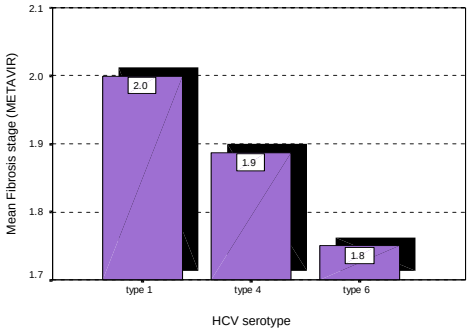

Fig. (9): A diagrammatic representation of mean fibrosis stages in the HCV –infected patients with the different serotypes showing no significant difference**.**

Serum AFP:

The mean AFP level in the studied population (72 cases) was 226.7 ± 416.5 ng/ml. In fibrotic cases, the mean serum AFP level was 83.5 ± 186.9 ng/ml (range 3-888, median 10.5 ± 23.7). When the levels of serum AFP were divided into two groups; normal and elevated, and the mean fibrosis scores of the two groups were calculated, a slight statistical difference was found in the fibrosis score between the two groups ($P< 0.05$). The distribution of the level of AFP in the different fibrosis stages was shown in figure (10). The mean AFP levels in mild fibrosis was 7.7 ± 2.9, while that in significant fibrosis was 93.2 ± 196.5 ng/ml. Therefore, there is a significant difference in the mean level of serum AFP between mild and significant fibrosis ($P = 0.023$). However, when the diagnostic value of serum AFP level in differentiationg mild from significant fibrosis was studied, serum AFP was found to have a bad discreminating power (area under the ROC curve= 0.349, Chart, 13).

In HCC cases, the mean serum level of AFP was 1004.5 ± 207.1 ng/ml (median 1081.5 ± 65.5, range 888 - 1750), while that in fibrotics was 83.5 ± 186.9 ng/ml (range 3-888, median 10.5 ± 23.7), indicating a positive relationship. At a cut off value 1000 ng/ml serum AFP level was found to have 90% sensitivity, 77% specificity, 93% positive predictive value and 71% negative predictive value when differentiating cancerous from non-cancerous hepatic lesions. Moreover, the area under the ROC curve (Chart 14) showed an excellent diagnostic power of serum AFP level for HCC (AUC = 949).

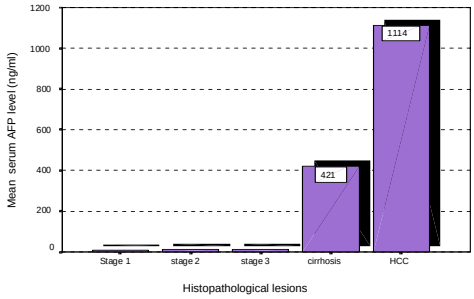

Fig. (10): Mean levels of serum AFP in HCV infected patients showing no significant correlation with the severity of liver fibrosis in spite of the increase in AFP concentration in cirrhotics. On the other hand there was a significant difference between cancerous and non-cancerous lesions in the AFP level.

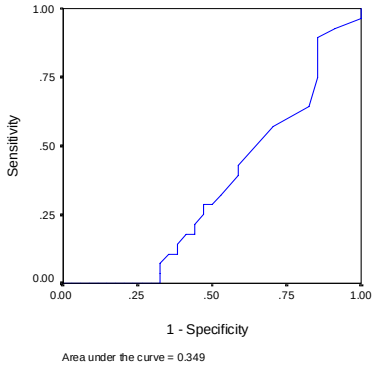

Chart (13): The ROC curve of serum AFP level when it was used to diagnose significant fibrosis showing a bad diagnostic power (AUC = 0.349).

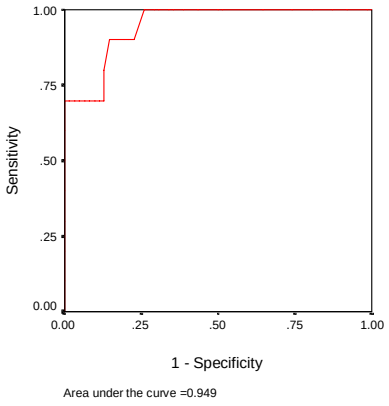

Chart (14): An excellent diagnostic power of the ROC curve of serum AFP level in diagnosing HCC in HCV infection (AUC = 0.949).

Table (3): Operating characteristics of indices of clinically significant fibrosis.

Indices	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Area under ROC curve
Age at biopsy	45	81	63	83	58	0.721
ALT	60	81	57	80	56	0.695
AST	130	88	72	84	71	0.815
AST/ALT	1.0	93	77	89	80.7	0.872
MMP-9	160	87	72	85.7	81.2	0.846

Table (4): Operating characteristics of indices of hepatocellular carcinoma.

indices	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Area under ROC curve
Age at biopsy	55	88	71	77	68	0.978
AFP	1000	90	57	93	71	0.947

4. Discussion

In the present investigation, the histopathological results indicated that, most patients were in stage 2 (28, 45.2%), while stage 1 was represented by few cases (7, 11.3%). In other words, most of the included patients (55, 88.7%) had significant fibrosis (i.e., METAVIR stages more than 1). These results indicated, therefore that, HCV infection has a rapid course of disease progression in the studied population. Similar results were reported in a similar Egyptian population by **Mangoud *et al.* (2004a)**. These authors attributed the progressive nature of the disease to the concomitant infection with other viruses like HBV. However, in this study patients with HBV concomitant infection were excluded. Hence, METAVIR stage 1 fibrosis was absent from the study of **Mangoud *et al.* (2004b)** (and METAVIR stage 2 replaced stage 1 in this study instead) due to the rapid course of disease progression caused by other concomitant infections.

In the present study, 10 cases (out of 82, 13.9%) were diagnosed as hepatocellular carcinoma. Several studies have shown that, HCV is positively related to HCC **(Kalamani *et al.*, 1991 and Simonetti *et al.*, 1992)**. Therefore, HCV infected individuals are regarded as a high-risk group for HCC. Several lines of evidence indicate a strong causal association between HCV and HCC. HCV-RNA can be found in the serum, liver, and tumor tissues of patients with HCC, but unlike HBV it does not integrate into host genome. Moreover, the age-standardized death rates owing to HCC are significantly correlated with the seroprevalence of HCV in the general population **(Deuffic & Poynard, 1999)**. Hepatitis C virus infection increases the risk for HCC probably by promoting fibrosis and cirrhosis; virtually all HCV-related HCC cases occur among patients with cirrhosis. With the exception of areas in the world where hepatitis B is endemic, it is uncommon to find HCC in the absence of cirrhosis **(Fattovich, 1998)**.

The choice of METAVIR fibrosis score for this analysis deserves comment, because there is no perfect fibrosis scoring system. METAVIR scores, which had been shown to be very reproducible for fibrosis evaluation (at least by the pathologists who created it; The METAVIR cooperative group**)** as well as more linear (i.e., no missing numbers) than the Knodell fibrosis score. Subsequently, more sensitive scores with greater numbers of categories have been published, but none as yet has become the standard for these types of studies. For ongoing and future studies, other scoring systems will be used and compared with previous methods, Knodell and METAVIR scores, to determine whether any of these is clearly superior. One of the most recent and more population specific score was that created by **Mangoud *et al.* (2004b)** through an Egyptian population screening study. However, the new concept stated by these authors needs further validations.

A percutaneous liver biopsy can be useful in patients with chronic hepatitis C virus (HCV) infection by providing information regarding the stage of fibrosis and grade of inflammation **(Garcia & Keeffe, 2001)**. However, patients with chronic hepatitis C are not always eager to have a liver biopsy. They frequently have anticipatory anxiety, which would be expected of a procedure that is associated with pain in 35% of patients, severe complications in 0.3% and death in 0.03% **(Piccinino *et al.*, 1986 and Cadranel *et al.*, 2000)**. Liver biopsy also adds significant direct costs (equipment, observation time, and time of a skilled clinician and pathologist) and indirect costs (time away from work and home) to the management of patients with chronic hepatitis C. Finally, a cost-effectiveness analysis suggested that, the best strategy in the management of chronic HCV infection is to offer therapy to all patients and not perform liver biopsies **(Wong *et al.*, 1998)**.

The use of biopsy to confirm HCC remains controversial for the following reasons; it can be difficult to distinguish large cirrhotic nodules from well-differentiated HCC or low-grade dysplastic nodules from HCC in either needle or wedge biopsies; liver biopsy carries a small risk of tumor spread along

the needle track; finally, fine-needle aspirates provide cells without some of the architectural abnormalities that are important in making a diagnosis **(El-Serag, 2002)**.

Other researchers have sought other markers that could signal liver damage without the need for biopsy. Therefore, liver biopsy would be less important where other clinical or laboratory tests available that could reliably predict the grade of inflammatory injury or stage of fibrosis. However, fibrosis is most important when considering the natural history of hepatitis C because fibrosis, not inflammation *per se*, leads to the sequelae of liver disease **(poynard *et al.*, 1997)**. Therefore, this study is a trial to find a non-histological marker(s) of fibrosis in the hepatitis C infected patients. The non-histological study included the historical and the immunochemical features of the studied cases. An alternative approach for the noninvasive prediction of fibrosis is the use of historical features, including advanced age at infection, male sex and alcohol consumption, which are all known to accelerate fibrosis progression **(Poynard *et al.*, 2001 and Myers *et al.*, 2001)**. This is a frequently used approach in the clinical setting. This approach has the advantage that, it is without cost and is relatively simple **(Alter, 1997)**, but it has yet to be validated. Furthermore, it cannot be used in patients who have received antiviral treatment because of the anti-fibrotic effects of current therapies **(Poynard *et al.*, 2000)**.

Considering the relationship between the age of the patient and the prevalence of HCV infection, anti-HCV positivity was more prevalent in older ages than in younger ones [51(70.8%) cases in patients with 40 years old or more versus 21 (29.2%) cases with younger ages]. The increase of anti-HCV prevalence with age has been reported in other studies in Egypt. **Darwish *et al.* (1992)** studying 90 volunteer Egyptian blood donors, reported that, anti-HCV among those 20 to 30 years old was 6% as compared with 37.5% among those older than 30 years, which is substantially lower than the estimates published in the study of **Abdel-Aziz *et al.* (2000)**. A study of 270 residents of another Egyptian village in the Nile Delta had a similar increase in anti-HCV prevalence after the age of 25 years from 12.7% to 36.7% **(Abdel-Wahab *et al.*, 1994)**. The rise in anti-HCV positivity with age in these studies could either be the result of continuous exposure, a cohort effect, or a combination of both. In Egypt, there is evidence for both continuous and cohort effects, reflecting both historical and continuing patterns of infection, with potentially different risk factors **(Frank *et al.*, 2000)**.

In the present study, liver fibrosis from portal tract enlargement (stage 1) to cirrhosis (stage 4) was almost linear according to age at biopsy (means; 29 in F1, 41 in F2, 49 in F3 and 54 in F4). Similar results were reported in a similar Egyptian population by **El-Shorbagy *et al.* (2004)**. In addition, **Poynard *et al.* (1997)** found that, the rate of fibrosis progression was low in individuals younger than 20 years, intermediate in those aged 21-40 years, increased in those aged 41-50 years, and highest in those older than 50 years. Although these investigators did not know why age is a risk factor, it may be that the host defense mechanisms against HCV is weaker in older people. The analysis of the impact of the historical features revealed that, older age at biopsy was an independent predictor of more advanced fibrosis. In discordance with other reports, older age at biopsy was not independently associated with significant fibrosis **(Poynard *et al.*, 2001)**. The reasons for this discrepancy remain unclear but may relate to the small size of the current study [62 versus > 2200 in the studies of **Poynard *et al.* (2001)**].

In the present study, patients with HCC were older (62 ± 4.2 years) than patients without HCC (43.9 ± 9.2 years). These results are in agreement with those reported in another study from Taiwan by **Peng *et al.* (1999)**. HCC is rarely seen during the first 4 decades of life except in populations in which HBV infection is hyperendemic. The incidence of HCC increases progressively with older age, reaching a peak between the ages of 70 and 75 years **(El-Serag, 2001)**. However, the increase in HCC cannot be explained solely by the effect of aging in the general population **(El-Serag, 2002)**. In the present study, a direct relationship exists between the HCC risk and the age at liver biopsy. In line with this observation is the study of **Tradati *et al.* (1998)**. Why patients of older ages are at higher risk of HCC is unknown. However, an increased exposure to environmental factors is responsible for cirrhosis or liver cancer or an increased vulnerability of the older liver to genotoxic agents are possible explanations.

The present study revealed that, the HCV infection was more prevalent in males than in females (61 versus 11). Similar observations were reported by other investigators (**El-Khoby *et al.*, 2000 and Frank *et al.*, 2000)**. The association between male sex and HCV positivity reflected the increased risk of exposure to the infection in males than in females. For example, males, who were frequently have schistosomiasis than females **(El-Khoby *et al.*, 2000)** and those more than 30 years of age who had risk of exposure to parenteral antischistosomal therapy (PAT) **(Frank *et al.*, 2000)**. The gender difference in anti-HCV prevalence being present in adults more than 30 years of age **(Frank *et al.*, 2000)** is further support for the present study. However, a history of therapy for schistosomiasis increased the risk of HCV infection by three times **(Frank *et al.*, 2000)**. The latter authors were unable to

explain the association between male sex and HCV prevalence. But, they suggested that, it may be due to the increased risk factors of HCV infection in males than in females like shaving at barber-shop, smoking goza in groups and PAT.

In the present investigation, although male sex was associated with HCV infection, yet it does not affect fibrosis. The difference in fibrosis stages between males and females was not significant (2.5 in males versus 2.3 in females, P= 0.84).

According to **Kew (2000),** serum aminotransferase concentrations are an indicator not of hepatocellular dysfunction but of hepatocellular damage. In the present study, the mean levels of AST were increased by the stage of liver fibrosis (36 U/L in F1, 84 U/L in F2, 182 U/L in F3 and 207 U/L in F4). A further increase in the mean level of serum AST concentration (125.6 U/L) was seen in the HCC cases. Although, ALT is considered to be more specific for liver disease than AST (because AST is found in more types of cells e.g., heart, intestine and muscle)**,** a notable increase in AST was shown in patients with viral hepatitis by **Gordon *et al.* (2000)** who found that, AST had a stronger correlation with liver histology, in particular hepatic fibrosis. More recently, **El-Shorbagy *et al.* (2004)** reported a parallel relationship between serum level of AST activity and the degree of liver disease in an Egyptian population infected with HCV.

Kaplan (1993) reported the commonly held view that, AST concentrations exceeding 300-400 U/L usually indicate acute hepatocellular disease such as hepatitis or drug toxicity, and points out that, similar values may sometimes occur with extrahepatic obstruction but are uncommon. He also declared that, the greatest elevations (> 1000 U/L) are seen in the early phase of viral hepatitis, in toxic injury, and with circulatory collapse, but the last of these is not considered a common cause. In an important British study **(Ellis *et al.,* 1978)** on serum enzyme concentrations in diseases of the liver and biliary tree involving over 1100 patients, circulatory collapse did not feature at all. **McIntyre & Rosalkis (1991)** stated that, AST concentrations greater than 20 times normal are strongly suggestive of viral or drug hepatitis but it was noted that, similar concentrations may sometimes be seen in shock and heart failure, usually with a very abrupt rise. The authors also remark on the occasional striking elevation of AST in extrahepatic biliary obstruction which may mimic hepatitis.

In the present study, the serum AST level at a cutoff value 130 U/L was found to have an 88% sensitivity and 72% specificity when it is used to differentiate between mild and significant fibrosis. While in HCC cases the AST concentration was found not to have any diagnostic value, in spite of its increase in HCCs when compared with non-cancerous cases. Raised AST concentrations, generally below 400 U/L, are also seen in intrahepatic and extrahepatic cholestasis, in cirrhosis, and in primary and secondary liver tumors **(Ellis *et al.,* 1978 and Reichling & Kaplan, 1988)**.

In the present investigation, the mean serum ALT level was increased by the progression of liver fibrosis. In agreement with this finding, the study reported by **El-Shorbagy *et al.* (2004)**. A number of studies have shown that, ALT does not necessarily predict liver disease. A patient can have moderate or advanced disease and have a normal ALT (**Mathurin *et al.,* 1998; Marcellin, 1999; Ghany *et al.,* 2000** and **Marcellin *et al.,* 2001**). According to **Mathiesen *et al.* (1999)** there is little information on the spectrum of pathological liver changes which can be found in patients with hypertransaminasaemia of unknown etiology. This is mainly because most histological studies in large series of patients were performed before the discovery of hepatitis C **(Berasain *et al.,* 2000).**

In the present study, 11 cases have normal ALT values in spite of having some degree of fibrotic changes. However, the mean fibrosis stage in ALT normal group was significantly lower than that in ALT elevated one (1.7 versus 2.7, P = 0.049). Controversies exist concerning histological findings in patients with normal ALT levels. Some authors have shown that, in this group of patients, liver histology is virtually normal **(Brillanti *et al.,* 1993)**. In contrast, other authors have shown that HCV, viraemia in persons with normal ALT is consistently associated with liver damage **(Alberti *et al.,* 1992 and Stanley *et al.,* 1996)**. These studies do not focus on fibrosis, which is clinically more important than the activity grade **(Poynard *et al.,* 2001)**. It can't be explained why patients remain asymptomatic (with normal ALT) despite having considerable amounts of HCV-RNA in the serum. However, **Okanoue *et al.,* (1996)** speculated that, asymptomatic patients may have had chronic hepatitis prior to entry and/or had developed transient elevation of serum transaminases in the past.

Mathurin *et al.* (1998) studied the rate of fibrosis progression in patients with persistently normal and elevated ALT values. These investigators found that, lower ALT values correlated with lower histologic activity scores, and that these differences were not related to viral load or genotypes. Moreover, the rate of fibrosis progression remained lower among these patients. Another study found that, HCV patients with persistently normal ALT levels not only had lower inflammatory and fibrosis scores than patients with abnormal ALT values, but that levels of viremia were also lower in this subset **(Jamal *et al.,* 1999)**. In

addition, in a study from Italy, none of 37 patients had a worsening of hepatic fibrosis on follow-up liver biopsy 5 years later **(Persico *et al.*, 2000)**. In a study from France, there was no significant changes in the Ishak fibrosis scores among 24 patients who had a second liver biopsy 3 to 5 years later **(Martinot-Peignoux *et al.*, 2001)**. On the other hand, in both of these studies, a proportion of patients (approximately 5% per year) developed abnormal ALT levels during follow-up. Thus, patients may have a change in disease activity over time and the lack of fibrosis progression during one period may not predict future lack of progression of disease.

Although the elevation of serum ALT and AST concentrations above the range of normal values is the most frequent feature of acute or chronic hepatitis, serum aminotransferase activity elevation is not specific (57% for ALT and 72% for AST), because it is seen in numerous liver disorders of various etiologies **(Hoofngle, 2002)**. It is also poorly sensitive (81% for ALT and 88% for AST), since ALT and AST can remain within the normal range for long periods of time in patients with chronic HCV infection, in spite of progressive liver disease **(Seeff, 2002)**.

In spite of lacking optimal sensitivity and specificity of serum transaminases levels in assessing disease progression, the present study supports the clinical usefulness of the enzymes as an alternative to liver biopsy, at least in those patients refuting this technique. However, the validity of this approach and the level above which the ALT elevations are predictive of more rapid progression require further delineation.

In the present study, mean levels of serum ALT and AST were higher in HCC groups than in the group without HCC (ALT: 155.7 versus 70.3; AST: 295.7 versus 125.6). This finding is in agreement with the report by **Sato *et al.* (1996)**. They compared cirrhotic patients with HBV or HCV infection and clearly demonstrated that, persistent elevation of ALT is an important factor for the development of HCV-related HCC. The persistently high levels of ALT was found by **Tarao *et al.* (2000)** to be also associated with more rapid recurrence of hepatocellular carcinoma in hepatoctomized patients with HCV-associated liver cirrhosis or hepatocellular carcinoma than those with persistently normal ALT levels. The authors found that, HCC was recurred within 3 years in 70.6% of patients with high (> 80 IU/ml) ALT levels, while it recurred in only 18.8% of low ALT group within the same period ($P<0.05$).

Overall fibrosis stages an AST/ALT ratio had a significant positive relationship. Thus, the present study agrees with the previous findings of **Correia *et al.* (1981); Williams & Hoofnagle (1988); Sheth *et al.* (1998) and Giannini *et al.* (1999)**. Moreover, this study confirms the usefulness of the AST/ALT ratio as a means for separating patients with mild fibrosis from those with severe fibrosis and cirrhosis as stated by **Myers *et al.* (2002)**. The mean fibrosis score in patients with AST/ALT ratio ≥ 1 was significantly higher than that of patients with <1 ratio (3.3 versus 1.8, $P< 0.001$).

Noninvasive determination of cirrhosis using the ratio of serum AST/ALT has also been previously examined **(Williams & Hoofnagle, 1988 and Sheth *et al.*, 1998)**. Typically, this ratio is > 2.0 in alcoholic liver disease and < 1.0 in viral hepatitis. **Williams and Hoofnagle (1988)** reported on the accuracy of the ratio in a small group of patients with non-A, non-B hepatitis. They found that, when the AST/ALT was > or = 1.0, the likelihood ratio for the presence of cirrhosis was 2.0. The AST to ALT ratio appeared to be more predictive in a separate population of patients with documented hepatitis B.

Considering the diagnostic power of the transaminases ratio for separating mild from significant fibrosis, the ratio at a cutoff value 1.0 was found in the present study to have a 93% sensitivity and 77% specificity. **Sheth *et al.* (1998)** found the AST/ALT ratio to be highly predictive of cirrhosis in their patients with hepatitis C. These authors found that, the AST/ALT ratio had 100% specificity, 100% positive predictive value, and 81% negative predictive value. The operating characteristics of this serum test were reduced in this study because of the dilution effect caused by the lower stages of fibrosis. **Reedy *et al.* (1998)** did not find the AST/ALT ratio to be clinically useful in a small population of patients with hepatitis C.

The mechanism responsible for raised AST/ALT ratio in liver diseases is not fully understood. However, **Fleisher & Wakim (1963)** demonstrated that, AST is electively taken up by the liver, while **Kanimoto *et al.* (1985)** stated that, plasma clearance of AST is predominantly carried out by sinusoidal liver cells. **Giannini *et al.* (1999)** suggested that, an increase in AST/ALT ratio in progressive degrees of disease depends on diminished liver cell uptake of AST due to impaired functional liver blood flow. Although the mechanisms of the AST/ALT ratio modifications in advanced disease are not yet clear, its documented correlation with progressive liver function impairment might suggest its use in clinical practice. The usefulness of evaluating the serum AST/ALT ratio has been highlighted in previous studies examining patients with liver diseases of different etiology (**Correia *et al.*, 1981; Gitlin, 1982 and Williams & Hoofnagle, 1988**). Therefore, the ratio of AST to ALT in serum may help in the diagnosis of some liver diseases. In most patients with acute liver injury, the ratio is 1 or

less, whereas in alcoholic hepatitis it is generally about 2 (**Cohen & Kaplan, 1979**). Moreover, **Sheth *et al.* (1998)** demonstrated that, AST/ALT ratio alone can be used as a diagnostic tool for identifying the appearance of cirrhosis in chronic hepatitis patients infected by hepatitis C virus. More specific tests of hepatocellular damage will undoubtedly become available in due course, but in the meantime, measurement of serum aminotransferase concentrations best fulfills this purpose. According to **Pontisso *et al.* (1999),** hepatitis C virus infection was assured in this study both qualitatively by using RT-PCR and quantitatively by using the real time PCR technique. Chronic infection with hepatitis C virus is characterized by persistent viraemia.

The amount of circulating HCV-RNA in the serum samples of the studied cases ranged from 140 to 9200 000 copies/ml. No correlation between viral load and degree of liver injury was found in the present study. Controversial reports have been published on this point; in some studies high titer viraemia was correlated with advanced stage of liver disease **(Gretch *et al.*, 1995 and Hagiwara *et al.*, 1993)**, while others found no correlation with either histology or aminotransferase activities **(Chayama *et al.*, 1993)**. In addition, in most studies in which the intrahepatic HCV RNA level was evaluated, no correlation with liver injury was shown **(Sakamoto *et al.*, 1994; Coelho-Little *et al.*, 1995 and McGuiness *et al.*, 1996)**. The present analysis can't determine why the amount of HCV-RNA was not correlated with liver histology. However, it should be noted that, the characteristics of the population studied are important variables for the interpretation of the results, since significant differences were only observed when extremely different clinical settings were considered, for example, asymptomatic HCV carriers versus end stage liver disease (**Gretch *et al.*, 1995 and Hagiwara *et al.*, 1993)**.

In the present investigation, the study population included patients with disease severity ranging from mild chronic hepatitis to liver cirrhosis; there was only a few cases with minimal features of hepatitis. It is possible that, the wide range in viral load (140-9 x 10^6) detected in individual patients does not allow identification of any difference, unless extreme situations are considered. On the other hand, the putative mechanism of liver injury is not yet fully clarified, while the contribution of a direct cytopathic effect of HCV to liver damage is still controversial. Several lines of evidence, including the existence of chronic HCV infections without clinically overt disease (**Brillanti *et al.*, 1993**) and the detection of diffuse viral antigens in the liver of immunosuppressed transplant patients (**Krawczynski *et al.*, 1992**) indicate that, immune mediated mechanisms, already described for hepatitis B virus infection (**Thomas *et al.*, 1988**) are likely to play an important role in the pathogenesis of hepatitis C. However, at variance with hepatitis B, where impairment of the virus specific T cell response has been observed (**Barnaba & Balsano, 1992**) in patients with hepatitis C. A valid T cell response to HCV proteins has been detected both in the liver (**Koziel *et al., 1992)*** and in the peripheral blood lymphocytes (**Ferrari *et al.*, 1994**). Whether the level of viral load is the result of immune surveillance or whether it acts as an independent variable awaits the development of reliable cell culture systems.

Most cross-sectional studies have reported the absence of correlation between serum HCV RNA levels and the activity or grade of liver disease **(Zeuzem *et al.*, 1996)**. Interestingly, patients with chronic HCV infection who have normal serum alanine aminotransferase (ALT) levels and nearly normal liver histology may have high serum HCV RNA levels **(Martinot-Peignoux *et al.*, 1994)**. However, serum HCV RNA levels are an indirect reflection of intrahepatic HCV replication, and the correlation between fibrosis and intrahepatic levels of HCV RNA has not been adequately investigated and should be included in prospective studies **(Gervais *et al.*, 2001)**. **Brillanti *et al.* (1993)** recorded that, HCV infection may persist for several years without biochemical or histological evidence of liver disease. Fluctuating ALT levels characterize chronic hepatitis C but the authors found the activities to be consistently normal during a 5 year follow-up period. Also, they did not found any histological changes at liver biopsy after 18-24 months of clinical observation. The investigators could not explain this absence of liver disease. But they proposed three hypotheses; these are, the patient may have been infected by non-virulent HCV strain **(Weiner *et al.*, 1991)**; the pathogenesis of HCV-induced liver disease is mediated by the host immune response, and the patients may have been tolerant to HCV infection **(Brillanti *et al.*, 1992)**. These findings demonstrate that, active HCV infection may persist for a long time in the absence of HCV-induced liver disease and thus support the existence of healthy carriers of HCV. Moreover **Navas *et al.* (1993)** detected plus and minus HCV-RNA in peripheral blood mononuclear cells and in liver biopsy specimens in HCV-infected patients with persistently normal ALT levels. These results suggested that, in some symptom-free HCV carriers, HCV may replicate in the liver without inducing histological lesions. Furthermore, HCV-RNA may replicate in the peripheral blood mononuclear cells of symptomless patients. The normality of the liver of these patients is probably intrinsically related to one type of HCV strain or a lack of recognition of HCV by the immunological system **(Navas *et al.*, 1993)**.

In the present study, only three types of HCV were detected in the studied cases (type 1 in 7, type 4 in 61 and type 6 in 4 cases). Therefore, type 4 is the most prevalent one in this population. A similar finding was observed by **Mangoud *et al.* (2004b)** in a similar Egyptian population. However, the type of HCV was found not to affect the stage of liver fibrosis (mean fibrosis stage is 1.3 in type 1, 1.3 in type 4 and 1.0 in type 6). The difference in mean fibrosis stage in cases infected with different HCV types was statistically not significant (P = 0.064), indicating no effect of HCV type on fibrosis progression.

The influence of viral genotype in the pathogenesis of the liver disease is not completely resolved. In several early studies, HCV genotype 1 (particularly 1b) was found to be associated with a more severe liver disease, including a higher frequency of cirrhosis and hepatocellular carcinoma **(Silini *et al.*, 1996)**. However, many of these studies did not control for important confounding factors, such as age, source, and duration of infection. Genotype 1b is more common among older than younger patients and has been commonly linked to spread by blood transfusion. In studies with adjustment for these variables, the association between genotype 1b and a more severe liver disease has not been found **(Ghany *et al.*, 2000 and Zeuzem *et al.*, 1996)**. Interestingly, the distribution of genotypes is not different in patients with chronic hepatitis C and normal serum ALT levels, as compared with those with increased serum levels **(Marcellin, 1999)**. Further studies are needed to better determination of the possible role of genotype in the outcome of HCV-related liver disease. On the other hand, several studies have shown a modest association between a high quasispecies heterogeneity and more severe liver injury in chronic hepatitis C **(Pawlotsky *et al.*, 1998)**. In one study, quasispecies heterogeneity was less in patients with normal ALT levels compared with those with elevated levels **(Asselah *et al.*, 2002)**. Quasispecies heterogeneity can also be confounded by features of gender, age, and duration of disease. Further studies are needed on the significance of quasispecies heterogeneity in the natural history of hepatitis C and its association with hepatic fibrosis.

In the present study, HCV type 4 is also more prevalent in HCC cases (6/10) than type 1 (3/10) and type 6 (1/10). In cross-sectional studies, HCV genotype 1 (1b in particular) is the most prevalent genotype worldwide and also is the most common genotype found among patients with HCC. However, all HCV genotypes have been described in HCV-related HCC. There was conflicting data as to whether genotype 1 is a risk factor for cirrhosis or HCC independent of older age **(Bruno *et al.*, 1997 and Lopez-Labrador *et al.*, 1997)**. It has been suggested that, the higher prevalence of these genotypes reported in some studies represents a cohort effect in which older persons (those at greatest risk for cirrhosis and HCC) were infected at a time when genotype 1 was most prevalent **(Lopez-Labrador *et al.*, 1997)**. Lastly, there is no evidence that either viral load or viral quasispecies are important in determining the risk for HCV progression to cirrhosis or HCC **(El-Serag, 2002)**.

In the present study, the levels of matrix metalloproteinase 9 was inversely correlated with the stage of liver fibrosis. Similar results were recently reported by **Mangoud *et al.* (2004b)**. Moreover, **El-Shorbagy *et al.* (2004)** reported a negative relationship between serum MMP-9 level and the severity of liver disease. The present study revealed a high sensitivity and specificity (87% and 72%, respectively) of this serum test in differentiating mild fibrosis from severe fibrosis that may reduce the need for liver biopsy in many patients especially when it was combined with transaminases ratio. Moreover, the serum MMP9 test has a fairly high positive and negative predictive values (86% and 82%, respectively) which supports the usefulness of its use in the clinical settings as a serum fibrosis marker.

Circulating levels of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) have been shown by several investigators to correlate with the development of cirrhosis **(Murawaki *et al.*, 1999; Muzzillo *et al.*, 1993 and Tsutsumi *et al.*, 1996)**. The results of the present study showed that, circulating concentrations of serum MMP9 change in the course of chronic hepatitis C. The changes in the concentrations of MMPs and TIMPs have been described by other investigators. **Lichtinghagen *et al.* (2000)** reported a slight decrease in MMP2 and MMP9 in patients with CAH but the decrease was almost doubled in patients with cirrhosis, while TIMP2 was increased in hepatitis and cirrhotic patients. In contrast to the present findings, plasma MMP9 concentrations have been reported to be within the reference intervals in patients with chronic liver diseases and even cirrhosis, but increased in patients with HCC **(Hayasaka *et al.*, 1996)**. The difference between the results of the present study and those reported by the later authors can possibly be explained by the fact that, the Japanese results were obtained in EDTA plasma, whereas serum samples were assayed in this study. **Jung *et al.* (1998)** found that, the Fuji-MMP9 antibody, which is used in the commercial assays used in this study and by the Japanese group, gives measurable MMP9 levels in EDTA plasma that may be up to 20-folds higher than that in serum.

Benyon & Arthur (2001) reported that, inactive metalloproteinases can be either activated through proteolytic cleavage, or inhibited by binding to specific inhibitors known as TIMPs (tissue inhibitors of metalloproteinases). So, progressive fibrosis will be associated with marked increases in TIMPs leading to a net decrease in protease activity of the MMPs and therefore promoting matrix accumulation by slowing down collagen breakdown **(Iredale, 1997)**. This assumption was assured by the findings of **Arthur (1997)** who reported an increase in serum and liver TIMP-1 in cirrhosis and that adds another support to the findings of the present study. The observations of **Arthur (1997)** are in agreement with those of **Murawaki *et al.* (1999)** and **Tsutsumi *et al.* (1996)**. Studies in animal models and human liver fibrosis indicated that, interstitial collagenolytic activity decreases in liver extracts in advanced fibrosis **(Okazaki & Maruyama, 1974; Takahashi *et al.*, 1980 and Maruyama *et al.*, 1982)** which would promote net collagen deposition. There is increasing evidence that, collagenase inhibition may arise from increased expression in fibrotic liver of endogenous MMP inhibitors (TIMPs). Expression of both TIMP-1 and –2 is increased in human and rat model fibrotic liver **(Rojkind *et al.*, 1979 and Han *et al.*, 1980),** and in human liver the degree of TIMP-1 expression correlates with extent of fibrosis **(Benyon *et al.*, 1996)** assessed by hydroxyproline content. In rat models of liver fibrosis, TIMP-1 is expressed early in fibrogenesis before apparent collagen deposition **(Iredale *et al.*, 1996)**. The resulting increase in TIMP:MMP ratio in liver may promote fibrosis by protecting deposited ECM from degeneration by MMPs.

In the present study, there was no statistical difference in the mean AFP serum level between mild and significant fibrosis (7.7 versus 16.7 ng/ml, P = 0.75). However, the receiver operating characteristic curve revealed a very good diagnostic power of serum AFP for HCC diagnosis (area under the curve = 0.949). When the predictive power of serum AFP was assessed in a prospective fashion, it was found to be higher in population-based versus clinic-based studies, as a consequence of the many false-positive results in patients with cirrhosis **(Sato *et al.*, 1993 and Colombo *et al.*, 1991)**. The clinical significance of an elevated AFP level in chronic hepatitis C remains to be identified. By using the most commonly reported cutoff value of a positive test result for hepatocellular carcinoma (AFP level > 1000 ng/ml) the test sensitivity was 90%, specificity 57%, positive predictive value 87% and negative predictive value was 59%. Data for AFP at higher cutoff values suggest that AFP, although not sensitive, can be highly specific for hepatocellular carcinoma. Therefore, a low AFP level (< 1000 ng/ml) would not be informative enough to stop further search for hepatocellular carcinomas but an AFP level higher than 1000 ng/ml would strongly suggest that, cancer is present, allowing for earlier counseling of patient **(Gupta *et al.*, 2003)**.

Although the National Cancer Institute recommended against screening for hepatocellular carcinoma, many physicians still screen high-risk populations with various strategies, including serum AFP, ultrasonography and computed tomography **(El-Serag, 2002)**. The use of AFP to detect these tumors has been widely debated **(Lin & Liaw, 2001; Sherman, 2001 and Johnson, 2002)**. Many conclude that, AFP is not a useful diagnostic test **(Sherman, 2001 and Tong *et al.*, 2001)**, but AFP continues to be commonly used. Patients with cirrhosis have a higher risk for cancer **(Tsukuma *et al.*, 1990)** but commonly have elevated levels of AFP thought to be unrelated to hepatocellular carcinoma **(Taketa, 1990)**, leading to an unknown effect on sensitivity and specificity. Some studies have shown that, AFP has different test characteristics in patients with hepatitis B virus than in those with HCV **(Tsai *et al.*, 1994 and Gupta *et al.*, 2003)**, but the sensitivities and specificities in the study of **Cedrone *et al.* (2000)** were within the range of values reported by other studies which included only patients with HCV.

With the recent development of improved resectional and ablative therapies, cirrhotic patients are now eligible for the treatment of HCC. The potential for cure of HCC depends on early detection **(Gogel *et al.*, 2000)**. Therefore, screening of high-risk population will be necessary to achieve early diagnosis. Most HCC screening protocols use ultrasound and serum AFP, although the use of AFP as a screening test is complicated by frequent false-negative and false-positive results. **Torzilli *et al.* (1999)** reported a sensitivity of 68% and specificity of 20% using AFP to diagnose early (less than 3 cm) HCC. In another study from China, serum AFP levels were elevated only in 60% to 70% of patients with small HCC lesions, and fewer than 20% of the elevated levels of AFP were due to HCC **(Lok & Lai, 1989)**. HCCs in symptomatic patients are generally large and demonstrate extrahepatic spread, thus rendering them unresectable **(Kassianides & Kew, 1987)**. Median survival in patients with clinically apparent HCC is less than 6 months, and 2 year survival is virtually nonexistent **(Gogel *et al.*, 2000)**. Early detection offers the best potential for curative intervention. Therefore, in contrast to certain other cancers, screening for liver cancer has become, at least among hepatologists, an accepted part of the management of patients with end-stage liver disease **(Collier & Sherman, 1998)**. A number of screening and surveillance programs have

been reported **(Lok & Lai, 1989 and Sherman *et al.*, 1995)**. However, the results range from very optimistic to downright pessimistic.

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A Note on Characterization by Renewal Variable

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Abstract: The concept of renewal variable associated with a non-negative random variable X is used to identify the distribution of X as well as its failure rate. Some illustrated examples are given.
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Keywords: Characterization, Renewal process, Mean residual life, Failure rate, Exponential, Pareto and Pearsonian distributions.

Introduction

Let X be a non-negative random variable (often represents the life of a unit in a certain process)with finite mean μ, cdf $F_X(.)$ and survival function $\bar{F}_X(.)$. A new random variable Y with density function $f_Y(.)$ can be defined (see, e.g., **Cox** [1] as follows:

$$f_Y(x) = \frac{\bar{F}_X(x)}{\mu} \qquad (1.1)$$

The random variable Y has many applications in life length studies (see, e.g., **Scheaffer** [2]) as well as in renewal Process (**Zacks** [3]). **Gupta** [4] has shown that for large values of X, the random variable Y represents the life of the process when an operating component is replaced upon failure by another possessing the same life distribution. **Pakes and Khattree** [5] have demonstrated that Y is related to the length biased sampling.

Moreover, several authors have used Y to characterize some probability distributions. **Huang and Lin** [6] have characterized the exponential distribution using a relationship between the k^{th} moments of Y and X. **Gupta** [2] has given an explicit formula of the cdf of X in terms of the failure rate of Y.

The main objective of this note is to identify the distribution of X as well as its failure rate function in terms of the mean residual life and failure rate functions of Y.

1- The Main Result.

The following Theorem determines the survival function of X as well as its failure rate in terms of the mean residual life function of Y.

Theorem 2.1

Let X be a non-negative continuous random variable with finite mean μ, failure rate $r_X(.)$, density function $f_X(.)$ and survival function $\bar{F}_X(.)$. Denote by Y, its associated random variable defined by (1.1). Assume that $g_Y(.)$ is the mean residual life function of Y, then

$$\bar{F}_X(x) = \frac{c}{g_Y^2(x)}\,(1 + \dot{g}_Y(x))\,\exp\left(-\int \frac{dx}{g_Y(x)}\right), \qquad (2.1)$$

For some constant c to be determined from $\bar{F}_X(0) = 1$

$$r_X(x) = \frac{1}{g_Y(x)} + \frac{2\dot{g}_Y(x)}{g_Y(x)} - \frac{\dot{g}_Y(x)}{1+\dot{g}_Y(x)} \qquad (2.2)$$

Proof. Using (1.1), the survival function $\bar{F}_Y(x)$, of Y will be:

$$\bar{F}_Y(x) = \mu^{-1} \int_x^\infty \bar{F}_X(z)\,dz \qquad (2.3)$$

By definition, the mean residual life function of Y (**Hall and Wellner** [7]) is given by:

$$g_Y(x) = \frac{\int_x^\infty F_Y(z)dz}{F_Y(x)} \qquad (2.4)$$

Using (2.3), we get:

$$g_Y(x) = \frac{\int_x^\infty \int_z^\infty F_X(t)dt\,dz}{\int_x^\infty F_X(t)dt}, \text{ i.e.,}$$

$$g_Y(x) \int_x^\infty \bar{F}_X(t)\,dt = \int_x^\infty \int_z^\infty \bar{F}_X(t)\,dt\,dz \qquad (2.5)$$

Differentiating (2.5) with respect to x, we get:

$$\frac{\bar{F}_X(x)}{\int_x^\infty F_X(t)dt} = \frac{1+\dot{g}_Y(x)}{g_Y(x)} \qquad (2.6)$$

Integrating both sides of (2.6) with respect to x, we get:

$$\int_x^\infty \bar{F}_X(t)\,dt = \frac{c}{g_Y(x)}\,\exp\left(-\int \frac{dx}{g_Y(x)}\right) \qquad (2.7)$$

For some constant c to be determined using $\bar{F}_X(0) = 1$. Differentiating (2.7) with respect to x, we get:

$$\bar{F}_X(x) = \frac{c}{g_Y^2(x)}\,(1 + \dot{g}_Y(x))\,\exp\left(-\int \frac{dx}{g_Y(x)}\right) \qquad (2.8)$$

To prove the 2^{nd} result, take the logarithm of both sides of equation(2.8),we get:

$$\ln \bar{F}_X(x) = \ln c - 2\ln g_Y(x) + \ln(1 + \dot{g}_Y(x)) - \int \frac{dx}{g_Y(x)} \qquad (2.9)$$

Differentiating (2.9) with respect to x and recalling that

$r_X(x) = \frac{f_X(x)}{\bar{F}_X(x)}$

We get:

$r_X(x) = \frac{1}{g_Y(x)} + 2\frac{\dot{g}_Y(x)}{g_Y(x)} - \frac{\dot{g}_Y(x)}{1+\dot{g}_Y(x)}$

Our proof is complete.

Remark (2.1)

Denote by $r_Y(.)$ and $g_Y(.)$, the failure rate and mean residual life function of the renewal variable respectively and recalling that (**Ruiz and Navarro** [8]) :

$r_Y(x)\, g_Y(x) = 1 + \dot{g}_Y(x)$,

then the 2^{nd} part of Theorem (2.1) can be written as follows:

$r_X(x) = 2\, r_Y(x) - (g_Y(x))^{-1}\ (1 + \frac{\dot{g}_Y(x)}{r_Y(x)})$

Examples

(1) If $g_Y(x) = k$ = constant, then equation (2.1) gives:

$\bar{F}_X(x) = \exp(-\frac{x}{k})$, $x > 0$

Also equation (2.2) gives $r_X(x)$ =constant, which is the well known result of the exponential distribution.

(2) If $g_Y(x) = \frac{b+x}{\theta-2}$, $0 < a \le x$, $\theta > 2$

Then equations (2.1) and (2.2) give:

$F(x) = (\frac{a+b}{b+x})^{\theta}$, $0 < a \le x$ $r_X(x) = \frac{\theta}{b+x}$

Which are the well known results for the general Pareto distribution with parameters a, b, and θ.

(3) If $g_Y(x) = \frac{b-x}{\theta+1}$, $a < x < b$, $\theta > 0$.

Then equations (2.1) and (2.2) give:

$\bar{F}_X(x) = (\frac{b-x}{b-a})^{\theta}$

$r_X(x) = \frac{\theta}{b-x}$

Which are the well known results for the 1^{st} type Pearsonian distribution with parameters a,b, and $\theta > 0$.

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Remarks

(1) Similar results for the uniform distribution with parameters a, b can be given. To see this, put $\theta = 1$ in the last example.

(2) Similar results for the beta distribution with parameters 1,m can be obtained. To this end, put θ= m, b =1 and a= 0 in the last example.

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Assessmenmt of Some Cardiovascular and Biochemical Parameters Induced in Rats by Chronic Noise Stress

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Abstract: The present study aimed to assess some cardiovascular and biochemical parameters induced in rats by chronic noise stress. Moreover, changes produced in the histological architecture of the heart and aorta were also investigated. For this purpose, forty healthy adult male albino rats weighting 200±30 grams were used in the study. They were divided equally into two main groups; noise group (n=20) which was exposed to chronic white noise stress (100 dBA) 6 hours daily for 30 days and control group (n=20) which was kept away from any stress source and was hold in the same conditions. After 30 days, animals exposed to chronic noise stress exhibited significant increase in the heart rate, systolic, diastolic, and mean arterial blood pressure associated with a significant decrease in serum Mg^{++} levels. There was a strong significant negative correlation between reduction in serum Mg^{++} and elevation in mean arterial blood pressure. A significant elevation in serum levels of ACTH, corticosterone, and leptin was detected after exposure to noise stress. Moreover, the data obtained indicated that, under these conditions of chronic and high noise exposure levels, there was significant increases in the serum levels of TC, TGs,VLDL and LDL-C and a significant decrease in the level of serum HDL-C. The histopathological examination of the heart tissue demonstrated that, exposure of rats to chronic noise stress has resulted in areas of hemorrhage inbetween the cardiac myocytes, necrosis and small areas of myocardial infarction. Microscopic examination of the aorta showed the presence of thickening of elastic fibers in the media with perivascular infiltration by acute inflammatory cells (neutrophils & eosinophils) and non-specific inflammatory cells.
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Key words: Rats – Noise – Cardiovascular – Biochemical – Histopathological – Parameters.

1. Introduction:

Noise is a type of unwanted sound pollution that penetrates the environment. Noise pollution can be caused by many sources including vehicles, factories, concerts, air conditioners, engines, machines, aircraft, ,helicopters, alarms, public address systems, industrial development and construction work, walkman –type headphones and power garden tools. In general, noise pollution refers to any noise irritating to one's ear which comes from an external source (**Mahmoud *et al.*, 2008**).

Selye (1979) defined loud noise as an environmental stress factor. Nowadays, a large number of people are exposed to potentially hazardous noise levels not only in work background (**Kawecka-Jaszcz, 1991; Lang *et al.*, 1992 and Zhao *et al.*, 1993**) but also during everyday life, i.e., in urban traffic, with electric household appliances, discos, etc. (**Parrot *et al.*, 1992 and Maschke, 2011**). Apart from the well-known hearing impairment, the noise bath causes slow but widespread injuries at several levels in human organs and apparati. (**Gloag, 1980**)

Noise is ubiquitous in every day interaction, and no one on the earth can escape the sound of noise wanted or unwanted (**Binhi, 1999**). Millions of persons are exposed daily to equivalent noise levels of at least 55 dB, 65 dB and 75 dB or more (**Elise *et al.*, 2002** and **Mahmoud *et al.*, 2008**).

Health risks attributed to noise have increasingly attracted political attention in recent years, this is due to continuous growth of noise encumbrance in the surroundings of daily life and progressive number of protests by noise plagued citizens or those representing their interests (**Maschke and Hecht, 2000**).

Noise can act as a non-specific stressor inducing stress reactions, anxiety disorders, insomnia, syndromes of immune disregulation, fatigue as well as hearing impairment (**Ising *et al.*, 1999**). A sound above 80 dB has been considered to produce ill effects on the health of animals and human beings, affecting functional ability, biochemical parameters, immunological system and histology. Some of stressor induced alterations have been attributed to an imbalance in autonomic system and involving hypothalamo-pituitary-adrenal axis activation; this is followed by changes in physiological function of the organism, including total peripheral resistance, cardiac output, and blood lipid metabolism (**Gehlot *et al.*, 2002**).

Acute exposure to maximum sound pressure level above 90 dB has the potential to stimulate the sympathetic nervous system to increase catecholamines

and cortisol secretions. However, if the noise disturbed activities such as conversation, concentration, recreation and sleep; acute increase of catecholamines and cortisol secretions were observed even at an environmental noise level > 50 dB (**Ising *et al.*, 1999 and Goyal *et al.*,2010).**

Chronic exposure to environmental and industrial noise may be a risk factor for cardiovascular disease (CVD) and the possible pathway that links noise exposure to CVD may be elevated serum lipid levels (**Saha *et al.*, 1996 and Maschke and Hecht, 2000**).

Exposure to noise causes many health problems such as hearing loss, sleep disturbance, and impairs performance as well as effecting cognitive performance. It also increases aggression and reduce the processing of social cues seen as irrelevant to task performance as well as leading to coronary heart disease, hypertension, higher blood pressure, increased mortality risk, serious psychological effects, headache, anxiety, and nausea (**Lenzi *et al.*, 2003; Stansfeld and Matheson, 2003; Abbate *et al.*, 2005 and Ravindran *et al.*, 2005**). Children chronically exposed to noise tend to have poorer reading ability and less cognitive capacity to understand (**Stansfeld and Matheson, 2003**). Some studies are being conducted on causation of exposure to noise near airports to the higher risk of developing hypertension, cardiovascular diseases and incidence of cancer (**Jarup *et al.*, 2005 and Visser *et al.*, 2005**).

Noise exposure of any kind that exceeds 90 dB has been reported to be a source of stressor (**Ravindran *et al.*, 2005**). A study showed that, working and reference memory error increased significantly following the noise-stress exposure, 100 dBA/4h per day for 30 days, when compared to control animals (**Manikandan *et al.*, 2006**). Acute as well as long term exposure to noise can produce excessive free radicals such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) (**Manikandan *et al.*, 2005**). Oxygen free radicals can attack protein, nucleic acids and lipid membranes thereby disrupting normal cellular functions and integrity (**Endo *et al.*, 2005 and Manikandan and Devi, 2005**).

Nervous system is relatively more susceptible to free radical damage (**Scarfiotti *et al.*, 1997**). According to **Ravindran *et al.* (2005),** neurotransmitters in discrete brain regions were found to be increased during noise stress even after 15 days of exposure. In addition to generating free radical species, it also leads to increase in radical induced lipid peroxidation end products such as malondialdehyde (MDA) which is an indicator of lipid peroxidation processes (**Derekoy *et al.*, 2001**).

Similar to other types of stress, noise stress has also been shown to increase levels of stress hormones like corticosterone and norepinephrine (**Agnes *et al.*, 1990 and Archana and Namasivayam, 1999**). Many studies indicated that corticosterone can stimulate the secretion of adiposite-derived hormone, leptin (**Slieker *et al.*, 1996 and Wabitsch *et al.*, 1996**). As noise increases corticosterone secretion, it may be proposed that, the exposure to stressors like noise could induce alterations in serum leptin levels. Such a possibility has not been fully investigated.

The present study was designed to investigate the influence of exposure of adult male albino rats to chronic noise stress on:

1. Some cardiovascular parameters such as the heart rate and the arterial blood pressure.
2. Serum leptin, ACTH, and corticosterone levels.
3. Serum lipid profile and magnesium ion concentration.
4. Histological architecture of the heart and aorta.

2. Materials and Methods

Animals

In the present study, forty healthy adult male albino rats weighing between 200 – 250 gm were enrolled. They were obtained from the laboratory animals' farm unit, faculty of Veterinary Medicine, Zagazig University, Egypt. All the animals were housed in the animal facility, 3 per open mesh-steel wire cage (29 cm x 22 cm x 14 cm), fed ad-libitum (allowed free access to food and water), kept under closely controlled hygienic and environmental conditions [12 hour reverse light/dark cycle, constant humidity and room temperature (20°-24°)]. The diet consisted of mixed commercial rat laboratory chow and was supplied in separate clean containers. Animals were allowed to adapt to the environment for at least 1 week prior to noise experiment to minimize all undesired stressors.

The animals were divided into 4 equal groups:

Group I: Consisted of 10 rats in which the arterial blood pressure (ABP) and electrocardiogram [ECG, to measure the heart rate] was recorded in vivo by using the MD4 osccillograph. This group served as a control group.

Group II : Consisted of 10 rats from which

A- Blood samples were collected and sera were separated for the determination of the levels of Mg^{++}, ACTH, corticosterone, leptin, total cholesterol, triglycerides, low density lipoprotein, very low density lipoprotein and high density lipoprotein.

B- The heart and the aorta were isolated and prepared for hsitopathological examination using the light microscope. This group served also as a control group.

Group III : Consisted of 10 rats which were exposed to chronic noise stress and immediately after the last exposure, they were used for the in vivo recording of the arterial blood pressure (ABP, mmHg) and ECG [to measure heart rate (HR, beats/min)] using MD4 Oscilograph. This group served as a noise exposure group.

Group IV : Consisted of 10 rats which were exposed to chronic noise stress and immediately after the last exposure,

A- The blood samples were collected and the sera were separated and used for the determination of the levels of Mg^{++}, ACTH, corticosterone, leptin, total cholesterol, triglycerides, low density lipoprotein, very low density lipoprotein, and high density lipoprotein.

B- The heart and the aorta were isolated and prepared for histopathological examination using the light microscope. This group served as a noise exposure group.

Noise-stress induction procedure:

When noise stress of any kind exceeds 90dB, noise becomes a stressor (**Ramsey and Flangan, 1982**). Noise was produced by two loud speakers mounted 40 cm a part on opposite sides of the cage (15 w) and driven by a white noise generator (range 0 – 26 KHz) installed (suspended) 30 cm above the cage. The noise level was set at an intensity of 100 dBA uniformly throughout the cage and monitored by a sound level meter (RAT-M Model RE-120, Germany). This noise level was delivered continuously to 20 males rats 6 hours daily for 30 days.

To avoid the influence of handling-stress on evaluation of the effects due to noise exposure, control rats (20 males) were kept in the above, described cages during the corresponding period of time without being exposed to noise **(Pellegrini *et al.*, 1997 and Lenzi *et al.*, 2003).**

Measurement of blood pressure and ECG recording

For calibration of the pressure, a mercury sphygmomanometer and 500 ml – capacity glass bottle with very light rubber stopper were used. Through one of the two holes in the stopper, an L- shaped rigid glass tube was forced to enter until it reached 0.5 cm from the bottom of the bottle and it was connected to the blood pressure transducer. Through the other hole, one limb of T- shaped rigid glass tube was forced just to pass through the rubber cover of the bottle and it was connected to the air pump of the sphygmomanometer.

Preparation of the recording system:

1) The ECG limb cable was attached to the FC 123 ECG facility coupler, which was fitted to one of the 4- channels of the oscillograph MD4 ***(Bioscience, Washington).***
2) The blood pressure transducer (PT 400) was connected to the FC 137 strain gauge coupler that was fixed to another channel of the oscillograph.
3) About 10cm length polyethylene tube with a clamp was connected to one side limb of the PT 400 transducer and the other limb of the transducer was connected through polythene tube with a clamp to the arterial cannula.
4) The 500 ml capacity glass bottle was filled with normal saline solution containing 16 l.u. herparin /ml to inhibit blood clotting **(Tuttle and Milts, 1975)**. The external limb of the L- shaped tube, previously fitted in the bottle stopper, was connected to the side limb of the transducer, all valves were opened; the pressure inside the bottle was raised by pressing on the pump, so that, the solution would be pushed to fill the connections to the transducer and the valve of the other limb of the transducer was reclosed. Calibration of the pressure was done by gradual elevation in the bottle by 10 mmHg increment, theraby, in the mercury manometer, starting from zero to 200 mmHg (10, 20, 30,……..etc) and record on the chart paper of the oscillograph. After that, all valves were closed and transducer was disconnected from the bottle.

Intra – arterial cannulation and recording of the systemic arterial pressure:

The arterial blood pressure of the animals was determined by employing the method of **Burden *et al.* (1979)**. after stabilization of anesthesia which was induced by intraperitoneal injection of ethylcarbamate (urethane) [1.2 gm / weight) **(Niu *et al.*, 2000)].**

Recording of the electrocardiogram [ECG] :

The ECG limb of the cable was attached to hypodermic needles inserted and fixed subcutaneously, in axilla (for each fore limb); just above the ankle (for each hind-limb). According to the manufacturer recommendation; the red lead was attached to the right arm, the yellow lead to the right leg; the white lead to the left arm; the green lead to the left leg.

The FC123 was switched on lead II that gives good signals for analysis of ECG. The selected paper chart speed for recording the ECG by the oscillograph was 25 mm/sec. [i.e 150 cm/minute]. Calculation of the heart rate/minute was carried out by counting the number of cardiac cycles (n) per fixed distance of the chart paper e.g. 5 cm and the heart rate/minute was then calculated by division of 150/5 multiplied by (n) **(Gay, 1995).**

Sampling of blood

Upon completion of all noise exposure regimens (immediately after the end of the last exposure), the animals were anesthetized with an intraperitoneal injection of intraval sodium (60 mg/kg body weight) and exanguinated. Approximately 5 ml of blood was

collected from each rat for hormonal assay, using ELISA method. The ELISA kits used were stored at 4ºC. In order to avoid variations in the results due to circadian rhythm of hormones, all blood samples were collected at the same time of the day.

Separation of serum:

The blood was collected from all the studied groups in clean centrifuge tubes and was allowed to clot over a period of 2 hours. Serum was separated by centrifugation of blood at 3000 rpm for 20 minutes. The supernatant serum was pipetted off using fine tipped automatic pipettes and was stored frozen at – 20ºC until assayed. The reagents and specimens were brought to the room temperature before use **(Chandralekha *et al.*, 2005 and Mahmoud *et al.*, 2008**).

Determination of serum Magnesium level:

Serum Magnesium was estimated according to the method described by ***Tietz (1995)*** using ELITECH MAGNESIUM CALMAGITE kits [SEPPM S.A.S. ZONE INDUSTRIELLE – 61500 SEES FRANCE].

Determination of the serum ACTH levels:

Serum ACTH levels were determined according to the immunoassay method of **Odell *et al.* (1989).** The ACTH Immunoassay is a two-site ELISA [Enzyme-Linked Immuno Sorbent Assay] for the measurement of the biologically active 39; amino acid chain of ACTH.

Kits for determination of ACTH: (SIEMENS-5210 Pacific concourse drive Los Angeles, CA 90045-6900-USA)

Determination of the serum cortieosterone levels:

Serum corticosterone level were determined by the application of the immunoassay method of **Vazquez-Palacios (2001)**. Cortieosterone ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding.

Kits for determination of corticosterone : (DRG instruments GmbH, Germany).

Determination of serum leptin levels:

The method used for hormonal assay of serum leptin level was Enzyme linked lmmuno sorbant Assay [ELIZA] with the aid of Micro ELIZA plate reader. Leptin concentrations were calculated according to **Considine *et al.* (1996).**

Active leptin ELISA kits: For the quantitative measurement of leptin in serum by leptin Enzyme – Linked immuno sorbent (ELISA) [DSL – 10 – 23100 Diagnostic Headquarters 445 medical center BLvd Webster, Texas 77598 – 4217 USA].

Determination of serum cholesterol:

Cholesterol serum level was estimated according to the method described by (**Tietz ,1995).**

The cholesterol CHOD – POD, Enzymatic colorimetric kits [SPINREACT, S.A. ctra. Santa Coloma 7E – 17176 SANT ESTEVE DE BAS (GI) SPAIN] and spectrophotometer or colorimeter measuring at wavelength 505 nm were used.

Determination of serum Triglycerides:

Triglycerides were estimated according to the method described by **Tietz (1995).**

The Triglycerides GPO – POD Enzymatic colorimetric kits

[SPINREACT , S.A Ctra Coloma, 7E – 17176 SANT ESTEVE DE BAS (GI) SPAIN] and spectrophotometer or colorimeter measuring at wavelength 505 nm were used.

Determination of serum high density lipoprotein cholesterol (HDL-C):

HDL-C was estimated according to the method described by **Tietz (1995)**. The HDL –C precipitating reagent kits [SPINREACT , S.A Ctra Coloma, 7E – 17176 SANT ESTEVE DE BAS (GI) SPAIN] and spectrophotometer or colorimeter measuring were done at wavelength 505 nm.

Determination of serum low density lipoprotein cholesterol (LDL-C):

LDL–Cholesterol was calculated according to the **Friedewald *et al.* (1972)** formula:

$$\text{LDL-C} = \text{Total cholesterol (TC)} - \frac{\text{Triglyceri des (TGs)}}{5} - \text{HDL- C}$$

Determination of serum very low density lipoprotein (VLDL)

VLDL was calculated according to the **Friedewald *et al.* (1972)** formula:

$$\text{VLDL} = \frac{(\text{TGs})}{5}$$

Statistical Analysis

All data were expressed as mean ± SE and statistically analyzed according to the methods described by **Kirkwood (1989)**. Differences were considered significant if $P < 0.05$. The statistical methods used in this study for analysis of the data include: Arithmetic mean ($\bar{X}$), Standard deviation (SD), Standard error of mean (SEM), Student's t-test. Correlation coefficient (r)

Histological and Histopathological studies:

After decapitation,small pieces of the heart and aorta were taken immediately from both control rats and those exposed to chronic noise stress, then fixed in

alcoholic Bouins fluid for 24 hours. Paraffin sections of 6 microns thickness were used. Dehydration of materials was carried out in an ascending series of ethyl alcohol, followed by clearing in terpineol for 2 days and then immersing quickly in 3 changes of benzol before embedding in molten wax. Paraffin sections were mounted on chemically clean glass slides without using any adhesive mixture and the standard procedure of routine histological staining using Haematoxylin (H) and Eosin (E) was used as described by **Humason (1979).**

3. Results

Changes in arterial blood pressure and heart rate after exposure to noise:

Animals subjected to chronic noise stress 6 hours daily for 30 days demonstrated a statistically significant increase ($P < 0.001$) in systolic (175 ± 4.97 mmHg), diastolic (130.5 ± 5.29 mmHg) and mean arterial blood pressure (145.497 ± 5.02 mmHg) when compared to the respective control values (systolic 123 ± 5.54 mmHg , diastolic 72.5 ± 3.18 mmHg and mean arterial blood pressure 89.3 ±3.95 mmHg) as shown in tables (1,2 & 3) and figs. (1,2 & 3) respectively .

Regarding the changes in the heart rate after chronic exposure to noise stress, it was found that, the mean value of heart rate was significantly increased in noise exposed group (351±11.00 beats/min, $P<0.001$) when compared to that of the control group (279±7.81 beats/min) as shown in table (4), figs. (4&5)

Changes in serum Mg^{+2} levels after exposure to noise:

Exposure of rats to chronic noise stress for 30 days resulted in a significant decrease ($P < 0.001$) in serum Mg^{2+} levels (0.871 ± 0.021 mM / L) when compared to the control group (0.988 ± 0.012 mM / L), as shown in table (5) and fig. (6). There was a significant strong negative correlation between reduction in serum Mg^{2+} and elevation of the arterial blood pressure (r= -0.921) as shown in table (6) and fig. (7).

Changes in serum ACTH and corticosterone levels after exposure to noise:

Exposure of rats to chronic noise stress for 30 days resulted in a significant increase ($P<0.001$) in serum ACTH and corticosterone levels (66.604±1.716 ng/ml; 57.186±2.1246 nmol/L respectively) compared to the respective control values (44.47±1.597 ng/ml; 33.325±1.2279 nmol/L respectively) as shown in tables (7&8) and figs. (8&9).

Changes in serum Leptin levels after exposure to noise:

Serum leptin concentrations were significantly ($P< 0.001$) higher in animals which were exposed to chronic noise stress for 30 days (8.52±0.3158 pg/ml) when compared to the control (6.05± 0.126 Pg/ml) (Table 9, Fig. 10).

Changes in serum lipid and lipoprotein levels after exposure to noise :

A. **Non exposure or control group :**

The mean serum TC level was (60.21±1.19 mg/dl) and the range was (51.00-64.00 mg/dl).

The mean serum TGs level was (53.21±1.088 mg/dl) and the range was (47.00-59.00 mg/dl) as shown in tables (10&11) and figs. (11&12).

The mean serum levels of lipoproteins VLDL, LDL-C and HDL-C were (10.642±0.217 mg/dl, 10.568±1.22 mg/dl and 40.00±1.29 mg/dl) respectively, as shown in tables (12,13&14) and figs. (13,14&15).

B. Noise exposure group :

The mean serum TC level was (70.61 ± 1.001 mg/dl) and the range was (63.5 – 74.2 mg/dl).

The mean serum TG_S level was 63.49 ± 0.98 mg / dl and the range was (58.2 – 68.00 mg/dl) as shown in tables (10&11) and figs. (11&12).

The mean serum levels of lipoproteins VLDL , LDL.C and HDL.C were (12.498 ± 0.237 mg/dl, 26.912 ± 1.28 mg/dl and 31.2 ± 0.986 mg/dl respectively), whereas their ranges were (11.2 – 13.6 mg/dl , 18.86 – 34.4 mg/dl and 26.00 – 36.00 mg/dl respectively), as shown in tables (12,13&14) and figs. (13,14&15).

It was found that, exposure of the animals to chronic noise stress resulted in a significant increase in serum TC, TG_S, VLDL and LDL.C levels ($P<0.001$) and a significant decrease in serum HDL.C levels ($P < 0.001$).

Histopathological changes in the heart and aorta after exposure to noise:

Comparing to the histological picture of **hearts** isolated from control rats (Fig. 16), the histopathological changes observed in **the hearts** isolated from rats exposed to chronic noise stress were the presence of: Areas of hemorrhage in between the cardiac myocytes (Fig. 17), homogenous pale pink areas of necrosis (Fig. 18), multiple areas of myocardial infarction (Fig. 19), multiple areas of hemorrhage inbetween cardiac myocytes (Figs. 20 & 21) and slitting of cardiac muscles (Fig. 22)

When comparing to the histological picture of the **aortae** isolated from control rats (Fig. 23), the histopathologhical changes observed in the **aort**e isolated from rats exposed to chronic noise stress were thickening or hypertrophy of elastic fibers in the media (Fig. 24), perivascular infiltration by nonspecific

inflammatory cells (Fig. 25) and acute inflammatory cells mainly neutriphils and eosinophils (Fig. 26).

Table (1): Changes in systolic blood pressure (mmHg) of studied groups

	Control group	Noise-exposed group
1	120	175
2	120	170
3	100	160
4	100	150
5	150	200
6	140	190
7	105	160
8	140	190
9	130	180
10	125	180
$\overline{X}$	123	175.5
SD	17.5	15.7
SE	5.54	4.97
t	7.056	
p	< 0.001	

Table (2): Changes in diastolic blood pressure (mmHg) of studied groups

	Control group	Noise-exposed group
1	70	130
2	70	110
3	60	120
4	60	110
5	90	150
6	80	140
7	65	120
8	85	140
9	75	150
10	70	125
$\overline{X}$	72.5	130.5
SD	10.069	16.74
SE	3.18	5.29
t	9.4	
p	< 0.001	

Table (3): Changes in mean arterial pressure (MAP, mmHg) of studied groups

	Control group	Noise-exposed group
1	86.66	145.00
2	86.66	130.00
3	73.33	133.33
4	73.33	123.33
5	110.00	173.33
6	100.00	156.66
7	78.33	133.33
8	103.33	156.66
9	93.33	160.00
10	88.33	143.33
$\overline{X}$	89.33	145.497
SD	12.478	15.87
SE	3.95	5.02
t	8.7939	
p	< 0.001	

Table (4): Heart rate (beats/min) changes in studied groups

	Control group	Noise-exposed group
1	270	360
2	270	390
3	240	360
4	2408	390
5	300	300
6	300	330
7	270	390
8	300	330
9	300	300
10	300	360
$\overline{X}$	279	351
SD	24.698	34.785
SE	7.81	11.00
t	5.337	
p	< 0.001	

Table (5) : Serum levels of Mg^{2+} (mM/l) in studied groups

	Control group	Noise- Exposed group
1	1.01	0.86
2	1.03	0.88
3	0.99	0.93
4	0.92	0.98
5	0.98	0.78
6	0.95	0.79
7	0.02	0.94
8	0.96	0.83
9	1.04	0.82
10	0.98	0.90
X-	0.988	0.871
SD	0.0379	0.066
SE	0.012	0.021
t	4.875	
p	< 0.001	

Table (6): Correlation coefficient between mean arterial blood pressure (mmHg) and Mg^{2+} serum levels (mM/l) in chronic noise stress exposed group

	MAP	Mg
1	145.00	0.86
2	130.00	0.88
3	133.33	0.93
4	123.33	0.98
5	173.33	0.78
6	156.66	0.79
7	133.33	0.94
8	156.66	0.83
9	160.00	0.82
10	143.33	0.90
r	-0.921^{**}	
P	P < 0.001	

Table (7): Serum ACTH (ng/ml) levels of studied groups

	Control group	Noise-exposed group
1	45.50	68.50
2	42.02	57.80
3	39.00	65.20
4	35.90	59.06
5	51.21	72.00
6	45.07	70.08
7	40.60	62.40
8	50.80	70.00
9	46.20	74.02
10	48.40	67.00
$\overline{X}$	44.47	66.604
SD	5.05	5.428
SE	1.597	1.716
t	9.4508	
p	<0.001	

Table (8): Serum corticosterone (nmol/l) levels of studied groups

	Control group	Noise-exposed group
1	34.20	58.20
2	33.50	56.50
3	29.00	50.30
4	25.80	44.06
5	38.00	65.60
6	35.60	61.10
7	30.05	52.05
8	36.02	62.22
9	34.00	57.80
10	37.08	64.03
$\overline{X}$	33.325	57.186
SD	3.88	6.7186
SE	1.2279	2.1246
t	9.73918	
p	< 0.001	

Table (9) : Serum levels of leptin (Pg/ml) in studied groups.

	Control group	Noise- Exposed group
1	5.7	7.1
2	5.9	7.2
3	6.1	7.4
4	6.0	8.2
5	6.1	9.4
6	6.3	9.1
7	5.6	9.5
8	5.5	9.3
9	6.6	8.4
10	6.7	9.6
X-	6.05	8.52
SD	0.4	0.998
SE	0.126	0.3158
t	7.1594	
p	< 0.001	

Table (10): Serum level of cholesterol (mg/dl) in studied groups

	Control group	Noise-exposed group
1	59.00	69.00
2	63.00	73.5
3	58.00	68.8
4	64.00	74.2
5	60.00	70.5
6	62.00	72.4
7	59.9	69.2
8	62.2	72.8
9	51.00	63.5
10	63.00	72.2
$\overline{X}$	60.21	70.61
SD	3.77	3.165
SE	1.19	1.001
t	6.688	
p	< 0.001	

Table (11): Serum level of triglycerides (mg/dl) in studied groups

	Control group	Noise-exposed group
1	51.00	62.2
2	55.00	66.00
3	49.80	59.6
4	56.30	67.00
5	59.00	68.00
6	55.00	64.00
7	53.00	63.40
8	52.00	62.00
9	47.00	58.20
10	54.00	64.50
$\overline{X}$	53.21	63.49
SD	3.44	3.11
SE	1.088	0.98
t	7.017	
p	< 0.001	

Table (12): Serum level of VLDL (mg/dl) in studied groups ($\frac{TG}{5}$) (**Friedewald *et al.*, 1972**)

	Control group	Noise-exposed group
1	10.2	12.44
2	11.00	11.2
3	9.96	11.92
4	11.26	13.4
5	11.8	13.6
6	11.00	12.8
7	10.6	12.68
8	10.4	12.4
9	9.4	11.64
10	10.8	12.9
$\overline{X}$	10.642	12.498
SD	0.688	0.75
SE	0.217	0.237
t	5.8	
p	< 0.001	

Table (13): Serum level of LDL (mg/dl) in studied groups [Tc-(HDL + $\frac{TG}{5}$)] (**Friedewald *et al.*, 1972**)

	Control group	Noise-exposed group
1	9.8	25.56
2	11.00	29.3
3	11.04	27.88
4	7.74	24.8
5	8.2	25.9
6	4.00	24.6
7	13.3	28.52
8	17.8	34.4
9	8.6	18.86
10	14.2	29.3
$\overline{X}$	10.568	26.912
SD	3.866	4.06
SE	1.22	1.28
t	9.244	
p	< 0.001	

Table (14): Serum level of HDL (mg/dl) in studied groups

	Control group	Noise-exposed group
1	39.00	31.00
2	41.00	33.00
3	37.00	29.00
4	45.00	36.00
5	40.00	31.00
6	47.00	35.00
7	36.00	28.00
8	34.00	26.00
9	43.00	33.00
10	38.00	30.00
$\overline{X}$	40.00	31.2
SD	4.08	3.119
SE	1.29	0.986
t	5.432	
p	< 0.001	

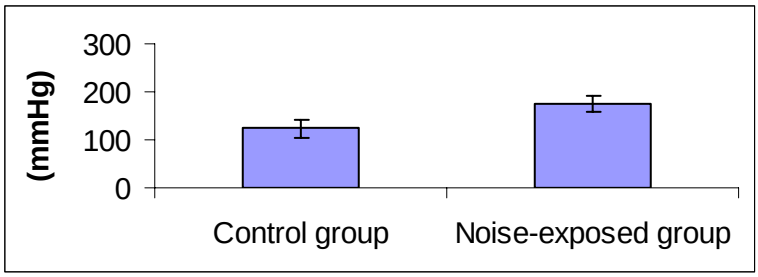

Fig. (1): The mean value ±SE of systolic blood pressure (mmHg) in chronic noise stress exposed group compared to the control group.

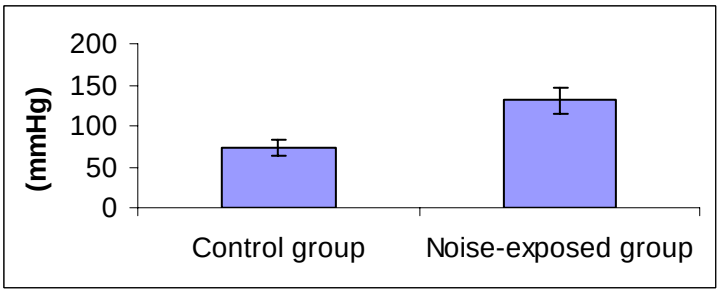

Fig. (2): The mean value ±SE of diastolic blood pressure (mmHg) in chronic noise stress exposed group compared to the control group.

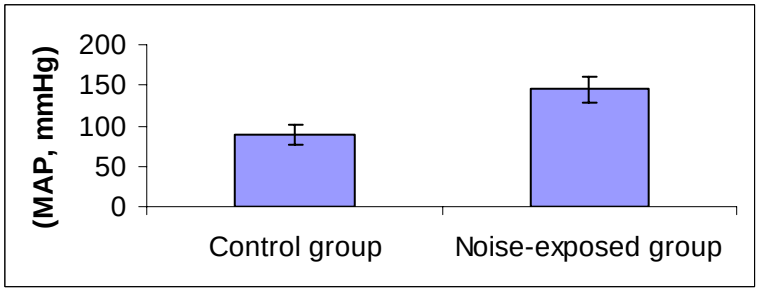

Fig. (3): The mean value ±SE of mean arterial pressure (MAP, mmHg) in chronic noise stress exposed group compared to the control group.

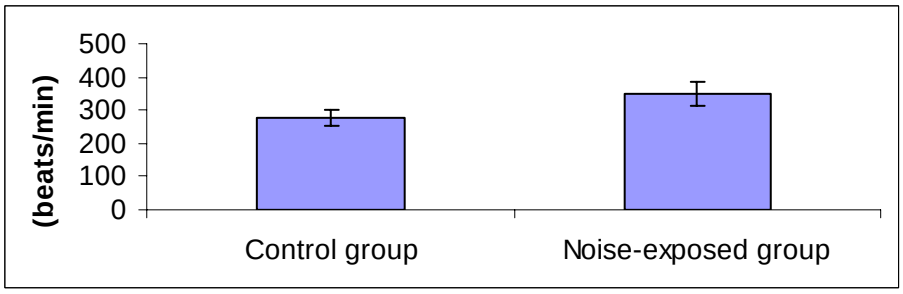

Fig. (4): The mean value ±SE of heart rate (beats/min) in chronic noise stress exposed group compared to the control group.

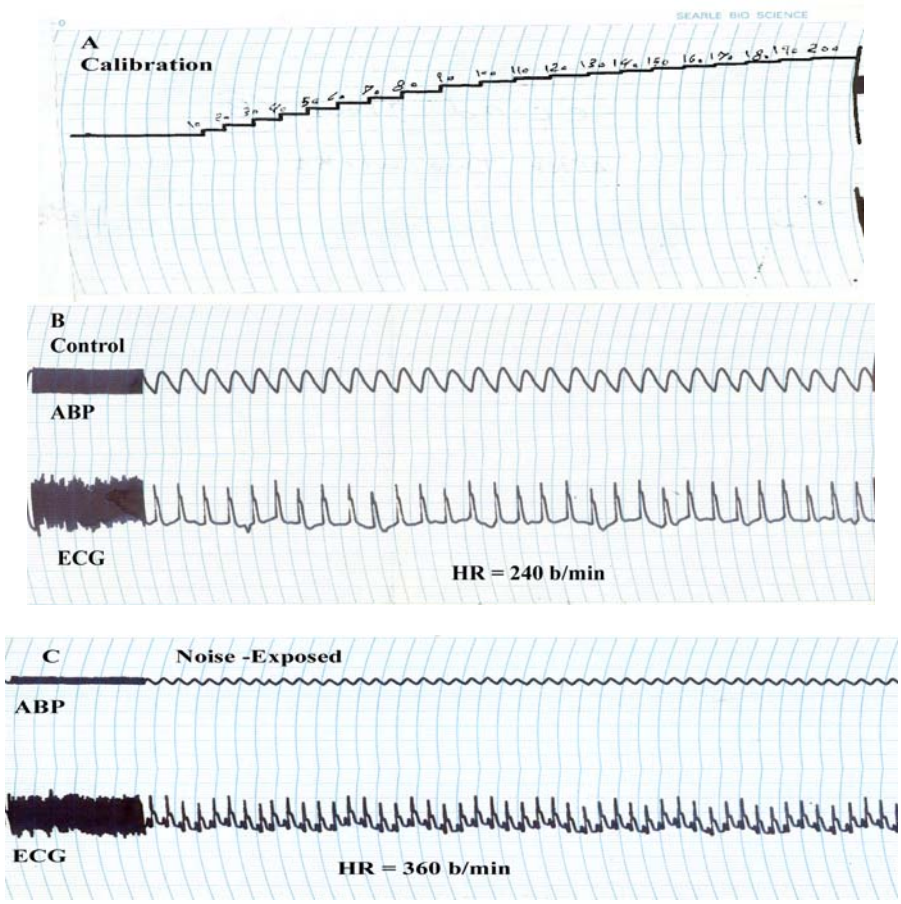

Fig. (5): Tracings illustrating the calibration used to measure the arterial blood pressure (A), a record of the arterial blood pressure and ECG in control group (B) and chronic noise stress-exposed group (C)

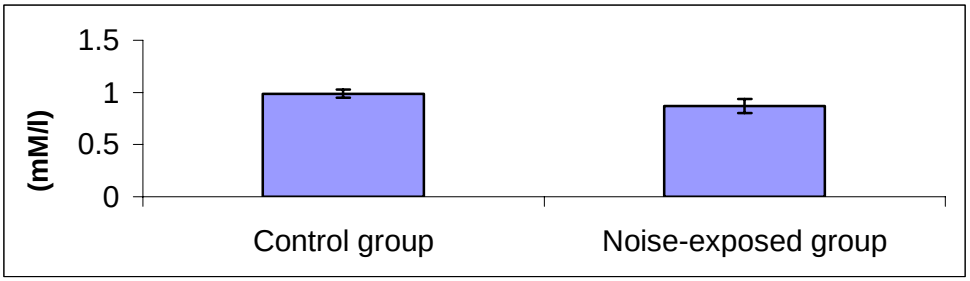

Fig. (6): The mean value ±SE of Serum Mg^{2+} (mM/l) levels in chronic noise stress exposed group compared to the control group.

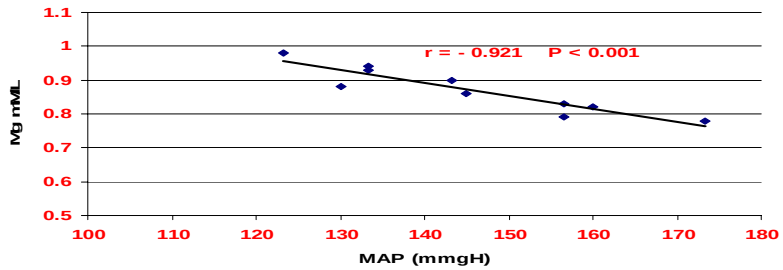

Fig. (7): Correlation coefficient between mean arterial blood pressure (mmHg) and Mg2+ serum levels (mM/l) in chronic noise stress exposed group.

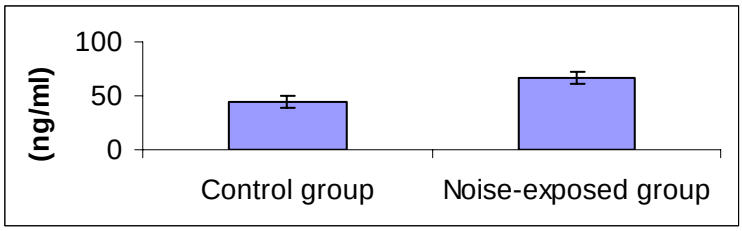

Fig. (8) The mean value ±SE of Serum ACTH (ng/ml) levels in chronic noise stress exposed group compared to the control group.

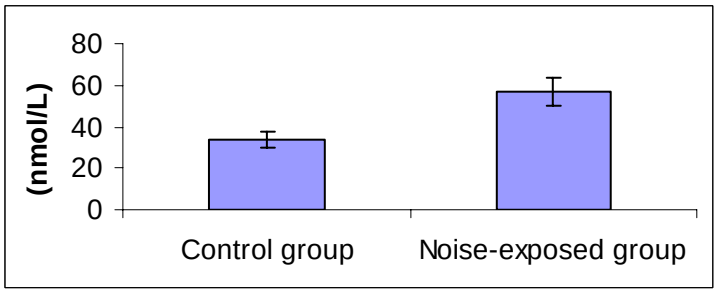

Fig. (9): The mean value ±SE of Serum corticosterone (nmol/l) levels in chronic noise stress exposed group compared to the control group.

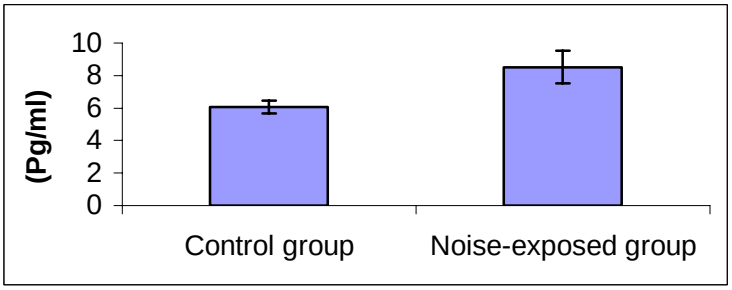

Fig. (10): The mean value ±SE of Serum levels of leptin (Pg/ml) levels in chronic noise stress exposed group compared to the control group.

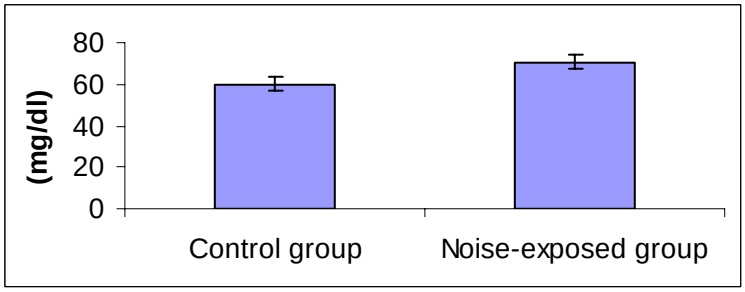

Fig. (11): The mean value ±SE of Serum level of cholesterol (mg/dl) in chronic noise stress exposed group compared to the control group.

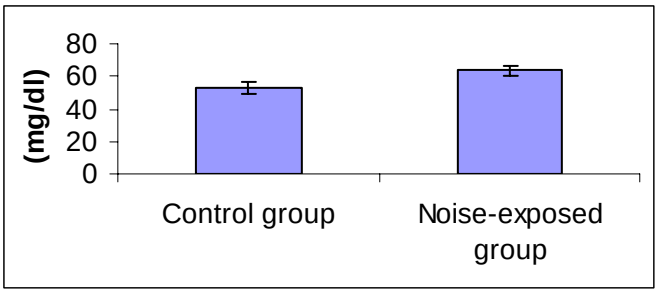

Fig. (12): The mean value ±SE of Serum level of triglycerides (mg/dl) in chronic noise stress exposed group compared to the control group.

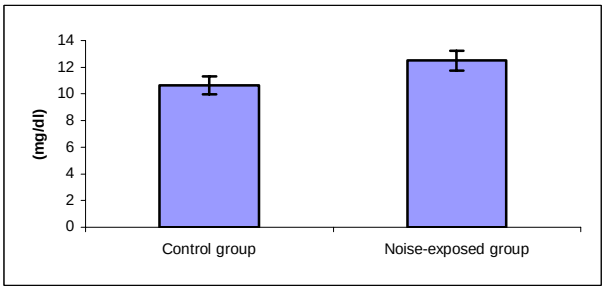

Fig. (13): The mean value ±SE of Serum level of VLDL (mg/dl) in chronic noise stress exposed group compared to the control group.

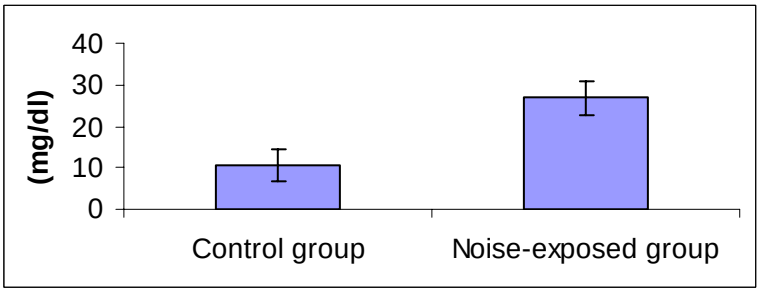

Fig. (14): The mean value ±SE of Serum level of LDL (mg/dl) in chronic noise stress exposed group compared to the control group.

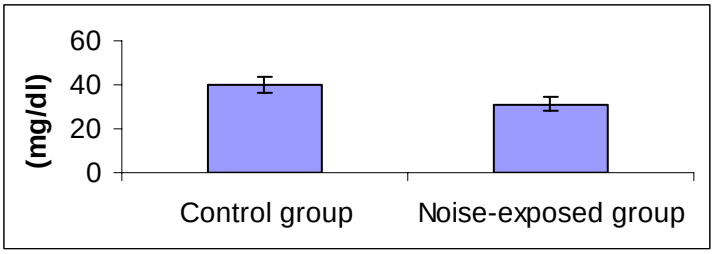

Fig. (15): The mean value ±SE of Serum level of HDL (mg/dl) in chronic noise stress exposed group compared to the control group.

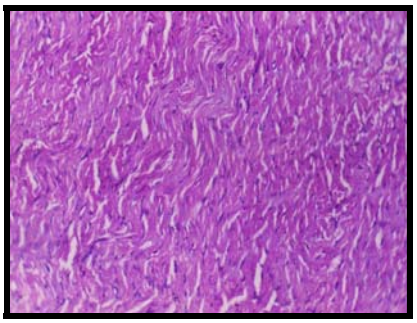

Fig. (16): A section in the heart taken from a control male albino rat showing normal arrangement and cross striation of myocardial cells; H&E X 100.

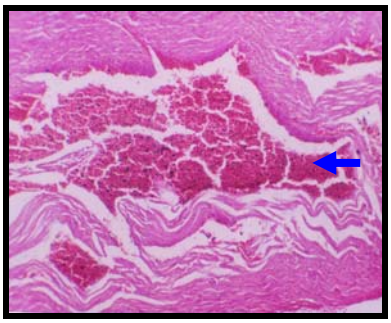

Fig. (17):A section in the heart taken from a male albino rat exposed to chronic noise stress showing areas of haemorrhage in between cardiac myocytes; H&E X 100

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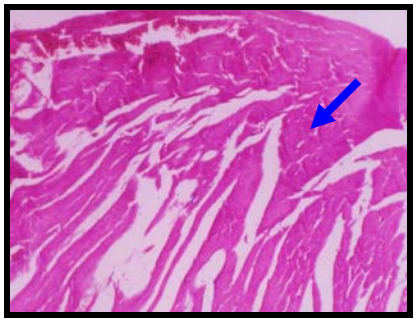

Fig. (18): A section in the heart taken from a male albino rat exposed to chronic noise stress showing homogenous pink areas of nucleated necrotic cardiac myocytes; H&E X 100.

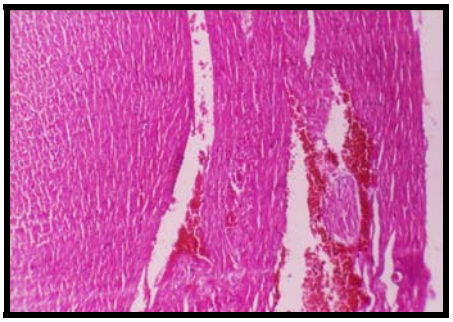

Fig. (19):A section in heart taken from a male albino rat exposed to chronic noise stress showing myocardial infarction; H& E X 100.

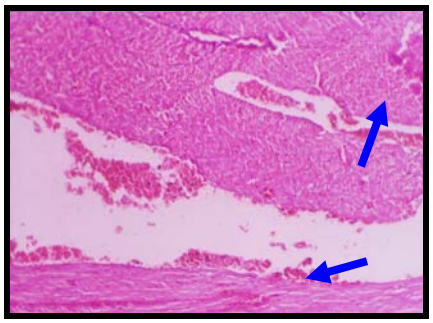

Fig. (20): A section in heart taken from a male albino rat exposed to chronic noise stress showing multiple areas of haemorrhage in between cardiac myocytes and multiple areas of myocardial infarction ; H & E X 100.

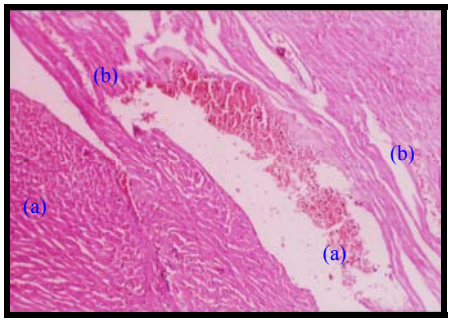

Fig. (21): A section in heart taken from a male albino rat exposed to chronic noise stress showing multiple areas of haemorrhage in between cardiac myocytes (a) and multiple areas of myocardial infarction (b);H & E X 100.

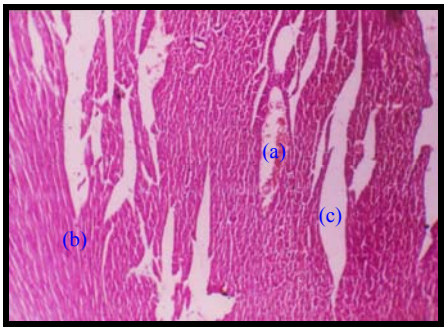

Fig. (22):A section in heart taken from a male albino rat exposed to chronic noise stress showing small areas of haemorrhage in between cardiac myocytes (a), small areas for myocardial infarction(b) and splitting of cardiac muscle(c); H&E X 100.

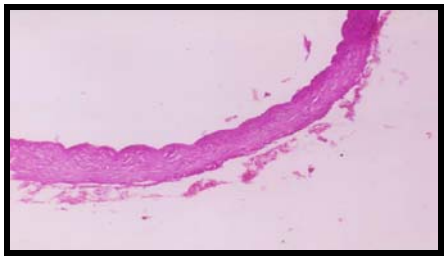

Fig. (23): A section in aorta taken from a control male albino rat showing normal wall thickness with normal intima, media and adventitia; H&E X 40.

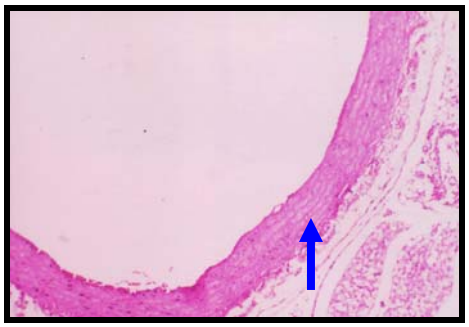

Fig. (24): A section in aorta taken from a male albino rat exposed to chronic noise stress showing thickening of elastic fibres in the media; H & E X 100.

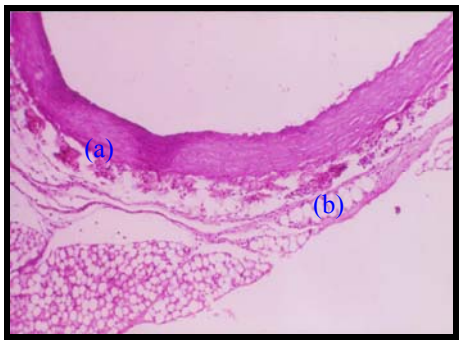

Fig. (25): A section in aorta taken from a male albino rat exposed to chronic noise stress showing thickening of elastic fibres in the media (a) with perivascular infiltration by non-specific inflammatory cells (b); H&E X 100.

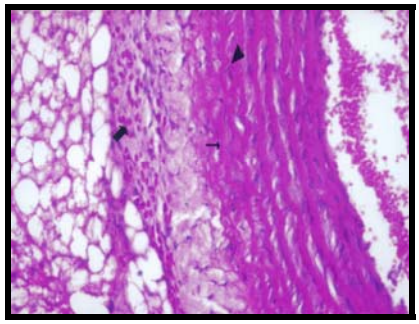

Fig. (26):A section in aorta taken from a male albino rat exposed to chronic noise stress showing hypertrophied eleastic fibres as well as perivascular infiltration by acute inflammatory cells mainly neutrophils and esinophils; H&E X400.

4. Discussion

Noise is considered a kind of stress, which produces significant physiological and biochemical changes in animals as well as in humans **(Borg, 1981).** The damaging effect of noise on hearing has been extensively studied **(Ogale, 1999).** However, very little information are available regarding the effect of noise on other body functions. The present study revealed that, exposure of adult male albino rats to chronic noise stress has resulted in a significant increase in systolic blood pressure, diastolic blood pressure, mean arterial blood pressure and heart rate which were recorded immediately at the end of the last exposure. These changes were accompanied with a significant increase in blood levels of ACTH, corticosterone, and leptin together with a significant decrease in serum levels of magnesium. These results are in accordance with those of many other investigators **(Archana and Namasivayam, 2000 and Chandralekha *et al*., 2005),** but at variance with those of others **(Fernandez *et al* .,2010)**

Research on the effect of noise exposure has focused not only on behavioral disturbances and mental diseases but also on chemical and physiological modifications of endocrine, cardiovascular, and nervous systems **(Alario *et al*., 1987).** In particular, noise has been recognized as one of the risk factors for cardiovascular diseases because it increases heart rate **(Linden *et al*., 1985)**, peripheral vascular resistances **(Bach *et al*., 1991)**, arterial blood pressure **(Altura *et al*., 1992 and Sawada, 1993)** and causes electrocardiogram disorders **(Tomei *et al*., 1992)**. In this respect, there is evidence for an enhanced activation of the sympathetic nervous system, as assessed by increased levels of circulating catecholamines **(Lagercrantz *et al*., 1990 and Goyal *et al* .,2010)**. Animal experiments and hormone plasma assays in humans have demonstrated that noise stimulus can increase catecholamine secretion **(Vogel and Jensh, 1988).** A previous study **(Breschi *et al*., 1994)** has shown that, a more prolonged exposure time induces a parallel enhancement of the sympathetic network.

Evidence is accumulating to suggest that, prolonged exposure to noise may induce long-term and permanent changes in cardiovascular function in animals and human subjects **(Babisch, 1998).** It was reported that, noise – induced hearing loss in animals and human subjects is associated with alterations in serum and perilymph magnesium (Mg) **(Joachims *et al*., 1987).** Some of these experiments indicated that, noise-induced hearing loss was associated with elevation of myocardial calcium content **(Ising *et al*., 1999),** vasoconstriction of the arterioles in the cochlea and the organ of corti (**Nakai and Matsutani, 1988)** as well as energy deficit of the cochlea hair cells **(Gunther *et al*., 1989).**

Several recent studies point to a causal relationship between decreased (Mg^{2+}) in blood or tissues and hypertension **(Gunther *et al*., 1989 and Altura and Altura, 1990)**. In the present study, we observed that, elevation of the arterial blood pressure was associated with a significant decrease in serum levels of Mg^{++} in animals exposed to chronic noise stress. Further more, a strong significant negative correlation was found between the reduction in serum Mg^{++} level and the elevation in mean arterial blood pressure (MAP). The incidence of hypertension is

often high in geographic areas with soft drinking water or Mg-poor soil **(Altura and Altura; 1990;).** Hypomagnesemia has been reported in a number of hypertensive patients **(Altura and Altura, 1990).** Considerable evidence indicates that, oral and parenteral administration of Mg^{+2} can lower blood pressure in hypertensive patients and animals **(Altura and Altura, 1990)** and can decrease blood pressure induced by stress **(Ising *et al.*, 1999)**. It has been demonstrated that, low dietary intake of Mg can result in hypertension in rats **(Altura *et al.*,1992)** and can aggravate hypertension in both spontaneously and deoxycorticosterone acetate-salt hypertensive rats **(Chrysant *et al.*, 1988).** A microcirculatory basis for these Mg-linked alterations has been suggested. **(Altura and Altura, 1990).**

It has been also suggested that environmental and/or occupational noise stress may be an important environmental factor in the etiology of hypertension **(Andren *et al.*, 1983).** A high positive correlation has been found between exposure of individuals to noise and high blood pressure in a number of retrospective epidemiological studies **(Cohen *et al.*, 1981).** According to **Eggertsem *et al.* (1987),** noise-induced elevation of arterial blood pressure in rats and human subjects has been associated with increased peripheral vascular resistance and cardiac hypertrophy**.** This peripheral vasoconstriction takes place in all types of microvessels, i.e., arterioles, venules and precapillary sphincters **(Alutra *et al.*, 1992).** There is evidence of increased activation of the sympathetic nervous system during noise stimulation **(Andren *et al.*, 1983*).*** Some investigators reported that the elevation of the blood pressure during noise exposure is independent of elevated levels of catecholamines, cortisol and prolactin or growth hormone **(Andren *et al.*, 1983).** In addition, central $\alpha 2$ – adrenoceptors are not involved **(Eggertsen *et al.*, 1987).**

Altura *et al.* (1992) demonstrated that, dietary deficiency of Mg can aggravate hypertension caused by noise-induced stress and that this relationship is associated with 1) intense vasoconstriction of terminal arterioles, precapillary sphincters, and venules in the microcirculation; 2) reduced capillary blood flow; 3) rarefaction of capillaries; 4) increased reactivity of intact muscular microvessels to neurohumoral agonists and 5) decreased responsiveness to the vasodilator histamine.

Several experimental and clinical studies have reported that, disturbances of the metabolism of Mg^{+2} may have profound effects on the contractile state of vascular smooth muscle, peripheral blood flow, and thus arterial blood pressure **(Altura and Altrua, 1990).** A number of recent studies in humans and experimental animals suggest that, Mg^{2+} may exert beneficial therapeutic actions via their circulatory actions **(Altura and Altrua, 1990). Peterson *et al.* (1977)** found an inverse correlation between serum (Mg^{+2}) and blood pressure. **Resnick *et al.* (1984)** noted an inverse relationship between diastolic blood pressure and intracellular free Mg^{2+} in erthrocytes. Elevation of extracellular Mg^{2+} has been demonstrated to produce vasodilation of arterioles, precapillary sphincters, and venules in the intact microcirculation of mesentery, skeletal muscle, and brain in a dose dependent manner **(Nishio *et al.*, 1989).** In addition, elevation of Mg^{2+} has been shown to attenuate, in a dose-dependent manner, constriction of these muscular, microvessels induced by a variety of neurohumoral agents **(Nakai and Matsutani, 1988).** According to **Altura *et al.* (1992),** the effects of ionized Mg^{2+} on vascular tone are reflections of this metal's influence on membrane permeability to Ca^{2+} as well as on binding, translocation, intracellular release, and on membrane stability**.** Studies have shown that Mg^{2+} sites in the blood vessels membrane act physiologically to regulate entery and exit of Ca^{2+} **(Altura and Altrua, 1990).** Lowering Mg^{2+} increases total exchangeable and intracellular Ca^{2+} fractions in blood vessels **(Altura *et al.*, 1987).** Mg^{2+} has been demonstrated to be a week Ca^{2+} channel antagonist **(Altura *et al.*, 1992),** which can act on voltage, receptor, and leak-operated membrane channels in vascular smooth muscules **(Altura *et al.*, 1992).**

In vitro and in vivo experiments clearly indicate that, when Mg^{2+} inlowered, Ca^{2+} influx and intracellular release are enhanced, causing contraction and an increase in basal tone **(Altura *et al.*, 1992).** It has also been shown that, reduction in serum Mg^{2+} attenuate endothelium-derived relaxant factors **(Altura *et al.*, 1987).** Several reports in experimental animals and in humans indicate that, noise –induced stress results in the release into blood stream of various stress hormones, i.e., catecholamines, prolactin, cortisol, growth hormone, and oxytocin. Most of these changes are, however, transient and do not seem to be associated with the Noise-induced sustained elevation of arterial blood pressure **(Eggertsen *et al.*, 1987).** It is distinctly possible that, alteration of the Mg^{2+} to Ca^{2+} ration induced by the noise stress in endocrine end organs is responsible for the transient release of the various hormonal secretions. The Mg^{2+} to Ca^{2+} ratio in such end organs and nerves is well known to excitation-secretion coupling mechanisms **(Mordes and Wacker, 1978).**

It was speculated that, Noise-induced stress releases cellular Mg^{2+} via intense vibrations bombarding various bodily tissues resulting in microtrauma to internal cellular and surface celleular membranes, may be similar to what has been noted in

patients and animals subjected to circulatory shock and body trauma **(Chaudry *et al.*, 1988).** An alternative possibility may be that Noise-induced stress releases catecholamines and adenosine $3^{/},5^{/}$ cyclic monophosphate, which may increase membrane permeability and thus allow Mg^{2+} to leak out of cells **(Altura *et al.*, 1992).**

The present study demonstrated that, exposure to chronic noise stress resulted in histopathological changes in the heart and aorta. The heart showed areas of hemorrhage inbetween cardiac myocytes, homogenous pale pink areas of necrosis and myocardial infarction. The aorta showed thickening of elastic fibers in the media with perivascular infiltration by non-specific inflammatory and acute inflammatory cells mainly neutrophils and eosinophils. These results are in agreement with those of **Kempen (2011)** and **Bluhm and Eriksson (2011).** Furthermore, our results are supported by the findings of **Soldani *et al.* (1997)** who examined the ultrastructure of the heart of albino rat by transmission and scanning electron microscopy after exposure to white noise (100 dBA) and found mitochondrial alterations, areas of enlargement in intercalated disc membranes and decreased density of sarcoplasm. In addition, **Paparelli *et al.* (1995)** showed structural modifications of rat myocardium after acute noise stress.

Our observations are also consistent with those caused by other stressors **(Lopes *et al.* 1992)** or with those observed in certain pathological conditions **(Ghadially, 1988). Ferrans and Roberts *(1972)*** described similar focal lesions following exogenous catecholamine administration. In a previous research **(Paparelli *et al.*, 1995),** it was pointed out that, noise exposure provokes a modification in the sympathetic innervation pattern depending on the increase in catecholamine synthesis. Catecholamines are released under various conditions, from alarm reactions to acute stress **(Selye, 1979),** and their action primarily concerns the cardiovascular system because they cause a rise in blood pressure and can cause a significant ischemic alteration **(Yamamura and Aoshima, 1980).** In humans exposed to short-term noise stress, the ischemic damage does not seem to be caused by direct coronary vasoconstriction but rather indirectly as a result of peripheral circulatory alterations such as arterial hypertension**.** Cardiac ischemia causes subcellular changes including mitochondrial alterations and an increase in the space at the level of the intercalated disc **(Ashraf and Halverson, 1978).**

Jennings *et al.* (1969) recorded a correlation between structural abnormalities of isolated mitochondria from the ischemic dog myocardium and a deficient Krebs citric acid cycle. Both oxidative phosphorylation and anerobic glycolysis contribute to the formation of ATP in the presence of low concentrations of oxygen. Hypoxia enhances glycolytic flux in myocardial tissue **(Burlington *et al.*, 1970),** and the ability to maintain cardiac performance during hypoxia has been related to this increased glycolysis**.** Therefore, ischemia seems to alter the cardiac fiber metabolism by increasing oxygen consumption or impairing its utilization. Thus, our structural observations may be related to a certain degree to hypoxia at the mitochondrial level, which induces loss of membrane stabilization with a consequent failure of the enzymatic pattern and a mild local deficiency of ATP that affects the Na^{+}-Ca^{+2} exchanger. Moreover, the alterations of mitochondria and sarcoplasmic reticulum, involved in maintaining a low concentration of Ca^{+2} in the cytoplasm close to the intercalated discs **(Forbes and Sperelakis, 1985),** could be resposible for structural changes observed in the junctions. Under stress conditions, some labile intracellular organelles such as mitochondria bear an intense biochemical activity **(Tomanek and Banister, 1972)** that can subsequently predispose to morphological changes. According to **Ising *et al.* (1999)**, chronic Noise-induced stress accelerates the ageing of the myocardium and thus increase the risk of myocardial infarction. The involved pathomechanisms included : **1)** Increased circulating levels of stress hormones such as catecholamines, cortisol, corticosterone, growth hormone, and prolactin which are associated with peripheral vasoconstriction, coronary spasm, increased peripheral resistance, hypertension and ischaemic heart disease**).**

In the present study, we found a significant increase in the circulating levels of ACTH, corticosterone and leptin in chronic stress-exposed group compared to the control group. These results are in agreement with those of other investigators **(Chandralekha *et al.*, 2005).**

Raised serum corticosterone levels following noise stress in rats have been reported before **(Archana and Namasivayam, 2000)**. In addition, elevation of glucocorticoid levels following many types of stressors is also well known. The precise mechanism for this remains unclear, but it may be related to altered activity of the hypothalamic-pituitary-adrenal axis secondary to noise stress and may involve alterations in the secretion of corticotropin releasing hormone (CRH), ACTH and proopiomelanocortin (POMC) gene expression. The increase in glucocorticoid secretion during stress appears to be important for the appropriate defense mechanism to be put into place.

In the present investigation, significantly higher levels of corticosterone were evident in rats exposed

to chronic noise stress for 30 days indicating poor or absent adaptation of the rats to noise stress. This is in contrast to what has been observed before by others investigators where a somewhat decreased corticosterone response to noise was observed on chronically stressed rats **(Archana and Namasivayam 2000).**

Several reports indicated that, glucocorticoids are capable of stimulating the synthesis and secretion of adipocyte-derived leptin **(Slieker *et al.*, 1996),** which regulates food intake and energy expenditure. According to **Slieker *et al.* (1996)**, leptin secretion is under the influence of hormonal and neural control**.**

The results of the present study indicated a significant elevation in leptin levels after chronic exposure to noise stress. **Heiman *et al.* (1997),** in earlier study examined the influence of exogenous administration of leptin on plasma corticosterone and ACTH in animals subjected to restrainst stress. They reported that, leptin was able to inhibit the release of corticotropin releasing hormone (CRH) from the hypothalamus in vitro and also blunted the plasma ACTH and corticosterone elevation due to restraint stress. They also speculated the possibility for reduction of leptin level during acute and chronic stress and thus facilitating the responsiveness of hypothalamic-pituitary-adrenal axis. However, they failed to demonstrate any reduction in leptin levels in their study on restraint stress and thus the speculation remains unsubstantiated. In fact, the data indicated an elevation of serum leptin levels after restraint stress though the levels were not statistically significant. In another study chronic subcutaneous leptin infusions have been shown to diminish responsiveness of the hypothalamo-pituitary adrenal axis in female Rhesus monkeys **(Wilson *et al.*, 2005).** Therefore it seems that, there is a significant interplay between leptin and the hypothalamo-pituitary adrenal axis.

The results of the present study in rats subjected to chronic noise stress clearly indicated simultaneous elevation of corticosterone and leptin levels. It appears that, the inhibitory effect of leptin on corticosterone secretion was somewhat absent during noise stress in this study. The reason for the variation between our observation and those mentioned in other studies is unclear but may be due to species variation or the different nature of stress. Nevertheless, our study suggests that, one arm of the hypothalamic-pituitary-adrenal axis appears disabled during noise stress, which permits for increase corticosterone secretion during stress.

Hence, continuous exposure to noise stress may have adverse effects on some of the vital physiological functions. **(Saha *et al.*, 1996)** in which the alterations in the levels of these two hormones (corticostrone & leptin) may play a significant contributory role, these two hormones have wide ranging effects on metabolism, growth and reproduction **(Chehab, 2000 and Goumenou *et al.*, 2003).**

Altura *et al.* (1992) recorded Ca^{2+}/Mg^{2+} shifts in the vascular walls of chronically noise-stressed rats**.** Besides an increased vasoconstriction under the action of noradrenaline. This effect was confirmed in humans by measuring the increase of the total peripheral resistance (TPR) during infusion of noradrenaline. The noradrenaline induced TPR increase was reduced by Mg –injections **(Ising *et al.*, 1992).** Further analysis of the experimental results led to an interaction model between chronic stress and intracellular electrolyte shifts **(Ising *et al.*, 1992).** Chronic stress caused a loss of extracellular and intracellular Mg^{2+} and an increase of intracellular Ca^{+2} **(Gunther *et al.*, 1978).** A decrease of Mg^{2+} was correlated to an increased physiological noise sensitivity **(Ising *et al.*, 1992).**

Ising *et al.* (1999), found a positive feedback mechanism between stress-caused by noise and/or other stressors and intracellular Ca^{2+}/Mg^{2+} shifts which may increase the cardiovascular risk. Since chronic noise stress significantly increased the ratio of Ca^{2+}/Mg^{2+} and the collagen content of the interstitial space of myocardium i.e. significant increase of cardiac fibrosis [which can be interpreted as accelerated aging **(Hermann *et al.*, 1994)** and increased Ca^{2+}/Mg^{2+}ratios were found in the myocardium of ischemic heart disease deaths. **Ising *et al.* (1999)** concluded that, chronic noise stress also accelerates the aging of the heart in humans. It was observed that, infarction size and complications during two weeks after the myocardial infarction depend upon the increase of circulating levels of catecholamines and the associated decrease in serum levels of Mg^{2+} **(Jeremias *et al.*, 1996).**

Another explanation for the histopathological changes of the cardiovascular system encountered in the present study may be the changes in mitochondrial ATP and free radicals. **Ceremuzynski *et al.*, (1991)** studied the injury of pigs' myocardium after 24h of immobilization stress. Electromicroscopic examination revealed microtraumate of the myocardium. Among other biochemical alterations they observed decrease of mitochondrial ATP and increased generation of free radicals which may be components of the stress-induced myocardial injury. In earlier experiments, infusions of adrenaline into healthy dogs resulted in marked decrease in myocardial ATP**.** Catecholamines i.e. adrenaline and noradrenaline stimulate the activity of c-AMP. Furthermore, catecholamines increased via c-AMP and thromboxane A2 the influx of Ca^{2+} into the smooth muscle cells thus increasing

the risk of a coronary artery spasm. **(Ceremuzynski *et al.*, 1991).**

Reactive oxygen species (ROS), also known as free oxygen radicals, are normal by products of cellular aerobic metabolism. These unstable molecules can impair cellular lipids, proteins and nucleic acids in DNA if the balance of corresponding antioxidants is disrupted **(Van Campen *et al.*, 2002).** Oxidative stress is a state where significant imbalance between oxidants and antioxidants occurs that leads to damage, dysfunction or cellular death **(El-Sayed and Gorbunov, 2003; Mercan, 2004).** Under normal conditions, sufficient concentrations of endogenous antioxidants as well as redundant protective systems exist to protect from environmental oxidant attacks. However, repeated exposure to environmental oxidants such as air pollution, smoking, disease states or blast overpressure (blast) exposure, can result in accelerated rate of antioxidant depletion tipping the balance from sufficiency to deficiency producing oxidative stress **(El-Sayed and Gorbunove , 2003).**

Manikandan *et al.* (2005) reported that, acute as well as long term exposure to noise can produce excessive free radicals (Reactive oxygen species) such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) and causes disorders involving extra-auditory organs such as nervous, endocrine, and cardiovascular system (CVS)**.** Oxygen free radicals can attack protein, nucleic acids and lipid membranes thereby disrupting normal cellular functions and integrity **(Endo *et al.*, 2005 and Manikandan and Devi, 2005).** According to **Scarfiotti *et al.* (1997),** nervous system is relatively more susceptible to free radical damage**. Ravindran *et al.* (2005)** reported that, neurotransmitters in discrete brain regions were found to be increased during noise stress even after 15 days of exposure**.** In addition, to generating free radicals species, it also leads to increase in radical induced lipid peroxidation end products such as malondialdehyde which is an indicator of lipid peroxidation **(Derekoy *et al.*, 2001). Demirel *et al.* (2009)** investigated the effect of exposure to chronic noise stress (20 days/ 4 hours, 100 dB-A)on oxidative stress parameters in rats. They observed an elevation in malondialdheyde, an indicator of lipid peroxidation as well as nitric oxide (NO) level and glutathione peroxidase (GSH-Px) activity by noise exposure. They suggested the presence of oxidative stress which may lead to various degrees of damages in the cells, mainly via lipid peroxidation pathways, leading to coronary heart disease, hypertension, increased mortality risk, serious psychological effects, headache, anxiety and nausea. **(Abbate *et al.*, 2005 and Lenzi *et al.*, 2003)** in addition to hearing loss, sleep disturbance, impairement of performance, impairement of cognition , increased error in working and reference memory and higher incidence of cancer **(Jarup *et al.*, 2005 and Manikandan *et al.*, 2006).**

As regards the changes in serum levels of total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in adult male albino rats exposed to chronic noise stress, the present study showed a significant increase in serum levels of TC, TG, LDL and VLDL while there was a significant decrease in serum levels of HDL. These findings are supported by the work of **Mahmoud *et al.* (2008)** who found that, textile factory workers exposed to high ambient noise level generated by big machines used in the factory for more than 6 hours daily at a strength of 90 deci-Bel (db) without using ear protectors (i.e. chronic and high noise exposure levels), showed a significant increase in the level of fasting serum TC, a non significant increase in the levels of fasting serum TGS, VLDL and LDL and significant decrease in the level of fasting serum HDL.

The noxious effects of sound stress have been the subject of numerous investigations. **Zhasminova *et al.* (1991)** studied serum lipids in workers of a mining enterprise who were affected by unfavorable occupational noise and found that, the miners have greater mean levels of triglycerides and potentially atherogenic lipoprotein cholesterol. The effect of stress caused by aircraft noise was studied on 14 female and 11 male volunteers, who were of age ranging from 21-42 years and of mean age of 25 years by **Marth *et al.* (1988)** and they found an increase in the serum levels of ACTH, TC and free fatty acids. **Maschke and Hecht (2000) and Gehlot *et al.* (2002)** reported in their studies a significant increase in the levels of serum TC, TGs and lipoprotein profile in workers exposed to high noise levels. **Tappila *et al.* (2001)** conducted a study on 406 paper mill workers exposed to noise levels of 91-94 dB, 124 forest workers exposed to noise levels of 96-99 dB and 176 shipyrad workers exposed to noise levels of 95-97dB and reported also an increase in the level of serum cholesterol. In another study done by **Jovanovic and Jovanovic (2004)** on 150 workers working in relatively silent environment found that, industrial noise caused an increase in the serum levels of TC, TGs and LDL.C and decrease in the level of HDL-C.

It is now well established that, there is a relationship between high cholesterol and low HDL-C levels and increased risks of cardiovascular and circulatory diseases. Whereas enhanced total cholesterol encourages atheroscelerosis, HDL-C is more likely to prevent the development of

atheroscelerosis. Total cholesterol should therefore always be considered together with HDL-C. In terms of the current state of knowledge, an increased level of TGs also has to be seen as an independent risk factor for heart disease, especially in connection with low level HDL-C. **(Maschke and Hecht, 2000).**

Ising *et al.* (1999) in a case control study with 395 myocardial infraction (MI) patients 31-65 years and 2148 controls found a significant increase in the rate of (MI) with the loudness of work noise, and they reported that, work noise appeared to be the second greatest external risk factor in (MI) after smoking. **Elise *et al.* (2002)** in a met-analysis study to investigate the relation between occupational noise exposure and blood pressure and/or ischemic heart disease found a statistically significant increase in systolic blood pressure. In another study carried out by **Melamed and Bruhis (1996)** to explore the effect of noise attenuation on urinary cortisol excretion, fatigue and irritability among 35 healthy industrial workers chronically exposed to high ambient noise levels (> 85 dB) without using ear protectors found an increase in urinary cortisol excretion at the end of work shift.

The alteration in the levels of lipids and lipoproteins found in the present study could be due to sound stress-induced hypothalamo-pitutitary adrenal axis and sympathetic system stimulation. The stimulation of the hypothalamo–pituitary axis and sympathetic system causes an increase secretion of their corresponding hormones (Glucocoricoids and Catecholamines). These hormones increased lipolysis, gluconeogenesis in liver and inhibition of insulin secretion **(Maschke and Hecht, 2000).** The excess glucocorticoids and catrcholamines increase also stored lipid mobilization leading to the activation of the enzyme lipase in fat cells. Consequently, there will be an increase in the release of fatty acids from these cells **(Murray *et al.*, 1993 and Spreng, 1998).** The excess released fatty acids undergo, metabolism and causing an increase of hepatic cholesterol contents which down regulates the activity of hepatic LDL-C receptor resulting in an increase in the levels of serum TC, LDL-C and TGs rich lipoproteins **(Mabuchi, 1996 and Mahmoud and Ahmed, 2003).**

Leptin directly inhibits intracellular lipid concentrations by reducing fatty acid and triglycerides synthesis and concomitantly increasing lipid oxidation **(Shimabukuro *et al.*, 1997).** This effect on lipid metabolism may be mediated by an inhibitory effect of leptin on acetyl-co-A carboxylase activity, the rate limiting enzyme in fatty acid synthesis. Inhibition of this enzyme leads to a reduction in malonyl coA, an inhibitor of carnitylacyl transferase-I and mitochondrial B- oxidation so that, inhibition of acetyl co A carboxylase will thus block fatty acid synthesis and favor mitochondrial fatty acid uptake and oxidation, resulting in lower intracellular fatty acid and triglyceride concentration **(Bai *et al.*, 1996)**. **Muoio *et al.* (1997)** showed that, leptin attenuates insulin's antioxidative lipogenic actions on muscle fatty acid metabolism without inhibiting insulin-stimulated glucose disposal. Hence, they concluded that, leptin and insulin had opposite effects on lipid metabolism, with leptin favoring lipid oxidation and insulin favoring lipid storage. On the other hand, **Emilsson *et al.* (1997)** have provided evidence that leptin can directly reduce insulin secretion**.**

Leptin causes an increase in noradrenaline turnover to the brown adipose tissue **(Collins *et al.* 1996)** suggesting that leptin increases the sympathetic outflow. **Sebiha *et al.* (2005)** reported that, leptin and neuropeptide-y (NPY) may be involved in the pathogenesis of arterial hypertension. They found that, both leptin and NPY showed a significant positive correlation with both systolic and diastolic blood pressure in preeclamptic women. Both hormones are involved in the regulation of sympathetic nervous system activity **(Michael and Rascher, 1995 and Haynes *et al.*, 1998)** and vascular remodeling and water electrolyte metabolism **(Zukowska *et al.*, 1997 and Oda *et al.*, 1997).** Moreover, intracerebroventricular or chronic (over 1 week) intravenous infusion of leptin was found to increase arterial blood pressure in rats **(Dunbar *et al.*, 1997 and Shek *et al.*, 1998)**. Thus, the participation of leptin in the pathogenesis of hypertension observed in the present study seems very likely.

In view of relevant results, the present study recommends the use of ear protectors which can reduce the noise level to minimize the stress and other effects of noise. People in general and workers of plants of heavy industries (eg. iron and steel & textile…. etc.) should be educated through radio, TV, newspapers about the grave effects of noise pollution. Further studies are necessary to examine the effects of noise on other biochemical, haematological and histopathological parameters.

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Effect of Bilateral Chronic Secretory Otitis media on Childhood Autistic Rating Score (CARS) Test.

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Abstract: The purpose of this work was to investigate the impact of bilateral chronic Secretory Otitis Media (S.O.M) on childhood autistic rating score in delayed language development children.**:** This study included 140 children having bilateral chronic secretory otitis , autistic feature and Delayed language development (DLD) in addition to 40 normal hearing, autistic feature children with DLD as a control. All children under the study came to Phoniatrics clinic in the period between 2007 to 2010, complaining from delayed language development with autistic features. Children in this study were classified into 2 groups; control and study groups. The study group was subdivided into 3 subgroups according to their hearing threshold level. All children were subjected to Childhood Autistic Rating Score (CARS) and Psychometric evaluation. Obtained results revealed that Children who had high hearing threshold level, found to be had high score at CARS with as, there was increase in the severity of CARS scores increasing hearing threshold level. In conclusion bilateral chronic Secretory Otitis Media can affect the severity of CARS results together with its obligate change borderline CARS score into autistic one.

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Keywords: childhood autistic rating score, chronic conductive hearing impairment, DLD, Autism.

1. Introduction

Autism firstly is described as a complex developmental disorder characterized by severe impairment in reciprocal social interaction and communication and by a pattern of repetitive or stereotype behavior (***American Psychiatrics Association, 2000)***. Autism Spectrum Disorder (ASD) is a diverse disorder that presents symptomalogy across sensory modalities such as auditory, tactile, speech and language, cognitive, and grossmotor. Specific patterns in sensory processing deficits have been found in children with ASD and suggest the importance of investigating specific modalities/domains of sensory function ***(Lane et al., 2010).***

Autistic disorder is subsumed under the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (*DSM-IV)* category, pervasive developmental disorders (PDDs), and a group of disorders that are distinguished from other psychiatric disorders by the presence of deficits in reciprocal social behavior, variously accompanied by deficits in communication ***(Fombonne et al., 2001),*** (and/or repetitive or stereotyped behaviors (***Beglinger and Smith, 2005)***. With the exception of Rett syndrome (a rare disorder caused by a point mutation on the X chromosome) PDDs affect males more commonly than females (prevalence ratio, 4:1 ***(Fombonne et al., 2001),***

The diagnostic criteria for autism require the presence of 6 symptoms from 3 categories, impaired reciprocal social interaction (at least 2 symptoms), impaired communication, and restricted, repetitive, or stereotyped behaviors. These criteria reflect the central role of deficits in social behavior in children with (ASDs), (***Beglinger and Smith, 2005)***. One of the earliest and most important predictor of Autism is the failure to develop joint attention (***Volkmar et al., 2005)***.

Abnormal behavioral responses to auditory stimuli are frequently reported in individuals diagnosed with autism ***(Egelhoff et al., 2005)***; Abnormal behavioral responses to sound can greatly impact how a child with Autism Spectrum Disorder (ASD) performs in common tasks such as going to school, being in public with his/her family and interacting with peers ***(Dunning, 2003). Ashburner et al. (2008),*** found that tactile sensitivities and auditory filter difficulties in children with ASD were associated with inattention, hyperactivity, oppositional behavior, and academic underachievement.

Otitis Media (OM) is the most prevalent disease during childhood, next only to common cold. It is estimated that chronic OM affects 65 million to 330 million people worldwide, and 60% of them (39 million to 200 million) show clinically significant hearing impairment ***(WHO, 2004).*** There is evidence

to show that major changes in brain organization take place in the first year of life though changes continue into adolescence. SOM in the first year of life leads to negative effects on brainstem signal processing even if it has occurred only for a short duration (maximum of 3 months). In such a situation, auditory cortical structures probably show compensatory changes through central gain to offset the prolonged central conduction time ***(Sandeep and Jayaram, 2008).***

Childhood Autism Rating Scale (CARS) is a test intended for diagnosis and evaluates the severity of autism from those with other developmental delay such as mental retardation. The child is rated from 1 to 4 in each item, ranging from normal to severe and yielding a final score indicating, non autistic, mild to moderate autistic or severely autistic.

The scale is used to observe 15 items; relation to people, imitation, emotional response, body use, object use, adaptation to change, visual response, listening response, taste-smell-touch response and use, fear and nervousness, verbal communication, non verbal communication, activity level, level and consistency of intellectual response, general impression ***(Eric et al., 1988)***. This study aimed to study the impact of bilateral chronic secretory otitis in Childhood Autism Rating Scale in delayed language development children.

2. Materials and Methods:

This study was conducted on tow study groups including 140 children; 104 boys and 36 girls They had Deayed Language Development (DLD) with different autistic features reported by their parents, presented to Phoniatrics, Audiology, Neurology Unit, Pediatric Department and Otolaryngology clinics over 36 months. Their age ranged from 36 months to 54 months with means age (48.3 ± 6.7). All of them had Delayed Language Development (DLD) with history of recurrent attacks of SOM. They were 104 boys and 36 girls. Exclusion criteria include craniofacial anomalies; severe mental retardation and sensory neural hearing loss.

Control group was selected to be age and gender matched with the study group. It includes 26 boys and 14 girls their ages ranged from 36 months to 57 months with mean age (45.9 ± 5.6). Also they had Delayed Language Development (DLD) with different autistic features but they had no history of ear diseases.

All patients were subjected to:

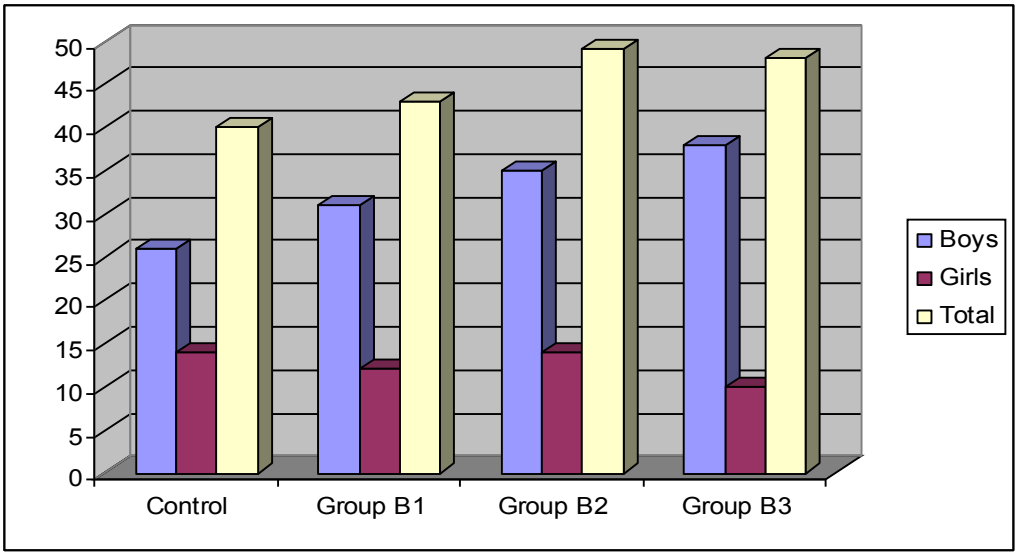
- Patient interviews (personal history, family history of consanguinity, hearing loss, DLD), pre-peri and postnatal history and developmental history
- E.N.T examination- language evaluation (eye contact, response to examiner, eye head coordination), assessment of passive and active vocabulary, Childhood Autism Rating Scale (CARS) and the degree of autistic was done as 30 serving as a cut-off for a diagnosis of autism, mild to moderate autism (30-37) and severe autism (≥ 37)
- Psychometric evaluation, using Stanford–Binet Intelligence Scales ***(Terman et al .,1960)***. & Vineland Adaptive Behavior Scales ***(Sparrow et al., 2005).***
- Neurological examination
- Audiological evaluation for threshold determination including (Immittancemetry; tympanometery & acoustic reflex), behavioral audiometry using free field test and play audiometry and auditory brain stem evoked potentials.
- Our study composed of 2 groups; control group (A), 40 normal hearing children and study group which subdivided into 3 (B1 ">15-25 dB HL", B2 ">25- 35 dB HL ", B3 >35 dB HL) subgroups according to hearing threshold level, The children were categorized into 4 groups.
- The results obtained were statistically analyzed with the help of SPSS software **(Statistical Analysis system).**

3. Results:

The results of this study revealed the incidence of delayed language development with different autistic features and bilateral conductive hearing loss affect male more than female as in table and figure (1)

Table (1): Gender and hearing level in all groups under the study.

Group / Gender	Control A	Study groups		
		B1 >15-25 dB	B2 >25-35 dB	B3 >35 dB
Boys	26	31	35	38
Girls	14	12	14	10
Total	40	43	49	48



Fig(1) Distribution of severity of hearing impairment.

In current study there were low numbers of children suffering from autism in control group than study groups and increase the number of severe cases with increase the degree of hearing impairment as showed in table and figure (2).

Table (2): Degree of autism in control and study groups.

Group / *CARS*	*Control Group*	*Group B1*	*Group B2*	*Group B3*
No-autistic < 30	36 (90%)	18 (41.9%)	15 (30.6%)	10 (20.8%
Mild to moderate autism 30 - < 37	4 (10%)	19 (44.2%)	21 (42.9%)	19 (39.5%)
Severe autism >37	0 (0%)	6 (13.9%)	13 (26.5%)	19 (39.6%)
Total	40	43	49	48

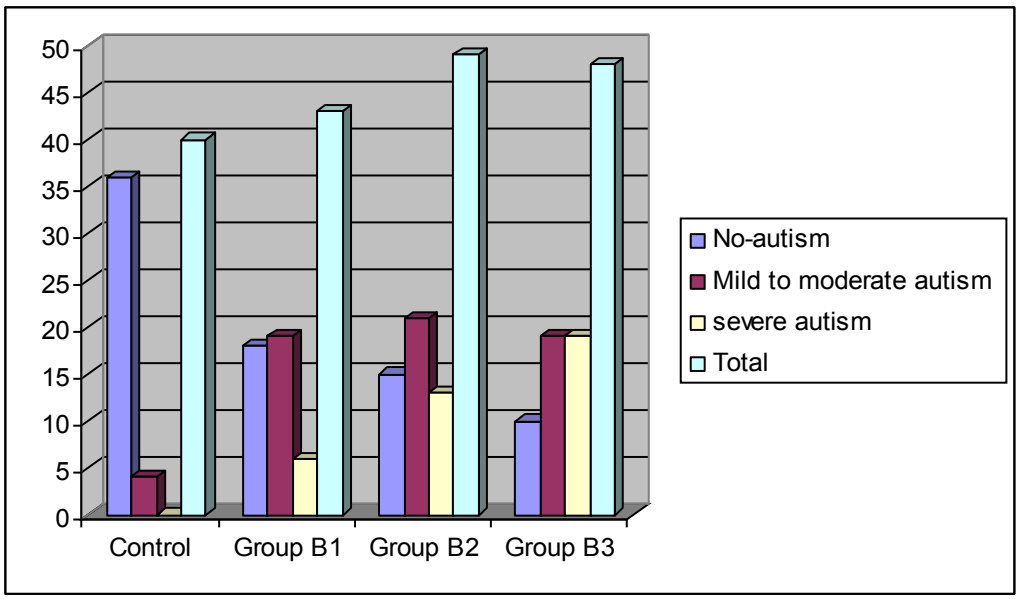


Fig. (2): The severity of autism in the different study groups.

Our results showed highly statistical significance difference between control group (A) and study groups (B1), (B2) & (B3) in Imitation , Emotional response, Object Use , Visual response and listening response parameters.

Also the results revealed highly significance difference in body use parameter in group (B1) & Adaptation to Change parameter in groups (B2) & (B3) and Taste, Smell, and Touch response and Fears parameters in group (B2) compared with the control group and level of intelligence in group (B3).

In current study there were significance difference in Adaptation to change, in group (B1) & Taste, Smell, and Touch response, parameters in group (B3) & verbal communication and Activity level parameters in groups (B2) & (B3) and General impression parameter. in group (B3) Finally there are high significance differences between control group and study group (B1) & (B2) and (B3) in total score of CARS.

4. Discussion:

The question of etiology in autism remains elusive primarily due to the fact that autism does not results from a single dysfunction, but is multi-faceted in nature. Investigations into etiology have ranged from identifying abnormalities in the genome to describing structural/functional brain abnormalities ***(Bomba and Pan,. 2004)***[.] abnormal behavioral responses to sound have been assumed to reflect a deficit in the auditory processing abilities of individuals diagnosed with autism Specific atypical behaviors were due to an overall hyper-responsiveness in the Autism Spectrum Disorder (ASD) group that resulted in hypersensitivity to sound, sensory defensiveness to tactile stimulation, sensory modulation dysfunction, aversion, and/or lack of habituation to sensory stimuli ***(Baranek et al., 2006).*** The predisposing factors for Auditory processing disorders APD include otitis media with effusion (sensory deprivation secondary to a peripheral disorder), neuro-maturational delay, and neurological insults to the central auditory system ***(DeBonis and Moncrieff, 2008).***

Secretory Otitis media (SOM) leads to significant reduction in the hearing sensitivity. The reduced auditory input, if in the early years of life when the auditory neural system is still maturing, may adversely influence the structural as well. It can also be said that if auditory processing is affected at the brainstem level because of early onset OM (reduced auditory input in the crucial periods of neural development), then, it may be said that auditory processing is also affected at the cortical level because it receives distorted input from the brainstem ***(Sandeep and Jayaram, 2008).***

. In present study the control group revealed 90% had normal CARS score (non autistic) and only 10%, 4 children had mild to moderate results while in study group only 30.7%, 43 patients out of 140, had non autistic scores and the remaining 69.3% had different degrees of autism from mild to severe degree. We attribute high incidence of autism in our study due to the impact of bilateral chronic Secretory Otitis media in auditory processing in brainstem and cortical level. The central auditory processing disorder is complicated and involved not only difficulty understanding what one hears or perceives auditorily, but also how one applies that information to different cognitive, social, and emotional tasks. Children with other leering difficulties, such as reading disorders, Attention Deficit Hyperactivity Disorders (ADHD) and mild to minimal hearing loss (loss in range of 20-30 dB) often experience similar lack of insight ***(ASHA, 1996; Keith, 1999).*** In agree with current study ***Smith and Jones, (2001)*** also reported that early-onset hearing impairment can seriously impede language development. Language can not develop normally without adequate speech stimulation so, conductive hearing loss can affect, listening response, relation to people, imitation, verbal communication, level of intellectual response, and also general impression, all these items involved can affect the CARS score. Even monaural occlusion during infancy can affect the organization of "auditory space maps" in superior colliculus, which lead to compensatory shifts in the auditory spatial tuning of superior colliculus neurons (***Knudsen1 et al., 1984; Knudsen et al., 1994; Schnupp et al., 1998).***

Chronic conductive hearing impairment can affect the central auditory processing function, ***Joseph et al (2003)*** found decrease ability to recognize speech in the presence of a masker in children with conductive hearing impairment. This was noticed before by ***Jerger et al (1983) &Gravel &Wallace, (1992)***[.] who reported poor recognition for words in sentences masked by competing talker in children with otitis media. The researches of ***Groenen et al (1996)*** **and** ***Petinou*** **et al** ***(2001)*** showed the association of poor perception of particular features in children with otitis media. So bilateral chronic SOM can affect the listening response and verbal and non-verbal communication in the CARS it may lead to false result for CARS score.

Miguel et al (2002)[)] studied ABR in autistic children ,they reported abnormal morphology in the response, there were prominence in amplitude of peak I over peak III and peak V in all examined

children .This may explain the hyperacusis and abnormal reaction to sounds often seen in autistic children and also it can show the delayed development in auditory pathway , because very early in life ,only wave I ,III,V are evident ,with wave I having much greater amplitude than that of wave V. Over time the relationship changes, with wave V becoming much more prominent than other waves in the first year of live.

Moreover we noticed that increasing the degree of autism with increasing the degree of hearing loss. So, hearing loss can increase the severity of autism or may be change the results of CARS, from non-autistic to autistic In conclusion Bilateral chronic SOM have an impact on CARS results. This is impact may be explained by the effect of bilateral chronic conductive hearing impairment in central auditory processing and may lead to central auditory processing dysfunction which affects items in CARS like listening response, verbal, non-verbal communication and also the level of activity. This CARS items were frequently affected in autistic and non-autistic children suffering from chronic hearing loss rather than control group. Also these CARS items are severely affected in children complain from bilateral mild chronic minimal hearing impairment.

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12/31/11

Effect of Hypertonic Saline on Adequacy of Resuscitation, Progression of Inflammation and Outcome of Critically Ill Septic Patients.

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Abstract: Background: Many studies discussed the use of hypertonic solutions (HTS) for treatment of septic shock; however, they do not refer to the possible prophylactic benefit of early use of such solutions (before development of severe sepsis or septic shock). **Aim of the work:** to evaluate the effect of early administration of hypertonic saline on adequacy of resuscitation, progression of inflammation and outcome of critically ill septic patients. **Patients and methods:** Thirty patients with sepsis were enrolled in our prospective study in El-helal hospital. Patients were divided into two groups: The study group(group A) (15 patients) with sepsis received 4ml/kg b.wt 7.5% hypertonic saline over 15 minutes plus standard medical therapy, compared to the control group(group B) (15 patients) with sepsis received standard medical therapy alone. Both groups were monitored as regard to hemodynamics (MAP, HR, UOP, CVP), respiratory parameters (R.R, ABG, CVSO2) and laboratory parameters (WBCs, CRP, TNF-α). **Results:** group A showed significant reduction in heart rate(P=0.049) and respiratory rate(P=0.001), occurrence of metabolic acidosis (p= 0.019), inflammatory markers (WBCs, CRP) (P=0.019, 0.034, respectively) , TNFα (p= 0.001), the rate of occurrence of septic shock (p = 0.006), need for mechanical ventilation (p = 0.006), the mean ICU length of stay (p = 0.001) , ICU mortality (p =0.032) and increase in CVSO2 (P = 0.034) compared to group B. **Conclusion:** HTS 7.5% has no inferior results on critically ill septic patients, but it has superior results in comparison to other fluids as it decrease inflammatory markers (WBCs, CRP), inflammatory mediator TNF-α and improve secondary outcome (occurrence of septic shock, need for mechanical ventilation, ICU mortality) with significant reduction of the mean ICU length of stay when given in early sepsis.

[Helmy Elgawaby; Mohamed Shehata; Sherif Sabri and Mohamed Soliman **Effect of Hypertonic Saline on Adequacy of Resuscitation, Progression of Inflammation and Outcome of Critically III Septic Patients.**] Life Science Journal 2011; 8(4):1148-1153]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 140

Keywords: Hypertonic saline, inflammation, critically- ill septic patients.

Abbreviations: MAP (mean arterial pressure), HR (heart rate), UOP (urine output), CVP (central venous pressure), R.R (respiratory rate), ABG (arterial blood gases, CVSO2 (central venous oxygen saturation),WBCs (white blood cell count), CRP (C reactive protein), TNF-α (tumor necrosis factor α).

1. Introduction:

Widespread activation of cells responsive to pathogens results in uncontrolled systemic inflammation. The release of inflammatory mediators induces vascular dilatation and increase in permeability with leakage of plasma components, extravasations and activation of leucocytes to tissues and organs [(1)]. The cytokines tissue necrosis factor-α (TNF-α) and Interleukin (IL)-1 are released first and initiate several cascades. TNF-α and IL-1 have been shown to be released in large quantities within 1 hour of an insult and have both local and systemic effects (2).

The infusion of several liters of isotonic fluids is associated with the adverse effects of extravasation into the interstial space. In sepsis, in particular, this may result in peripheral and/or pulmonary edema (3). Several studies have been performed that used small volume resuscitation which is defined as a rapid infusion of hypertonic solution (NaCl 7.5%) at a dose of 2-4 ml/kg into a peripheral vein and have some demonstrated promising beneficial effects (4). Most of the studies found that HTS infusion caused a rapid and significant increase in oxygen delivery, elevated cardiac output, increased oxygen extraction and redistribution of fluids from the perivascular to the intravascular space (5).

Improvement in myocardial contractility by HTS may be related to direct hyperosmolar effect, restoring transmembrane potentials or decreasing myocardial edema (6).

A large number of very interesting experiments highlighted that HTS resuscitation may decrease susceptibility to post-traumatic sepsis; modulate trauma and sepsis-induced immune dysfunction, inflammatory response and apoptosis (7) .

During small volume resuscitation by HTS, the intracellular fluid is primarily mobilized from microvascular endothelial cells and erythrocytes; this produces a reduction in hydraulic resistance and an

improvement in tissue perfusion(8). HTS may also improve immune function with control of neutrophils migration; reduce pro-inflammatory mediators and free radicals with increased antibacterial activity and decreased susceptibility to bacterial toxins (**9**).

Data have been reported which indicate that HTS augments interleukin-10 induction by lipopolysaccharide in the bacterial cell-wall and reduces tumor necrosis factor α level. These actions may explain the lesser degree of injury following HTS administration. However, because HTS reduces but does not completely abrogate proinflammatory pathways, there is an adequate balance between proinflammatory and anti inflammatory cytokines, thus maintaining the ability to fight bacteria efficiently (10).

Aim of the work:

To evaluate the effect of early administration of hypertonic saline on adequacy of resuscitation, progression of inflammation and outcome of critically ill septic patients.

Patients' Population and data collection:

Thirty patients with sepsis were prospectively enrolled in our study, which performed in ICU of El-helal hospital (Cairo, Egypt).

A-Inclusion criteria:

-Age between 30 and 50 years old, both sexes.
-Evidence of SIRS: 3 or more of the following:
-Fever of more than 38 ^{o}C or less than 36 ^{o}C.
-Heart rate of more than 90 bpm.
-Respiratory rate of more than 20 breaths per minute or a $PaCo_2$ level of less than 32 mm Hg.
-Abnormal WBCs count (>12,000/μL or <4,000/μL or >10% bands)
-Presence of documented infection (11).

B. Exclusion criteria:

-Patient with septic shock or end-organ dysfunction (altered organ function such that normal physiology cannot be maintained without support)
-Patients on circulatory support or mechanical ventilation.
-Patients with pre existing severe organ system dysfunction.
-Patients with poorly controlled blood sugar or uncontrolled blood pressure.

Our patients were divided into two groups;

(Group A)Study group (n = 15): These patients will be administered 4 mL / kg of hypertonic saline 7.5 % over 15 minutes every 24 hours. Maintenance of the same hemodynamic parameters will be achieved with isotonic fluids when needed, in addition to the same lines of treatment of Control group.

Group B (Control group, n = 15):

These patients will be managed with isotonic solution (Ringer acetate, or normal saline) to maintain the following hemodynamic values (central venous pressure 8 – 12 mm Hg, mean arterial pressure ≥ 65 mm Hg, urine output > 0.5 mL / kg / h) in addition to the other lines of treatment of septic patients in ICU.

Both groups are subjected to:

I- Full history from patient relatives and full clinical examination.

II- Hemodynamic monitoring (MAP, HR, UOP, CVP):

Will be measured at the start of the study (base line) then every 2 hours for 48 hours.

III-Respiratory parameters monitoring (RR, ABG, CVSO2):

Will be measured at the start, then every 24 hours for 48 hours.

IV-Laboratory parameters (CRP, WBCS, TNF-α):

Will be measured before resuscitation and after 48 hours.

All patients were followed up during period of hospitalization from the date of ICU admission for.

Need for mechanical ventilation.
Occurrence of septic shock and need for circulatory support.
Length of stay in ICU.
Death.

2. Method of statistical analysis:

The sample was selected by simple random sample so all members of the population have an equal chance of being selected as part of the sample. Every patient with sepsis admitted to the hospital and matching the inclusion and exclusion criteria. Data collection using textular, tabular and graphical method. Our primary data is master tables and our secondary data is the statistical results. Data was statistically analyzed using SPSS (statistical package for social science) program version 13 for windows and Epi info program version for all the analysis a p value < 0.05 was considered statistically significant Data are shown as mean, range or value and 95% confidence interval (95% CI) and frequency and percent.

3. Results:

Patient characteristics on admission:

In randomised controlled trial, 30 patients, 16 (53.3%) males and 14 (46.7%) females with sepsis were enrolled in the study with mean age 41.8 ± 3.7 years.

Patient divided randomly into two groups:

(Group A) Study group: 15 patients with sepsis received hypertonic saline plus standard medical therapy (SMT),

(Group B) Control group: 15 patients with sepsis received standard medical therapy (SMT) alone.

A. Comparison of baseline characteristics between both groups according to demographic data (age, gender), vital signs (MAP, HR, R.R), hemodynamic monitoring parameters (CVP, UOP), ABG, CVSO2 and laboratory parameters (CRP, WBCs, TNF-α):

There were no statistically significant differences in baseline characteristics among the two groups of patients regarding demographic data (age, gender) and patient characteristics before treatment as regard to (vital signs, hemodynamic monitoring parameters(CVP, UOP), ABG and Laboratory parameters), **(Table1).**

Table (1): Comparison of baseline characteristics on admission between both groups

Characteristic	HTS(study group)=15	SMT (control group)=15	*P*-value
Age(yrs)	41.8 ± 4.1	41.7 ± 3.4	0. 9
Male gender%	53% (8)	53% (8)	1. 0
MAP	88.3 ± 3.6	86.3 ± 3.9	0.162
HR(beats/min)	113.3 ± 3.6	113 ± 4.1	0.816
RR(breaths/min)	26.1 ± 3.2	25.4 ± 2.6	0.500
CVP	7.45 ± 0.01	7.45 ± 0.009	0.377
UOP	72.4 ± 10.2	72.3 ± 11.2	0.980
PH	27.8 ± 2.4	27.1 ± 2.4	0.465
HCO_3(mmole/L)	21.4 ± 1.2	21.1 ± 1.5	0.492
PaO_2(mmHg)	9.9 ± 0.4	9.9 ± 0.4	1.000
$PaCO_2$(mmHg)	106.7 ± 14.8	98.3 ± 17.6	0.172
SPO_2	94 ± 2.4	94.2 ± 2.2	0.787
$CVSO_2$	65.2 ± 2.4	65.3 ± 2.07	0.899
CRP	84.2 ± 11.2	82.8 ± 10.4	0.726
WBCs	21.2 ± 4.8	22.8 ± 5.0	0.401
TNF-α	289 ± 207	172 ± 89	0.06

(N.B).Reference range of TNF-α (10-50 pg/ml), ideal range (<8.1 pg/ml)

B. Primary outcome:

♦ **Mean changes in patient characteristics 48 hours after treatment:**

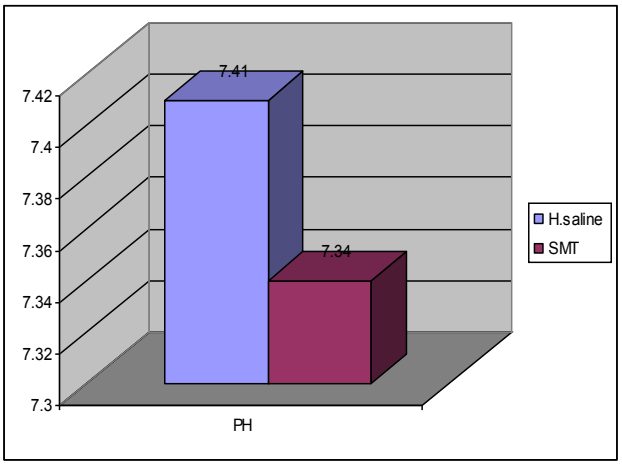
Comparison between two groups regarding mean changes in vital signs, hemodynamic monitoring parameters (CVP, UOP), ABG, CVSO2 and laboratory parameters (CRP, WBCs, TNF-α) 48h after treatment:

Showed significant reduction in heart rate (HR) , respiratory rate (RR), significant changes in PH , $PaCO_2$ and HCO_3 , significant changes of $SCVO2$ and significant reduction of CRP, $WBCs$ and $TNF\alpha$ in **(group A)** which receive hypertonic saline plus standard medical treatment compared with **(group B)** which receive standard medical therapy alone(Table 2; Figs. 1-4).

Table (2) Comparison between two groups regarding mean changes in vital signs, hemodynamic monitoring parameters (CVP, UOP), ABG, $CVSO_2$ and laboratory parameters (CRP, WBCs, TNF-α) 48hrs after treatment.

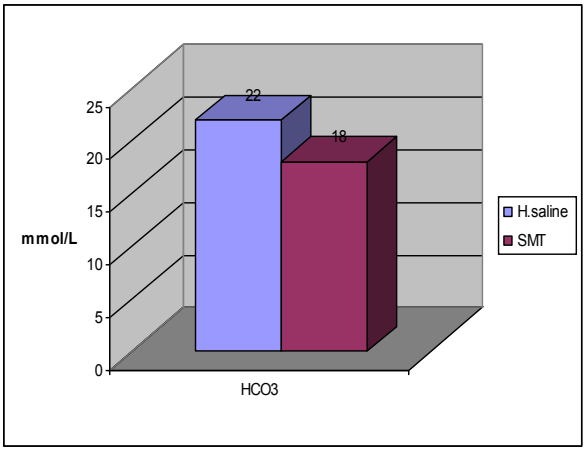
Characteristic	HTS(study group)=15	SMT (control group)=15	*P*-value
MAP	89.67 ± 3.52	87 ± 4.14	0.068
HR(beats/min)	91 ± 9	100 ± 14	0.049*
RR(breaths/min)	18 ± 1	22 ± 2	0.001*
CVP	9.9 ± 0.4	10 ± 0.3	0.667
UOP	105 ± 14	101 ± 17	0.571
PH	7.41 ± 0.02	7.34 ± 0.10	0.019*
HCO_3(mmole/L)	22.5 ± 1.5	18.7 ± 4.4	0.006*
PaO_2(mmHg)	75.8 ± 6.6	75.8 ± 6.6	0.477
$PaCO_2$(mmHg)	35.6 ± 3.0	29.6 ± 4.6	0.001*
SPO_2	95 ± 1	94 ± 2	0.415
$CVSO_2$	66.1 ± 1.5	62.7 ± 5.4	0.034*
CRP	59.6 ± 8.1	71.5 ± 18.4	0.034*
WBCs	14.6 ± 4.2	19.5 ± 6.2	0.019*
TNF-α	12.4 ± 9.8	171.7 ± 89.4	0.001*

Significant *p*- value<0.05.



P value 0.01

Figure (1): PH 48hrs after treatment



p value 0.006

Figure (2): HCO_3 48hrs after treatment

C. Secondary outcome:

There was statistically significant reduction in the rate of occurrence of septic shock, need for mechanical ventilation, ICU mortality and the mean ICU length of stay in group A(study group) patients compared to group B (control group) , (Table 3; Fig. 5).

Table (3): Secondary outcome

Characteristic	HTS(study)**N = 15**	SMT(control)**N = 15**	P **- value**
Septic Shock No %	0 (0%)	6 (40%)	0.006*
Mechanical ventilation No %	0 (0%)	6 (40%)	0.006*
ICU **mortality No %**	0 (0%)	4 (26%)	0.032*
Mean ICU **length of stay (days)**	9.8 ± 3.8	17.2 ± 5.0	0.001*

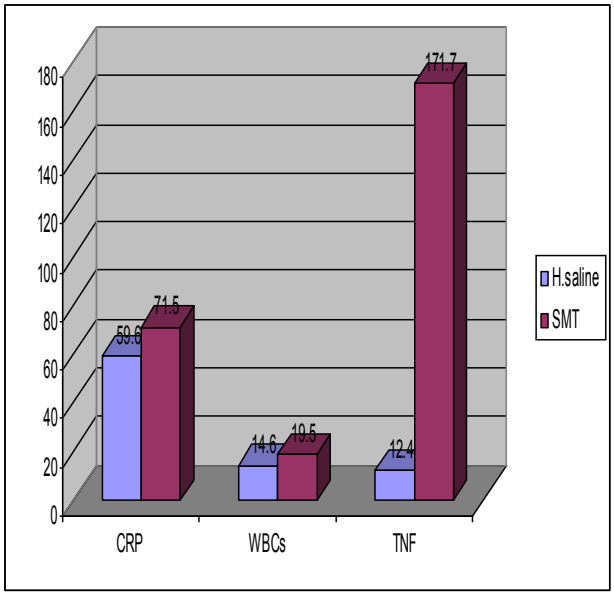


Figure (4): Laboratory data 48hrs after treatment

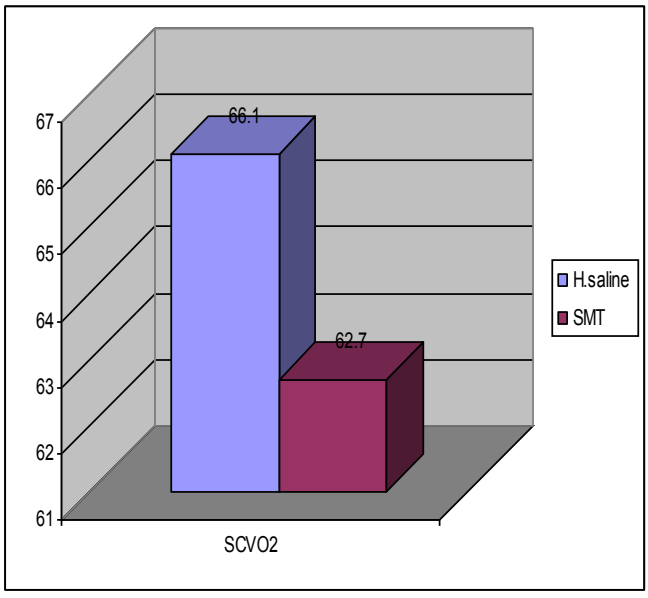


Figure (3): SCVO2 48hrs after treatment

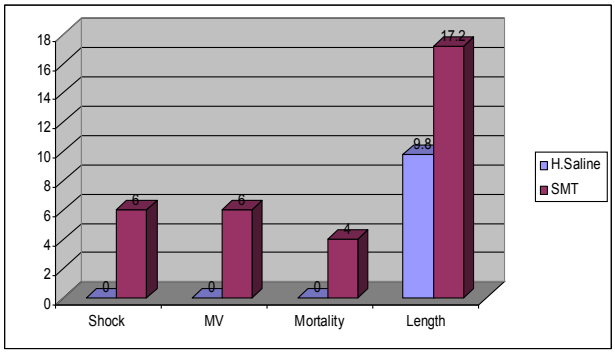


Figure (5): Secondary outcome and complication

4. Discussion:

In their review, Oliveira and coworkers discussed the use of hypertonic solutions for treatment of septic shock; however, they do not refer to the possible prophylactic benefit of early use of these solutions (before development of severe sepsis or septic shock) (8).

In our study we gave HTS 7.5% early in sepsis before development of multiple organ dysfunction syndrome or septic shock unlike other studies which were always looking for the effect of HTS 7.5% on septic patients after development of multiple organ dysfunction syndrome or septic shock, and this explains the differences in results according to hemodynamics , respiratory parameters, laboratory parameters (WBCs, CRP, TNFα) and secondary outcome (ICU mortality or ICU length of stay) because of delayed use of HTS 7.5% in other studies.

Our study supported that HTS 7.5% when given in early sepsis avoid progression of inflammation (from sepsis to septic shock), and improve outcome of critically ill septic patient. This was proved in our study which showed significant reduction in heart rate (20% versus 11%) and respiratory rate (31% versus 12%) (P=0.049, 0.001, respectively) , significant reduction in occurrence of metabolic acidosis((p=0.019), significant reduction of WBCs (29% versus 17%; P=0.019), CRP(29% versus 13%; P=0.034) and TNFα (95% versus 0% reduction; P=0.001) and significant reduction in the rate of occurrence of septic shock(zero versus 40% septic shock; P=0.006), need for mechanical ventilation(zero versus 40% mechanical ventilation; p = 0.006), ICU mortality(zero versus 26% mortality; p =0.032) and the mean ICU length of stay(10 days versus 17; p = 0.001) in study group(group A) which receive hypertonic saline plus standard medical treatment compared to control group (group B) which receive standard medical therapy alone. These results explained by **early improvement in hemodynamic status** (6), cardiac contractility may also improve (12), **immune modulating effect** (13) and reducing tumor necrosis α factor level (10).

Since, the prediction of outcome is one of the major problems associated with critical illness. Investigations have been performed on the potential use of TNF-α and other proinflammatory mediators as prognostic indicators for severity of disease and for mortality in previously healthy immunocompetent patients with well-documented sepsis or severe sepsis and was found that non survival from sepsis or septic shock had been mainly associated with higher levels and persistant high serum TNF-α, Also patient with an early haemodynamic deterioration associated with higher levels of TNF-α (14). This come in agree with our study which showed that septic shock, need for mechanical ventilation, ICU mortality and prolonged ICU length of stay associated with higher levels and persistent high TNF-α which observed in group B(control group), not received HTS .

Also in a study performed by **Chih-Chin *et al.*, 2008** who investigated the effect of (HTS 7.5% 4ml/kg) on the hemodynamics (MAP, HR) and 18 hours mortality results from the development of multiple organ dysfunction syndrome) on 128 rats having sepsis induced by cecal ligation and puncture, and the animals observed another 18 hours. The result was that hypertonic saline prevented circulatory failure, alleviated multiple organ dysfunction syndrome, and decreased the mortality rate (15). This comes in agree with our study which showed significant reduction in the rate of occurrence of septic shock, *ICU* mortality (P=0.006, 0.034, respectively).

In a study performed by **Gurfinkel et al., 2003** who compared the effect of hypertonic saline (HTS 5ml/kg) and isotonic saline (IS) solutions on tumor necrosis factor-alpha and survival benefit on Wister rats having endotoxic shock. The result was that, patients treatment with HTS have decrease in tumor necrosis factor-alpha (p < 0.0001) and lower mortality with (p < 0.01) (16). This comes in agree with our study which showed that the early use of HTS significantly decreases TNF-α (p = 0.001) and decreases ICU mortality with (p =0.032).

While **Maciel et al., 1998,** who investigated the effect of HTS 7.5% on MAP, and mortality in patients with septic shock on 14 patients, and shows that no significant difference in MAP between the two groups and there was no survival benefit of HTS (5). This comes in agree with our study which showed that there was no statistically significant difference in patient MAP among the two groups (p 0.068), but this was not in agreement with our study which showed that HTS 7.5% decrease mortality due to sepsis with significant p value 0.032.This is can be explained by delayed use of HTS in this study.

Conclusion and recommendation:

HTS 7.5% has no inferior results on critically ill septic patients, but it has superior results in comparison to other fluids as it decrease inflammatory markers (WBCs, CRP), inflammatory mediator TNF-α and improve secondary outcome (occurrence of septic shock, need for mechanical ventilation, ICU mortality) with significant reduction of the mean ICU length of stay when given in early sepsis. So we recommend further studies to evaluate prophylactic benefit of HTS when used early (before development of severe sepsis or septic shock).

Limitations:

Small number of patients of both study and control group.

Cardiac output and ejection fraction are not used as comparing parameters.

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3/2/2012

Assessment of the Role of Interleukin-18 in diagnosis of Hepatocellular Carcinoma related to Hepatitis C Virus infection

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Abstract: Background: Hepatocellular carcinoma (HCC) accounts for 90% of primary liver neoplasms. Representing one of the most common cancers and is responsible for up to 1 million deaths annually worldwide. Egypt has the highest prevalence of Hepatitis C Virus (HCV) worldwide and has rising rates of Hepatocellular carcinoma (HCC). The prognosis of most patients is unsatisfactory due to rapid clinical deterioration after the initial diagnosis. Therefore, it is very important to detect HCC and the recurrence at its earlier period. Alpha Feto protein (AFP) has been the most widely used plasma marker for diagnosis, surveillance and as a prognostic indicator of HCC patients' survival. Several studies indicated that high plasma levels of AFP are related to poor prognosis, as well as histological grades of malignancy. However, it has been recognized that AFP has a low sensitivity in detection of HCC, and that AFP level often increases in the absence of HCC. Thus the identification of novel biochemical markers for HCC remains an important goal for many laboratories around the world. Interleukin 18 (IL-18) plays a critical role in the host defense against intracellular microbe's infection and also it induces autoimmune diseases and propagating inflammatory process also it was found that, IL-18 could play a key role in the pathogenesis of HCC. **Methods:** This study was conducted on a total number of 120 patients admitted to Hepatology and Gastroenterology Department in Faculty of medicines, Ain Shams University. The patients of this study were subdivided as follows. Group I: included 20 normal healthy subjects (as controls). Group II: included 100 patients with Hepatocellular carcinoma confirmed by pathology, cytology, imaging (computer tomography and ultrasound) and serum α -fetoprotein. **Results:** The mean level of IL-18 was significantly higher in HCC patients (238.69±145.5 pg/ml) compared to the controls (52.8±13.32 pg/ml), P <0.001). There was significant positive correlation between IL18 and Tumor size. **Conclusion:** IL-18 could be used as an additional non invasive marker for monitoring the degree of disease severity in Hepatocellular carcinoma.
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Key words: HCC, HCV, AFP, IL-18.

1. Introduction

Hepatocellular carcinoma (HCC, also called malignant hepatoma) is a primary malignancy of the hepatocyte, the major cell type in the liver **(Motola-kuba *et al.*, 2006)** generally leading to death within 6-20 months. The disease is often clinically silent until it is well advanced or tumor diameter exceeds to 10 cm. Hepatocellular carcinoma frequently arises in the setting of cirrhosis, appearing 20-30 years following the initial insult to the liver. However, 25% of patients have no history or risk factor for the development of cirrhosis. HCC with more than 250000 new cases annually and a 5year survival rate of less than 5% is the five leading causes of cancer death in the word (**Dong *et al.*, 2009**). Egypt has the highest prevalence of HCV worldwide and has rising rates of hepatocellular carcinoma (HCC). Egypt's unique nature of liver disease presents questions regarding the distribution of HBV and HCV in the etiology of HCC. **Lehman and Wilson (2009**) reported prevalence for HBV and HCV to be 6.7% and 13.9% among healthy populations, and 25.9% and 78.5% among HCC cases. Detection and characterization of all hepatic focal lesions are critical especially in patients with liver cirrhosis, as those patients are at high risk to develop hepatocellular carcinoma (**Zhou *et al.*, 2006).** Serum α-fetoprotein (AFP) is the only marker that has been widely used for screening and diagnosis of HCCs. However, development of false-negative or false-positive rates with (AFP) was as high as 30%-40% for patients with small hepatocellular carcinomas (**Wei *et al.*, 2006**). Liver biopsy is the traditional gold standard method to establish the diagnosis and to determine the extent of inflammatory changes and the extent of fibrosis and cirrhosis. However this procedure has many disadvantages, it is invasive, coasty and difficult to standardize (**Bonny *et al.*, 2003).** Patients with chronic HCV are often anxious regarding undergoing a liver biopsy. Biopsy results show significant variability up to 40% for fibrosis diagnosis which can lead to a

wrong diagnosis, indeed the result depends on the representatively of the punctured sample (**Andriulli *et al.*, 2001).** That is why there has been increasing interest in noninvasive assessment of liver fibrosis by the use of surrogate serum markers (**Saadeh *et al.*, 2001).** Besides these features, a number of biological markers including cytokines and growth factors have been demonstrated to be increased in the sera of patients with HCC and may be associated with a poor prognosis. Interleukin-18 (IL-18), originally known as interferon-γ (IFN-γ)-inducing factor (IGIF), is a cytokine that shares structural and functional properties with interleukin-1(IL-1). This cytokine is mainly produced by activated macrophages, but may also be expressed by Kupffer cells, T cells, B cells, keratinocytes, astrocytes, and osteoblast **(Tangkijvanich *et al.*, 2007).** IL-18 has multiple biological activities via its capacity to stimulate innate immunity and both Th1 and Th2 mediated response. It also exerts anti-tumor effects that are mediated by enhancement of NK cell activity, reduction of tumorigenesis, induction of apoptosis and inhibition of angiogenesis in tumor cells **(Tangkijvanich *et al.*, 2007). Chiac *et al.*, 2002** approved that IL-18 play a key role in the pathogenesis of HCC and its levels can be utilized as a possible marker in the diagnosis of HCCs and so, this study was aiming to evaluate IL-18 as non invasive marker of the severity of liver damage in HCC patients and comparing these results by the results of AFP.

2. Study Population

This study was conducted on a total number of 120 patients admitted to Hepatology and Gastroenterology Department in Faculty of Medicines, Ain Shams University. The patients of this study were subdivided as follows. Group I: included 20 normal healthy subjects (as controls), age and sex matched, Group II: included 100 patients with Hepatocellular carcinoma (HCC). All HCC patients were newly diagnosed and none had received any form of anti-cancer therapy before collection of blood samples for biochemical analysis. Diagnosis of HCC was confirmed by pathology, cytology, imaging (computer tomography and ultrasound), and serum a-fetoprotein (AFP).

Blood samples

Ten ml of venous blood were withdrawn from each patient in dry sterile vacutainers. After centrifugation, the serum was tested for: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), G-glutamyltranspeptidase (GGT), total bilirubin, direct bilirubin, albumin and glucose concentrations were assayed using Beckman CX4 chemistry analyzer (USA, supplied by the Eastern Co. For Eng. & Trade-Giza, Egypt). AFP and viral markers were measured using Abbott, Axyam (USA, Supplied by al kamal company Cairo, Egypt). II-18, test kit was purchased from Medical & Biological Laboratories CO., LTD Nogoya, Japan. Level of II-18 (pg/ml) was calculated by interpolation from a reference curve generated in the same assay with reference standards of known concentrations. This assay was performed in duplicate according to the manufacturer's instructions.

Statistical analyses

Statistical analysis was performed using the statistical package for social sciences (SPSS, USA). Data are expressed as means ± standard error. The chi-square test was used for the comparisons of proportions. A $p < 0.05$ was considered significant.

3. Results

This study was conducted on 100 patients and 20 healthy volunteers as controls. The Group (1), which included 20 subjects with male to female ratio 9/11, and Group (2), which included 100 patients with Hepatocellular carcinoma with male to female ratio 60/40, was illustrated in table (1). The mean age of control groups was 33.1±8.16 years versus 58.8±9.77 years in HCC group. There was a significant increase in serum levels of Albumin, Total Bilirubin, Direct Bilirubin, ALT, AST, GGT, INR, Creatinine and Glucose were detected in HCC patients when compared to normal healthy controls, while Albumin was lower in HCC group when compared with group I ($P < 0.001$; Table 3). IL18 and AFP are significantly higher in patients with HCC (G2) than in healthy normal subjects (G1), ($P <0.001$).

Table (2) was revealed, a significant positive correlation between IL18 and Tumor size), while there is no significant correlation between AFP and tumor size. By comparing receiver-operating characteristic (ROC) curves for both IL18 and AFP, we found that the areas under the curves were 0.704 and 0.296, respectively and IL18 curve is closer to the upper left corner than that for AFP as shown in Figures (1&2) which means the higher the overall accuracy for IL18 over AFP. Also by comparing the validity and reliability of Il -18 compared to α-Fetoprotein regarding the exclude of control from our calculations, serum AFP performs moderately well as a biomarker of HCC but it showed lower sensitivity 71% compared to serum IL18 (100%).

Table (1): Comparison between all groups according to gender

		Groups		
		Group I	Group II	Total
sex	Female	11	37	48
		55.0%	37.0%	40%
	Male	9	63	72
		45.0%	63.0%	60%
Total count		20	100	120

Table (2): Correlation between IL- 18, AFP & Tumor size

		Tumor size
IL-18	Pearson Correlation	0.583**
	Sig.(2-tailed)	0.000
	N	100
AFP	Pearson Correlation	0.101
	Sig.(2-tailed)	0.318
	N	100

**Correlation is significant at the 0.01 level (2-tailed).

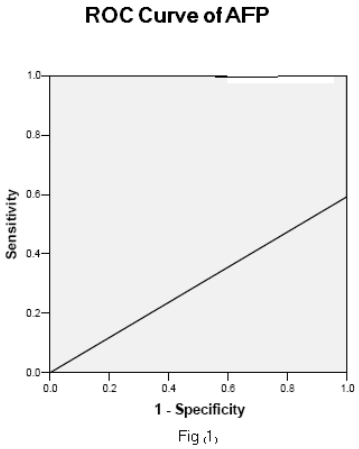

Fig (1)

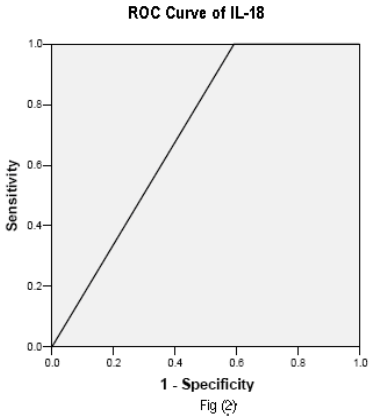

Fig (2)

Table (3): Clinical and biochemical characteristics among studied groups.

Variables	Normal control N=20	HCC N=100	*P*-value (Control vs HCC)
	Mean±SD	Mean±SD	
Age	33.1±8.16	58.8±9.77	0.000 (Significant)
Glucose(mg/dl)	101.8±13.9	211.0±150.2	0.002(Significant)
ALT (IU/L)	30±6	65.7±18.5	0.000 (significant)
AST (IU/L)	32±9	154.8±67.2	0.000 (significant)
T. Bil (mg/dl)	.74±.20	2.71±1.0	0.000 (significant)
D. Bil (mg/dl)	.5±.067	.77±.409	0.000 (significant)
Albumin (g/dl)	3.85±.21	2.74±.53	0.000 (significant)
INR (%)	1.0±.07	1.27±.17	0.000 (significant)
GGT (IU/L)	34.2±9.5	218.7±122.7	0.000 (significant)
TG (mg/dl)	156±30.6	195.1±30.9	0.000 (significant)
Cholesterol(mg/dl)	161.85±20.63	201.69±40.76	.000 (significant)
AFP(ng /ml)	5.86±2.02	316.65±291.60	0.000 (significant)
IL-18(ng/ml)	2.8±13.32	238.69±145.5	0000 (significant)

ALT, Alanine aminotransferase, AST, Aspartate aminotransferase, INR, International normalization ratio of prothrombin time, GGT, Gamma Glutamyltransferase, TG, Triglycerides, Glu, Glucose, Chol, Cholesterol, AFP, Alpha fetoprotein, IL- 18, Interleukin 18, P-value: (comparison between patients with HCC & control group).

4. Discussion

HCC is the fifth leading cause of cancer death in the world with a 5 year survival rate of less than 5% (**Dong *et al.*, 2009)**. Egypt has the highest prevalence of chronic hepatitis c virus infection world wide ranging from 6% to more than 40% among regions and demographic groups, and has rising rates of hepatocellular carcinoma development (**Leman and Wilson, 2009**). Many patients are diagnosed in the late stage and cannot tolerate hepatectomy because of advanced cancer or poor liver function reserve (**Huang *et al.*, 2010)** because this disease is often clinically silent until it is well advanced or tumor diameter exceeds 10 cm. Given the poor prognosis and lack of effective therapies for hepatocellular carcinoma, prevention programs are desperately needed. Surgical resection is the treatment of choice for patients with HCC when the tumor is small and limited to one lobe of the liver. There is increasing interest in non invasive markers to assess inflammatory activity and degree of fibrosis in chronic hepatitis C infection **(Finotto,2004).** Some tumor markers, such as glypican-3, gamma-glutamyltransferase II, alpha-l-fucosidase, transforming growth factor-beta1,tumor-specific growth factor, have been indicated to be available supplementaries to AFP in the detection (**Zhou *et al.*, 2006)**. Some other markers, such as vascular endothelial growth factor and interleukin-8 could also be used as available prognostic indicators and the simultaneous determination of AFP and these markers may detect the recurrence of HCC at its earlier period (**Zhou *et al.*, 2006**). IL-18, previously known as interferon-gamma-inducing factor, is a pleiotropic pro-inflammatory cytokine that is expressed mainly by peripheral blood mononuclear cells and macrophages. In the liver, besides its expression in Kupffer cells, IL-18 can also be synthesized by injured hepatocytes (**Fantuzzi and Dinarello, 1999)**. IL-18 plays a critical role in the host defense against intracellular microbe's infection and also it induces autoimmune diseases and propagating inflammatory process **(Gracie, et al., 2003).** IL-18 increases the susceptibility of liver endothelial cells to undergo apoptosis **(Mohran, 2011).** Elevated levels of interleukin-18 (IL-18) were described previously for chronically HCV-infected patients with different disease severities (chronic hepatitis C, liver cirrhosis and HCC) with an association between IL-18 plasma concentration with the outcome of chronic HCV infection (**Bouzgarrou *et al.*, 2008).** While other investigators describe increased levels of inflammation associated interleukins IL-15, IL-17, IL-18 and IL-18, and their binding proteins tumor tissue. Furthermore, RT-PCR and Western blot analysis revealed that IL-18 was up regulated in tumor tissues and contributed to tumor progression through their proangiogenic effect. In this study, regarding the results of serum alpha fetoprotein (AFP) levels, it was found that, the mean values of AFP levels were significantly increased in HCC group (GII) patients as compared to control group (GI) with the highest values towards HCC patients. This is due to AFP is a well-recognized tumor marker for HCC and elevated serum AFP concentration is found in approximately 60% of HCC patients in agreement with **Goldman *et al.*(2009**).

To validate the up regulation of IL-18 levels in HCC patients, these levels in serum were measured and compared to those of controls. In this study, it was found that serum IL-18 levels were significantly higher in patients with HCC than in controls (the mean level of IL-18 cases was 238.69± 145.5 pg/ml versus the mean IL-18 level in controls 52.8±13.32 pg/ml, p <0.001). These data are in accordance with earlier findings observed by **Mc Guineness *et al.* (2000)** who noted that IL-18 mRNA was up regulated in chronic and cirrhotic HCV patients. The same findings were observed by **Ludwiczek *et al.* (2002**) who found that disease progression from non cirrhotic to cirrhotic disease was accompanied by an increase in plasma IL-18 level, he also found that the deterioration of cirrhosis from Child-pugh stage A to B and C further increased IL-18 levels. Also, **Bouzgarrou *et al.* (2008)** found that patients with cirrhosis and HCC presented a higher increase in plasma IL-18 concentration than chronically infected patients. Our data revealed that, there was a significant positive correlation between IL18 and Tumor size), while there was no significant correlation between AFP and tumor size so, we recommended that simultaneous determination of IL-18 and AFP might significantly increase the Sensitivity and specificity in the diagnosis of HCC.

Conclusion:

It can be concluded that IL-18 levels are elevated in hepatocellular carcinoma patients than in healthy subjects. IL-18 level is significantly increased with the increase the tumor size and its concentration may predict the degree of hepatocellular damage. Thus IL-18 could be used and nominated as an additional non invasive marker for monitoring the degree of liver damage.

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Type-A Nucleophosmin (Npm1) Gene Mutation as a Prognostic Marker in Myelodysplastic Syndrome Patients with Normal Karyotypes

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Abstract: Background: MDS are stem cell disorders characterized by impaired hematopoiesis, and variable risk of AML. MDS can be primary or secondary with several risk factors incriminated. Increased apoptosis, genetic aberrations and autoimmune disorders are the key mechanisms incriminated in disease pathogenesis. NPM1, a shuttling protein that has several functions, is a commonly investigated marker in AML. NPM1 gene mutations occur frequently in AML, and are strongly associated with normal karyotypes. Exact molecular factors underlying progress from MDS to secondary AML are largely unknown. **Aim of this work**: was designed to investigate the prognostic value of nucleophosmin (NPM1) exon 12 mutation type A in adult patients with myelodysplastic syndrome and normal karyotype. **Subjects and method:** This study included 30 subjects divided into two groups: **Patient group**, 30 adults with de novo MDS and normal karyotype, their age ranged 17-85 years with a mean±SD 47.70±18.31years**.** The diagnosis of patients was described according to the revised WHO classification. Accordingly, **12** patients (40.0%) had refractory cytopenia (RC), 9 patients (30.0%) suffering from refactory cytopenia with multilineage dysplasia (RCMD), 4 patients (13.3%) had refractory anemia with excess blast type I(RAEB-I) and 3 patients (10.0%) classified as (RAEB-II) and **2** (6.6%) diagnosed as unclassified MDS (MDS-u). According to International Prognostic Scoring System (IPSS), the patients were classified into low risk (15 patients, 50%), intermediate-1 risk(10 patients, 33.33 %) intermediate-2 risk (5 patients, 16.66 %), High risk (0 %). **Control group**; 10 apparently healthy adult volunteers of matched age and sex. Age range from 19 to75 with a mean ±SD 43.25±20 years. **Results:** By using of reverse transcription PCR (RT-PCR), Two (6.6%) patients were positive for a nucleophosmin gene mutation (***NPM1-mutA***), one case with RAEB-I and one case had RAEB-II. NPM1 mutA was restricted to patients with intermediate risk, while no healthy individual was positive for it. **Conclusions; (*NPM1-mutA*)** is a rare finding in adult patients with de novo MDS and normal karyotype, and appears to be restricted to those patients with intermediate risk of progression to AML. None of these patients had a disease that progressed to AML. We concluded that NPM1 mutA may be a favourable early molecular event that confers some protection against evolution of AML, and thus might be a good prognostic factor in a disease that lies on the verge of AML, but this needs to be confirmed with further Studies on large cohort.

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Key words; NPM-1, MDS , RT-PCR, Myelodysplastic, Nucleophosmin.

1. Introduction:

Myelodysplastic Syndromes (MDS) are a group of clonal disorders characterized by tri-lineage defects in hematopoiesis, including the erythrocytic, granulocytic, and megakaryocytic series. They are considered premalignant conditions that often progresses to acute myeloid leukemia (AML) with several prognostic factors involved [(1)] . MDS refer to a heterogeneous group of disorders due to defect in stem cells, characterized by increasing bone marrow failure, with qualitative and quantitative abnormalities in one or more of the three marrow cell lineages (erythroid, myeloid and megakaryocytic), expressed in the form of peripheral cytopenia(s) with variable natural history characterized by increased morbidity and mortality [(2)]. Chromosomal aberrations are present in half of all de novo MDS patients with several of them noted in AML as well, suggesting a common origin of at least a fraction of these two diseases [(3)]. Nucleophosmin gene is the most commonly mutated one in AML with normal karyotype. However, its role in MDS is less well studied [(4)].Nucleophosmin (NPM1) is a nucleo-cytoplasmic shuttling protein that plays a key role in a variety of cellular processes including promotion of ribosome biogenesis, maintenance of genomic stability, regulation of transcription, and modulation of tumor-suppressor transcription factors [(5)] . Nucleophosmin has been implicated in the pathogenesis of several human malignancies and has been also described both as an oncogene and a tumor suppressor gene, depending on cell type and protein levels [(6)] . NPM1 mutations may be involved in the pathogenesis of MDS, but further studies will be required to confirm this presumption [(7)] , and assessment of NPM1 mutation status is potentially useful for predicting progression to AML [(8)] . **Aim of this work;** was designed to study the prognostic value of nucleophosmin (NPM1)

exon 12 mutation type A in adult patients with de novo myelodysplastic syndrome with normal karyotype.

2. Subjects and Methods

This study was carried out in Clinical Pathology and Internal Medicine Departments of Zagazig University Hospitals. The study protocol was approved by the ethical committee of Faculty of Medicine, Zagazig University. This study included two groups: **Patient group**; Included 30 adults with newly diagnosed MDS and normal karyotype, their age ranged 17-85 years with a mean±SD 47.70±18.31 years. They included 16 males and 14 females. Diagnosis was based on Revised WHO (2008) , and all patients were monitored during the study for complications. Accordingly, there were 4 different types of MDS. Refractory cytopneia (RC) was seen in 12 cases (40.0%). RCMD was seen in 9 cases (30.0%). RAEB was seen in 7 cases (23.3%), of which 4 cases (13.3%) were of RAEB-I subtype, and 3 cases (10.0%) were of RAEB-II subtype. Finally, there was two MDS-u cases (6.6%). **Control group**; 10 apparently healthy adult volunteers of matched age and sex. Their ages ranged from 19 to 75 years with a mean ±SD 43.25±20 years, they included 4 males and 6 females.

Informed consents were obtained from all the participants who *subjected to the following*: Full history taking and clinical examination. Radiological studies , including; chest X-Ray, CT scan and pelvi-abdominal ultrasound (if indicated).

Laboratory investigations include; CBC, liver function test, kidney function test , serum LDH, ferritin levels. **Bone marrow aspiration** for patients, followed by Leishman Staining**,** stress on the percentage of blast cells, myelodysplastic features and bone marrow cellularity, Myeloperoxidase cytochemistry , conventional cytogenetic study (karyotyping) using G-banding technique.

Special investigation;

Reverse transcriptase polymerase chain reaction (RT-PCR) of NPM1 exon 12 mutation-A, in the following sequence;

A) RNA extraction;

One ml (bone marrow aspirate for patients, peripheral blood for volunteers), samples collected on sterile EDTA vacutainers (BD) were subjected to RNA extraction kit "(Easy Nucleic Acid Isolation) ESNA, Blood RNA" manufactured by Omega Bio-Tek Incorporation (USA). *Principle;* ESNA, Blood RNA kit is designed for purification of total RNA from fresh whole blood or bone marrow aspirate. The kit uses the reversible binding properties of HiBind matrix, a new silica based material, combined with the speed of mini-column spin technology. Red blood cells are selectively lysed and white cells collected by centrifugation. After lysis of WBCs under denaturing conditions that inactivate RNases, total RNA is purified in the HiBind spin column. 2-mercaptoethanol is the key denaturing agent of RNases. A specially formulated high salt buffer system allows RNA molecules greater than 200 bases to bind to the matrix. Cellular debris and other contaminants such as hemoglobin are effectively washed away and high quality RNA is finally eluted in Diethylpyrocarbonate (DEPC)-treated sterile water.

B) Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR):

We performed each sample using "Illustra Ready to- Go RT-PCR Beads" supplied by General Electric Healthcare (USA) in order to amplify fragments of NPM1 exon 12 mutation. Ready-to-go RT-PCR bead utilize Moloney Murine Leukaemia Virus (MMuLV) reverse transcriptase and *Taq* DNA polymerase to generate polymerase chain (PC) product from an RNA template. Each bead is optimized to allow the 1st strand cDNA synthesis and PCR reactions to proceed sequentially as a single tube, single step reaction. First strand cDNA generated with the RT-PCR beads is designed to be used directly as a template for PCR [9] . In this procedure the double stranded RNA: cDNA heteroduplex made during first strand synthesis is heat-denatured to allow the cDNA strand to be used as a template for polymerization in PCR [10]**.** The specificity of the PCR amplification is based on two amplification primers which flank the cDNA segment to be amplified and hybridize to complementary strands. Repeated cycles of denaturation, primer annealing and primer extension by *Taq* DNA polymerase can result in exponential amplification of the target cDNA. For amplification of NPM1 gene, we used the forward primer NPM-A, and the reverse primer NPM-REV-6, and their sequences were as follows: **Forward→**5'CCAAGAGGCTATTCAAGATCTC TCTC3'.**Reverse→**5'ACCATTTCCATGTCTGA GCACC-3'. The forward primer contains an intentional mismatch at the third nucleotide from the 3' end to improve specificity, while the reverse primer is specifically designed to exclude amplification of NPM1 pseudogenes [11]. As internal PCR control, we used Abelson Tyrosine Kinase (ABL) gene amplification. ABL gene is a house keeping gene that, with successful amplification, should be detected in all samples. The same RT-PCR conditions were used, but with specific forward primer ABL-A2B-5' and reverse primer ABL-A3E-3'. Their sequences were: **Forward→**5'GCATCTGACTTTGAGCCTCAG3' .**Reverse→**5'TGACTGGCGTGATGTAGTTGCT T-3'. Primers were supplied as lypholized agents by Metabion International AG. All primers were reconstituted with purified sterile water to the

concentration of 100 pmol/µl according to manufacturer guidelines. Each reaction tube was flicked gently to mix, then RNA template & primers were added to each dissolved bead as follows: Total RNA volume →6µl. Forward NPM1primer →1µl. Reverse NPM1 primer →1µl. Forward primers →1µl. Reverse primers →1µl. DEPC water →40µl. **Total volume →50**µl. Finally, caps were closed & reactions were transferred to thermal cycler. A negative control reaction to test for DNA contamination was prepared by reconstituting the bead to 50µl without addition of template RNA or primers, and then the bead was incubated at 95°C for 10 minutes to inactivate the MMuLV reverse transcriptase. For a control reaction to test performance of PCR beads, we added 50µl of DEPC-treated water to the rabbit globin control mix bead, then transferred the entire contents to a tube containing an RT-PCR Bead.

Amplification Protocol; The following temperature scheme was performed for samples and negative control reactions [11] . For amplification, we used the thermal cycler "Gene Amp PCR System 9700" supplied by Perkin Elmer (Singapore): Hold (1) Preheating 95°C →7 min. Hold (2) 35 amplification cycles of ; 95°C →30 sec (Denaturation). 67°C → 45sec (Annealing). 72°C → 45sec (Elongation). Hold (3) Final extension 72°C → 7 min

C) Detection by Agarose Gel Electrophoresis;

5µl of the PCR products (DNA sample)were electrophoresed on 2% agarose gels after mixing 1:1 with "loading dye" ethidium bromide staining. Detection of 320-bp product indicated the presence of NPM1 exon 12 mutation A. ABL product detection at 258-bp was used as an internal control of successful extraction and amplification. 10µl of the DNA ladder composed of ten chromatography-purified individual DNA fragments (in base pairs): 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100, were loaded directly into the gel and run in parallel with sample**.** The ladder was ready to use, being premixed with 6X DNA loading dye for direct loading on gel [12] **.** DNA fragments migrated through the gel at various rates depending on their sizes. After finishing the procedure, the gel was viewed and photographed over an ultraviolet trans-illuminator (Wealtec) using UV illumination for sample visualization.

Statistical Analysis

The data were tabulated and statistically analyzed using Microsoft Office Excel 2010 and Statistical Package for Social Sciences version 20 (SPSS: An IBM Company). Data were summarized using the arithmetic mean, standard deviation (SD), range for numerical variables. For qualitative data, the frequency and percent distribution were calculated and for comparison between groups, chi square test and the student **"t"** test was used. P values <0.05 indicate significant results.

3. Results

Thirty subjects included in this study, 10 control and 30 patients presented with anemic manifestations (fatigue, pallor or fainting) being the most common (65.0%), followed by repeated infections or fever proved to be related to infection (15.0%), bleeding (10.0%), follow up (5.0%) and routine check (5.0%). Splenomegally was found in 15.0% of patients, while hepatomegally was found in 10.0% of them (Table 1).

There was a significant decrease in the HB levels, total leucocytic count (TLC) and platelets count in the patient group in comparison to the control group. The mean±SD of HB level, TLC and platelets count were (In patient group; 9.66±2.20 g/dl, 5.05±2.08x10^3/µl, 160.45±120.01x10^3/µl, respectively and in control;13.41±1.05 g/dl, 8.13±2.17x10^3/µl , 245.00±60.3x10^3/µl respectively). Bone marrow blasts: In the patient group, BM blast count ranged between 0.5-19%, with mean±SD of 4.03±5.23(Table 2).

As regards LDH and Ferritin, there was a significant increase in patient group in relation to control group. The mean±SD of LDH (Iµ/ml) was, (In patient; 427.9±188.68 and in control; 106.4±21.18) and Ferritin (ng/ml) was, (In patient 2325.75±3080.03 and in control; 116.6±36.54), (Table 3)

Prognosis;

According to calculation of their prognostic score based on International Prognostic Scoring System (IPSS)**,** [13] 15 cases (50.0%) were classified as low risk patients, 10 cases (33.33%) as intermediate-1 risk, 5 cases (16.66%) as intermediate-2 risk, and none as high risk (Table 4). According to the WHO Classification-based Prognostic Scoring System (WPSS), 14 cases (46.66%) were classified as very low risk patients, 3 cases (10.0%) as low risk, 7 cases (23.33%) as intermediate risk, 6 cases (20.0%) as high risk, and none as very high risk (Table 5).

Nucleophosmin mutation A;

Nucleophosmin mutation A was detected in 2 MDS cases (6.6 %) and none of the healthy volunteers (0.0%)**.** Using gel electrophoresis, the amplification product of ABL gene was detected as a band at 258-bp, while the product of mutated NPM1 gene was detected as a band at 320-bp (Figure 1). Cases positive for NPM1 mutA were 76 and 65 years old male and female, respectively. Both patients were diagnosed with RAEB (subtypes I and II, respectively), with BM blast counts of 7% and 13.6% at diagnosis, respectively. Both patients also belonged to the intermediate risk (1 and 2, respectively) category of IPSS system,

and to the high risk category of WPSS system, neither transformed to AML and not needed frequent blood transfusion (Table 6).

Disease Progress:

Five cases (16.6%) died during the study, one of them (3.3%) following AML transformation. Cause of death couldn't be identified in others as they died out of hospital.

Table (1): Clinical data and presentation of patients.

Clinical data	Number	Percent
Presentation		
Anemic manifestations	13	65.0%
Infection	3	15.0%
Bleeding	2	10.0%
Follow up	1	5.0%
Routine check	1	5.0%
Clinical examination		
Splenomegally	3	15.0%
Hepatomegally	2	10.0%
Lymphadenopathy	0	0.0%

Table (2): Statistical comparison of hematological data between patient and control groups.

Parameter	Patient Group	Control Group	*t-test (t)*	*P value*
Hemoglobin (g/dl)				
Mean±SD	9.66±2.20	13.41±1.05	-5.054	0.001
Range	3.5-14.0	11.7-14.9		
Platelet count ($x10^3$/ µl)				
Mean±SD	160.45±120.01	245.00±60.33	-2.568	0.016
Range	20-454	156-340		
TLC ($x10^3$/ µl)				
Mean±SD	5.04±2.08	8.13±2.17	-3.773	0.001
Range	1.9-8.1	4.2-10.7		
Bone marrow blasts (%)				
Mean±SD	4.03±5.23	-	-	-
Range	0.5-19.0	-	-	-

Table (3): Statistical comparison of LDH and Ferritin levels between patient and control groups

Parameter	Patient Group (No. = 20)	Control Group (No. = 10)	*P value*
LDH (IU/L)			
Mean±SD	427.9±188.68	106.4±21.18	0.001
Range	239-877	75-150	
Ferritin (ng/ml)			
Mean±SD	2325.75±3080.03	116.6±36.54	0.005
Range	38-12696	82-190	

Table (4): Prognosis of patient group according to **IPSS.**

Prognostic group	Number	Percent
Very low risk	14	46.66%
Low risk	3	10.0%
Intermediate risk	7	23.33 %
High risk	6	20.0%
Very High	0	0.0%

Table 5: Prognosis of patient group according to WPSS.

Prognostic group	Number	Percent
Low risk	15	50.0%
Intermediate-1 risk	10	33.33%
Intermediate-2 risk	5	16.66%
High risk	0	0.0%

Table (6): Characterization of NPM1 mutA positive patients.

Case	Age	Sex	Diagnosis	BM blasts%*	LDH	Ferritin	Frequent transfusion	IPSS group	WPSS group
1†	76	Male	RAEB-I	7 %	299	12696	Yes	Int -1	High
2	65	Female	RAEB-II	13 %	533	4350	Yes	Int -2	Very low

***At diagnosis, † Dead**

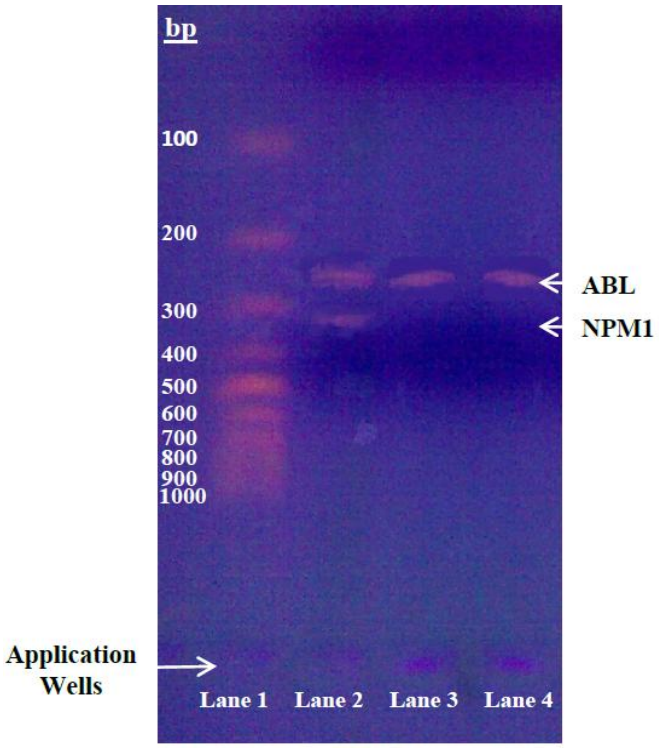

Figure 1: Gel electrophoresis of PCR products for NPM1 mutation A

- Lane 1: molecular weight marker (100-1000 bp);
- Lane 2: DNA of patient sample positive for NPM1 mutA;
- Lane 3: DNA of patient sample negative for NPM1 mutA;
- Lane 4: DNA of control individual negative for NPM1 mutA.

4. Discussion

Myelodysplastic syndromes are a collection of stem cell disorders characterized by impaired hematopoiesis resulting in low peripheral blood counts with a variable risk of progression to AML. According to etiology, MDS are broadly classified into de novo and therapy related MDS [(14)]. NPM1 is a phosphoprotein belonging to the nucleoplasmin family of chaperones, mainly localized in the nucleolus where it exerts many of its functions, with a proportion of the protein continuously shuttling between the nucleus and the cytoplasm. However, work on NPM1 mutations in MDS is less than needed considering its rarity in MDS and data about NPM1 role in disease fate and evolution are still elusive [(15)]. Hence, this study aimed to investigate the role of NPM1 mutation A as a prognostic marker in adult MDS patients with normal karyotype. All patients were followed up during the study period for dependence on blood transfusion, rise of complications and progression to AML. At least two months follow-up period was necessary to enable assessment of need for regular transfusion as a determining factor in WPSS prognostic score.

As regards clinical presentation, our patients were presented mainly with anemia (easy fatigability, fainting attacks or pallor), being found in 13 of twenty patients (65%). Kelaidi *et al* [(16)] stated that the need for RBCs transfusion was the second most common cause (31.4% of patients) for hospitalization of patients diagnosed later with MDS. Chevassut and Mufti.,[(17)] also stated that anemic manifestations are the most common presenting feature in MDS patients, being found in up to 80% of them. Other less common presentations in our study included infection (15%) and bleeding (10%), in addition to follow up and routine check (5% each). These data are fairly compatible with Chevassut and Mufti [(17)] statement that infections or bleeding can be found in up to 20% of cases. Splenomegaly and hepatomegaly

were found in 15% and 10% of cases, respectively. Although reported to be rare event by Chevassut and Mufit.,[17], these higher figures can be attributed to either the small sample size or more importantly to the high incidence and endemicity of hepatic diseases in Egypt especially hepatitis C [18] . Regardless of the exact figures which vary from a locality to another, our results seem consistent with the currently circulating literature, but studies on larger cohorts are needed to confirm percentage of each presenting feature.

Our study revealed that most common type of MDS was RA (40.0%), followed by RCMD (30.0%) and RAEB (23.3%). Considering the wide spread use of FAB classification in most medical records utilized in literature, Neukirchen *et al.*,[19] showed much different distribution of cases compared to our study, where the most common MDS type was RCMD (56.48%) followed by RAEB (24.07%), while RA came third with (11.57%). This difference is most probably due to our small sample size. However, other studies using FAB classification revealed incidences that match our findings, such as that of Germing *et al.*,[20] with RA comprising 52% of MDS cases and RAEB comprising 33.0% of cases.

As regards laboratory data, there was a significant decrease of hemoglobin, TLC and platelet count in patients in comparison to control. This was expected since cytopenias are diagnostic features that discriminate MDS from normal state. LDH and Ferritin levels were also significantly higher at diagnosis in MDS patients than in control individuals, but there was a wide overlap in their ranges among different IPSS and WPSS groups. This goes hand in hand with the results of Varma and Varma [21].

Upon establishment of MDS diagnosis, BM aspirate samples were used in the same day of aspiration for detection of NPM1 exon 12 mutation A. It was found that only two (6.6%) of our patients had NPM1 mutation A at diagnosis. Due to the small percent of patients positive for nucleophosmin mutation-A, it wasn't statisically possible to study its relation to prognosis with certainty, and data acquired were mainly descriptive. One of the patients positive for NPM1 mutA was diagnosed with RAEBI, and the other with RAEB-II MDS, and both were of intermediate-risk category according to IPSS system. It was obvious that NPM1 mutation-A appeared to be a rare finding in de novo MDS patients, and this was in agreement with the findings of [7,8,22 ,23] who reported positivity rates for NPM1 mutations to be 5.2%, 2.8%, 8.3% and 3.9%, respectively. Our results was in consistency with Zhang *et al.*,[7] who concluded that, two cases (5.2%) out of 38 cases of newly diagnosed MDS were found to have NPM1 exon 12 mutation A, one diagnosed with MDS-RA and the other with MDS-RAEB-I. Both however, had abnormal karyotypes of intermediate type according to WPSS system. The MDS-RA patient had a good survival prediction of >24 months, but the other died during the study. Our results was in agreement with **Bains *et al.*,**[8] who studied large cohort on MDS patients, (2.8%) had mutation A, out of 107 cases, which showed normal cytogenetics at diagnosis. Bains *et al.*,[8] also stated that NPM1 mutations were restricted to cases of intermediate- and high-risk MDS, with no significant differences in the frequencies according to sex, both of which are consistent with our findings. Comparing our results to those of Gritsaev *et al.*,[22] their positivity rate was in consistency to our results , with 5 out of 65 FAB-MDS patients (7.6%) with variable karyotypes. On the other hand, Xiao and Li.,[23] found NPM1 mutation A in 9 out of 232 (3.9%) de novo MDS patients with predominantly normal karyotypes.

As regards disease progress, four out of twenty patients (13.3 %) died during this study, one of them following AML transformation, identified during follow up examination of BM aspirates and performance of immunophenotyping. The cause of death in the rest of them was unknown. Bains *et al.*,[8] reported that no case with NPM1 mutation alone had a disease that progressed to AML. Since we didn't screen for other molecular markers, our results can't be conclusive in this, although none of our cases positive for NPM1 progressed to AML either.

5. Conclusions

Nucleophosmin exon 12 mutation A is a rare finding in adult patients with de novo MDS and normal karyotype, and appears to be restricted to those patients with intermediate risk of progression to AML. None of these patients had a disease that progressed to AML. We concluded that NPM1 mutA may be a favourable early molecular event that confers some protection against evolution of AML and thus might be a good prognostic factor in a disease that lies on the verge of AML, but this needs to be confirmed with further studies on large cohort.

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Tenacibaculosis in Picasso Tigger Fish (*Rhinecanthus Assasi*) and Black Damsel Fish (*Neoglyphieodon Meles*) of Red Sea at Hurghada, Egypt

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Abstract: This study reported the first isolation and identification of *Tenacibaculum maritimum* (*T. maritimum*) in Egypt from Picasso Tigger Fish (*Rhinecanthus assasi*) and Black damsel fish (*Neoglyphieodon meles*) in the indoor aquarium of National Institute of Oceanography and Fisheries (NIOF) in Hurghada. The disease onset started after exposing the fish to catching and indoor rearing stress, the diseased fish manifested off food, lethargic and had external body lesions in the form hemorrhagic ulcers, ulcerated mouth and fin rot, in addition to 55 and 65% mortalities rate among the two fish species respectively. The pathogen was recovered from the body surface lesions and internal organs of the examined fish. Eleven isolates were isolated and identified as *T. maritimum* on the basis of morphological and cultural characters, API20E system tests and conventional biochemical tests. It is pathogenic strain caused clinical signs such as lethargic, off food and body surface lesions as white areas with hemorrhagic ulcers on all experimentally infected fish and 60% mortality. The experimentally infected fish could be treated by repetitive enrofloxacin at rate 30 ppm immersion bath for 1 h during three consecutive days.

[Mohamed A. A. Abd El-Galil and Mahmoud Hashiem **Tenacibaculosis in Picasso Tigger Fish (*Rhinecanthus Assasi*) and Black Damsel Fish (*Neoglyphieodon Meles*) of Red Sea at Hurghada, Egypt.** Life Science Journal 2011; 8(4):1166-1171]. (ISSN: 1097-8135). http://www.lifesciencesite.com.143

Keywords: *Tenacibaculosis*, *Tenacibaculum maritimum*, Picasso Tigger Fish (*Rhinecanthus assasi*), Black damsel fish (*Neoglyphieodon meles*)

1. Introduction

Marine *Tenacibaculosis* is a serious bacterial disease affecting a great variety of marine fish especially cultured species, both adult and young are susceptible but the young fish are seriously affected (**Toranzo *et al.*, 2005)**. It is an ulcerative disease causes massive mortalities and severe economic losses in marine fish cultures in Japan (**Wakabayashi *et al.*, 1986**), Scotland (**Bernardet *et al.*, 1990**), Spain (**Alsina** & **Blanch, 1993**), France (**Bernardet *et al.*, 1994**) and North America (**Ostland *et al.*, 1999**).

Among the cultured fish, *Tenacibaculosis* has been reported in sole, *Solea solea* (L.) (**Bernardet *et al.*, 1990**); Senegalese sole (*Solea senegalensis*) (**Cepeda and Santos, 2002**); Japanese flounder (*Paralichthysolivaceous*) (**Baxa *et al.*, 1986**); turbot (*Psetta maxima*) (**Avendaño -Herrera *et al.*, 2004a**). Also, it is recorded in Atlantic salmon (*Salmo salar* L.,), Rainbow trout (*Oncorhynchus mykiss*), Striped trumpeter (*Latrislineata*), Greenback flounder (*Rhombosolea tapirina*) (**Soltani *et al.*, 1996** and **Handlinger *et al.*, 1997**) and sea bream and sea bass (**Toranzo *et al.*, 2005)**.

It is caused by *T. maritimum* (formerly *Flexibacter maritimus*) (**Suzuki *et al.*, 2001**) which is a filamentous gram negative bacterium and primarily attacks skin, mouth and fins of fish causing severe necrotic and ulcerative lesions on the body surface (**Baxa *et al.*, 1986** and **Toranzo *et al.*, 2005**).

It has been noted that, 55 and 65% mortalities occurred in Picasso Tigger Fish and Black damsel fish in the indoor aquaria of The National Institute of Oceanography and Fisheries (NIOF) at Hurghada, Egypt. The diseased fish were lethargic, off food and had body surface lesions as white area with hemorrhagic ulcers, eroded and haemorrhagic mouth and ulcerative skin lesions. A systemic disease can be also established in the form congestion of spleen and intestine.

The diagnosis of this disease is usually carried out by clinical signs, isolation and biochemical characterization (**Bernardet *et al.*, 1990; Pazos *et al.*, 1993**). Marine agar (MA), Anacker and Ordal agar (AOA) (**Anacker** & **Ordal, 1959**), Huso- Shotts medium (**Chen *et al.*, 1995**) and *Flexibacter maritimus* medium (FMM) has been advocated for the isolation of *T. maritimum* from infected fish **(Pazos *et al.*, 1996)**. However, *Flexibacter maritimus* medium (FMM) has been proposed to be the most appropriate medium for the successful isolation of *T. maritimum* (**Avendaño -Herrera *et al.*, 2005**).

The present study was designed to isolate and characterize the causative agent of *Tenacibaculosis* in Picasso Tigger Fish (*Rhinecanthus assasi*) and Black damsel fish (*Neoglyphieodon meles*) in the indoor

aquaria of National Institute of Oceanography and Fisheries (NIOF) at Hurghada. Moreover, pathogenicity assay and treatment trials with antibiotic were conducted.

2. Material and Methods

Fish :

Forty five clinically diseased and moribund Picasso Tigger Fish and Black damsel fish were collected from the indoor aquaria of The National Institute of Oceanography and Fisheries (NIOF) at Hurghada and subjected to clinical examination (**Kimberley, 2004)** and bacteriological isolation.

Bacteriological samples

Bacteriological samples from external lesions, liver and kidney of several clinically diseased fishes were directly inoculated onto freshly prepared plates of Flexibacter maritimus medium (FMM) (**Pazos *et al.*, 1996**) and Huso- Shotts medium (**Chen *et al.*, 1995**), the two media were prepared with 50% sea water and supplemented with antibiotic (tobramycine). All the inoculated plates were incubated at 25°C for up to 72hrs. Pure colonies of the presumptive *T. maritimum* isolates were picked up and inoculated on sloped FMM and preserved for further studies.

Identification of the isolates

The pure isolates were exposed to conventional bacteriological tests and API20E assay. API20E (BioMèrieux) was used according to the manufacturer's instructions and sterile 50% sea water was used as a diluents and the strips were incubated at 25°C for 24hrs. The eleven isolate were identified according to **Suzuki *et al.* (2001), Buller (2004), Mouriño *et al.* (2008)** and **Avendaño-Herrera (2009).**

Water quality

Water samples were taken from the investigated indoor aquaria and their water source from the red sea (control sample) in dark brown clean and bottles and subjected to DO, pH and total ammonia determination. Water temperature was determined in the aquaria.

Pathogenicity assay

Twenty Black damsel fish were acclimated for one week then subdivided into two equal groups each of 10 fish. The fish of the first group were experimentally infected by bath immersion in sea water bath for 18hrs (**Avendaño-Herrera *et al.*** (**2006a**) with *T. maritimum* suspension containing $1.5x10^6$ cellmL^{-1}.The fish of the second group were submitted to the same procedure without bacteria and used as control. Each fish group was preserved in 110L glass aquarium at water temperature 24±2°C and observed for 14days, the clinical signs and mortalities were recorded.

Treatment trial

Two groups of black damsel fish (each of 10 fish) were subjected to experimental infection with *T. maritimum* as had described in the pathogenicity assay. At the second day of infection, the fish of the first group were subjected to repetitive enrofloxacin treatment at rate 30 ppm immersion bath for 1 h during three consecutive days (**Avendaño -Herrera *et al.*, 2008)** and fish of the second group were left as infected control. The fish of the two groups were subjected to daily inspection for 14 days after treatment and the clinical signs and mortality were recorded.

3. Results

Clinical signs

Generally the diseased fish were off food, lethargic and had external body lesions, the diseased Picasso Tigger fish showed hemorrhagic ulcers **(Photo - 1)** and eroded and ulcerated mouth **(Photo - 2)** but the diseased Black damsel fish showed ulcerated skin lesions surrounded with white batch of necrotizing tissues **(Photos – 3- 5)** and fin rot, **photo (5).** 55 and 65% mortalities were reported among the two fish species respectively.

Photo (1) Picasso Tigger fish showed congested ulcer on the skin.

Photo (2) Picasso Tigger fish showed eroded and ulcerated mouths.

Photos (3 & 4) Black damsel fish showed haemorrhagic ulcer surrounded with necrotizing tissues

Photo (5) Black damsel fish showed haemorrhagic ulcer and fin rot

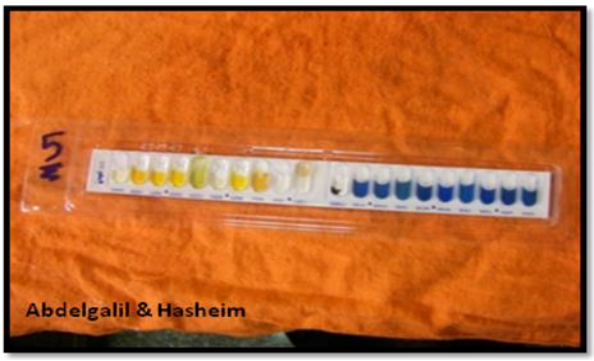

Photo (6) API20E results

Isolates identification

The eleven isolates were biochemically similar and identified through their morphology, conventional biochemical tests and API20E system tests as *T. maritimum* (Table, 1 and Photo, 6).

Water quality

The examination of water sample from the indoor aquaria revealed deterioration in the water quality in the form of increase total ammonia and decrease dissolved oxygen with alkaline pH comparing with the criteria of the control water sample from the red sea (Table 2).

Pathogenicity assay

The experimentally infected black damsel fish showed lesions similar to those of naturally infected fishes such as off food, lethargy, skin hemorrhagic ulcers, eroded and ulcerated mouth and these clinical signs started from the third day post-infection. By the end of the observation time (14 days) the mortalities of the experimentally infected

fish reached 70% and *T. maritimum* could be reisolated in pure culture from the experimentally infected fish.

Treatment trial

The enrofloxacin treated fish of the first group showed no mortality or clinical signs during the experiment time while all fish of the second group (no treated infected control) showed typical clinical signs as mentioned above and 60% mortality started from the third day post-infection.

Table (1): Shows the results of the conventional biochemical and API20E tests of the *T. maritimum* suspected isolate.

Serial number	Tests	Result	
		Tested isolate	Standard *T. maritimum*
1	Colony shape	Uneven edge	Uneven edge
2	Colony colour	Pale yellow	Pale yellow
3	Gram stain	-ve rods	-ve rods
4	Motility test	Motile	Motile
5	Oxidase test	+ve	+ve
6	Catalase test	+ve	+ve
7	Nitrate reduction	+ve	+ve
8	Congo red reduction	+ve	+ve
9	KOH	-ve	-ve
10	Hydrolysis of Esculin	-ve	-ve
API20E			
12	ONPG (Ortho NitroPhenyle Galactopyranosidase)	-	-
13	ADH (Arginine Dehydrolase)	-	-
14	LDC (Lysine Decarboxylase)	-	-
15	ODG (Ornithine Decarboxylase)	-	-
16	CIT (Citrate test)	-	-
17	H2S (H_2S production test)	-	-
18	URE (Urase test)	-	-
19	TDA (Tryptophane Deaminase)	-	-
20	IND (Indole test)	-	-
21	VP (Vogus proskauer)	-	-
22	GEL (Gelatine liquefaction test)	-	±
23	Glu, Man, Ino, Sor, Rha, Sac, Mel, Amy, Ara	-	-

List of abbreviation

*(GLU) Glucose *(MAN) Mannitol * (INO) Inositol test * (SOR) Sorbitol *(RHA) Rhaminose *(SAC) Sucrose *(MEL) D-Melibiose *(AMY) Amygdaline * (ARA) L-Arabinose

Table (2): Shows water quality criteria

Item	Unit	Tested water sample	Control water sample
Water temperature	°C	24°C	27°C
pH values	-	9.2	7.7
Dissolved oxygen	mgL^{-1}	3.1	4.5
total ammonia	mgL^{-1}	0.4	0.03

4. DISCUSSION

This study reported the first isolation of the marine pathogen *T. maritimum* from Picasso Tigger Fish (*Rhinecanthus assasi*) and Black damsel fish (*Neoglyphieodon meles*) of red sea at hurghada, Egypt. The affected fish showed the classical clinical signs such as off food, leathergic, skin hemorrhagic ulcers (sometimes surrounded by white batch of necrotizing tissues), eroded and ulcerated mouth and fin rot. Similar lesions were recorded by Baxa *et al.* (1986); Santos *et al.* (1999); Toranzo *et al.* (2005) and López *et al.* (2009) in different fish species.

The *T. maritimum* virulence was exaggerated and the disease's clinical signs started by stressors including exposing the wild investigated fish to fishing and confinement in indoor aquarium, deteriorated water quality as high ammonia ($0.4mgL^{-1}$) and low dissolved oxygen ($3.1mgL^{-1}$) at temperature around 25°C and these results are in accordance with Magariños *et al.* (1995) who stated that the prevalence and severity of the disease

increased at temperatures above 15 °C with variable number of environmental stressors and host-related factors as skin surface condition and Mouriño *et al.* (2008) who stated that the presence of highly virulent strains of Flexibacter sp. is associated with the presence of organic matter dissolved in water. Such stressors are the main predisposing factors for the chronic immune suppression of marine fish (Santos *et al.*, 1999).

The eleven isolates had similar morphological and biochemical characterizations and identified as *T. maritimum.* Their biochemical tests were positive for cytochrome oxidase, catalase, motility, congo red reduction and nitrate reduction tests and they were negative for all tests of API20E system. These findings come in agreement with the results of *T. maritimum* identification established by Suzuki *et al.* (2001), Buller (2004), Mouriño *et al.*, (2008) and Avendaño-Herrera (2009).

The experimentally infected black damsel fish showed lesions similar to those of naturally infected fishes such as lethargy, external body lesions in the form hemorrhages and then ulcers on the skin, eroded and ulcerated mouth associated with 60% mortality, similar clinical signs were reported by Handlinger *et al.* (1997). These findings clearly demonstrated the potential pathogenicity of the isolated strain and confirmed the effectiveness of bath immersion challenge model to estimate the virulence of *T. maritimum*. Results of immersion challenge carried out in the present study demonstrated that *T. maritimum* can spread through water.

The treatment of the experimentally infected fish was successfully conducted by using repetitive 30 ppm enrofloxacin immersion bath for 1 h during three consecutive days, the treated fish showed no external lesions and no mortality recorded between them and these findings come in contact with the results reported by Avendaño-Herrera *et al.* (2008).

In conclusion, this study reported the first isolation and identification of the marine pathogen *T. maritimum* in Egypt from Picasso Tigger Fish and Black damsel fish reared in indoor aquarium of National Institute of Oceanography and Fisheries (NIOF) in Hurghada city. *Tenacibaculosis* could be treated by repetitive enrofloxacin immersion bath (30 ppm) for 1hr during three consecutive days. Further studies should be conducted on the molecular identification, immunity and control by using natural and safe alternatives such as probiotics or plant extracts.

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12/31/11

Efficiency of some plant extracts, carbohydrates and inorganic salts as anti-adhesion agents against the adhesion of *Staphylococcus* strains to HEp-2 cells

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Abstract: Some Staphylococcus strains are well adapted to humans. It can live as a commensal bacterium but it can initiate severe infection at various body sites. Its structural components secrete products which can efficiently target human tissues and evade host defense mechanisms. So it continues to cause invasive, life-threatening infections despite the availability of effective antimicrobial agents. *Staph. aureus* produce variety of diseases like soft tissue infection (wound infection, boil, eczema, blister and scalded skin syndrome), pneumonia and osteomyelitis. The present study was conducted to the screening of different substances (plant extracts, carbohydrates and inorganic compounds) as anti adhesion agents of Staphylococcal strains to human epithelial cells and trials for improvement of the anti adhesion characters of positive compounds. Clinical samples were collected regardless the type of infection and the sex of patients. This study was carried out over a period of 7 months from September 2009 to May 2010. Pus, urine, sputum and stool were collected to isolate samples. The obtained results showed that; among the plant extracts tested for their anti-adhesion potency the highest effect was recorded to the extract of *Nigella sativa* followed by the extract of *Trigonella fosnum* and *Eucalyptus globules*. Clear antiadhesion potencies were recorded also to glucose, arabinose, galactose, xylose and fructose (monosaccharides), sucrose, maltose and lactose (disaccharides), starch and cellulose (polysaccharides) and the inorganic salts NaCl and $CaCl_2$.
[Mohamed T. Shaaban, Sobhy E. El Silk[*] and Mona A. Tayel. **Efficiency of some plant extracts, carbohydrates and inorganic salts as anti-adhesion agents against the adhesion of *Staphylococcus* strains to HEp-2 cells**. Life Sci J 2011;8(4):1172-1182] (ISSN: 1097-8135). http://www.lifesciencesite.com.144

Key word: Staphylococcus**,** adhesion, antiadhesion, plant extracts, carbohydrates

1. Introduction

Staph. aureus is a common human colonizer and pathogen that causes infections ranging from skin and soft tissue to invasive diseases such as pneumonia, osteomyelitis, and endocarditic (**Allison *et al.*, 2010**). *Staph.s aureus and Staph epidermidis* are major causes of infections associated with wounds, indwelling catheters, and cardio-vascular and orthopedic implant devices (**Eidhin *et al.*, 1998**).

Bacterial adhesion to human epithelial cells (HEp-2) is a key step in infections, allowing subsequent colonization, invasion and internalization of pathogens into tissues. Antiadhesive agents are therefore potential prophylactic tools against bacterial infections (**Janecki and Kolodziej, 2010**).

Anti-adhesion therapy and anti-adhesin immunity are meant to reduce contact between host tissues and pathogens, either by prevention or reversal of adhesion of the infectious agent. It is well established that adhesion of enteric, oral and respiratory bacteria is required for colonization and for subsequent development of disease (**Ofek *et al.*, 2003**).

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. In the developing countries, they used as a primary source of medicine (**Chitme *et al.*, 2003**). About 80% of the people in developing countries use traditional medicines for their health care (**Kim, 2005**). The natural products derived from medicinal plants have to be a source of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals (**Enzo, 2011**).

Sugars are vital components of infecting microbes and host cells, and are involved in cell signaling associated with modulation of inflammation. Indeed, sugars are the molecules most commonly involved in cell recognition and communication. In skin, they are essential to epidermal development and homeostasis. They play important roles in microbial adherence, colonization and biofilm formation, and in virulence (**David *et al.*, 2007**).

Inorganic antimicrobial agents are being increasingly for control of microorganism in various areas, especially in dentistry. Particle size of mental oxides had an impact on their anti-microorganism activity. There is growing interest in nanoscale particles since materials exhibit unique properties which offer considerably from those of macroscopic materials. Inorganic nano mental oxides including MgO, ZnO and CaO have been shown anti-microorganism activity (**Shi *et al.*, 2010**).

2. Material and Methods

1. Material

A) The tested bacterial strains

The study involved 3 *Staphylococcal* strains, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* NCTC 6571 that were obtained from El Nasr pharma company, Cairo; *Staphylococcus epidermids* and *E. coli* were obtained from Tanta Cancer Center and Faculty of Medicine, Tanta University respectively.

B) Plants tested

Extracts of three plants namely *Nigella sativa* (family: Ranunculaceae), *Trigonella fosnum* (family: Fabaceae) and *Eucalyptus globules* (family: Myrtaceae) were used in the study.

C) Media used for tissue culture cultivation:

(1): Rosewell park memorial institute medium (RPMI-1640 medium) (**Moore and Woods, 1976**), the medium was used for culturing and maintenance of the human tumor cell lines.

(2): Dulbecco's modification of Eagle's medium (DMEM) (**Dulbecco and Freeman, 1959**), medium was used for cultivation of human epithelial cells

(3): Transport Medium, (Dulbecco's modification of Eagle's medium (DMEM)) supplemented with fetal bovine serum 10 %, gentamicin sulphate (Sigma co.) 100 µg/ml and amphotericin B 10 µg/ml (Aliquot in 10 ml lots and store at - 20 ºC)

D) Media used for isolation and cultivation of bacteria:

(4): Nutrient broth medium (Shirling and Gottlieb, 1966).

It contains beef extract (3 gm), peptone (5 gm, for nutrition), NaCl (5 gm) and distilled water (1000 ml).

(5): Nutrient agar medium (Shirling and Gottlieb, 1966).

It composed of nutrient broth plus 20 gm agar.

(6): MacConkey 's agar(**Oxoid Manual,1981**), Composed of peptone (20 gm, for nutrition), Lactose (10 gm as test sugar), neutral red (0.05gm, indicator that changes pink in the presence of acid which is produced as a result of lactose fermentation), bile salts (1.5 gm, sodium taurocholate to inhibit the growth of non intestinal bacteria) and agar (20 gm, solidifying agent) and crystal violet (0.001 gm); it used to differentiate between Lactose fermenters (L.F) group which give rose pink colonies include the coliform group and Non-lactose fermenters (Non L.F) group which give pale yellow and include the *Salmonella* and *Shigella*.

(7): Manitol salt agar (**Chapman, 1945**) used as a selective and differential medium as the growth of *Staphylococcus aureus* appears yellow while *Staphylococcus epidermidis* appears white.

(8): Blood agar (**Oxoid Manual, 1981**)**,** 100 ml of sterilized nutrient agar media in a flask was cooled to 40°C – 45°C before adding 5% defibrinated blood.

(9): Tryptic Soy Broth. (**Smith and Dell, 1990**), it contains casein peptone (17 gm), dipotassium hydrogen phosphate (2.5 gm), glucose (2.5 gm), sodium chloride (5 gm) soya peptone (3 gm)

2. Methods

Identification of *Staph. epidermids* and *E. coli:*

Three *Staphylococcal* strains were obtained in addition to twenty five bacterial isolates isolated from Tanta Cancer Center (10 samples) and the Faculty of Medicine, Tanta University (15 samples) from different human infected tissues during the period of 3 months from March 2010 to May 2010, the medical samples included pus, sputum, urine and stool.

The twenty five bacterial isolates were identified through studying their morphological, physiological and biochemical characteristics.

Morphological identification (Gram staining) revealed that, the isolates under study were classified as fifteen isolates belonging to Gram positive bacteria, while the other ten bacterial isolates were belonging to Gram negative bacteria and with concern to the best grown isolates; it was found that the isolate no. 7 (Gram positive cocci, from pus) and isolate no. 22 (Gram negative bacilli, from stool) were subjected to further studies concerning their identification into the species level.

They were tested for growth on mannitol salt agar as a specific and differential media for *Staphylococcus* ; they exhibited a heavy growth for isolate No. 7 and no growth for isolate No. 22 this may refers that isolate No. 7 might be *Staphylococcus epidermdis* as small white colonies.

Analytical profile index (API) 20 E: (bio-Merieux SA, Montalieu Verica, and france).

It is an identification system using standardized and miniaturized biochemical tests, used for biotyping to delineate different species.

Preparation of Human laryngeal Epithelial cells (HEp-2 cells).

Human epithelial cell lines used for testing anti-adhesion activity, were obtained frozen in liquid nitrogen (-180 °C) from the American Type Culture Collection. The cell lines were maintained in the Tissue Culture Unite (TCU), The Holding Company for Production of Vaccines Sera and Drugs (VACCERA) Cairo, Egypt by serial sub culturing (**Andrei *et al*., 2000; Leivo *et al*., 2000**).

Cultivation of Human Epithelial cells:

HEp-2 cells were maintained in 10 ml (DMEM) containing 2.5 % fetal bovine serum (FBS) with 1% antibiotics (Ampicillin or chloramphencal or gentamicin). The culture were incubated in humidified

atmosphere with 5% CO_2 incubator, sub-cultivated once every 4 days and harvested from sub-confluent monolayer by washing with 5 ml Hank's balanced salt solution (HBSS). 1 ml of 0.25 % trypsin EDITA were added, incubated for 15 minutes then washed with DMEM and only used adherent cells (**El silk *et al*., 2003**)

Investigation of the adhesion of bacterial strains on eukaryotic human epithelial cell lines (HEp-2) (Adhesion assay).

Adherence of the Staphylococcus strains to human epithelial Cells (HEp-2) was studied using [3-(4, 5- Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] MTT assay. The principle of this assay is depend on cleavage of MTT by bacterial enzymes to formazan that was used to measure the adherence of bacterial strains to a fixed eukaryotic cell lines at 492 nm. (**Elsilk *et al*., 2003**)

Evaluation of the efficiency of different substances as anti-adhesion agents for the pathogenic bacterial strains.

From confluent HEp-2 cells in a 96-well microtiter plate the medium was removed and cells were fixed by adding 100 µl of 3.7 % paraform aldhyde at room temperature for 30 min. After washing three times with 50 µl saline solution (0.9 % NaCl), 2 ml of this fresh culture were used.

a) Evaluation of plant extracts as anti-adhesion agents.

Fresh leaves and aerial parts of the three different plant species *Nigella sativa, Trigonella fosnum and Eucalyptus globules* were collected during spring 2010 from different localities. The identification of the plant material was confirmed at Pharmacognosy Department, Faculty of Pharmacy, and University of Tanta. The plants were dried at room temperature and were then milled by electric miller. 100 gm was added to 500 ml of 70% ethanol, left in room temperature for 24 hrs. The mixture was then filtered, kept overnight and then evaporated until dryness. (**Al-Bakri *et al*., 2010**).

b) Evaluation of carbohydrates and inorganic compounds as anti-adhesion agents.

Different carbohydrate compounds including; monosaccharides (glucose, fructose, xylose, arabinose and galactose); disaccharides (sucrose, maltose and lactose), polysaccharides (cellulose and starch) and inorganic compounds including (NaCl & $CaCl_2$) were tested for acting as anti-adhesion agents as the following:

Two ml fresh culture of the tested bacterial strains were centrifuged and suspended in 1ml broth medium containing 1% of the tested compound, compared with control sample (without compound addition).

The tubes were incubated at 37° C for one hour, centrifugation for 5 minutes and then wash the pellet by 100µl HBSS. Centrifuged again and the pellet was resuspended in broth medium. The plates were inoculated with 100µl of cells treated by the compounds; the plates were then centrifuged for 10 minutes at 700 rpm and incubated for one hour at 37°C. The plates were washed five times with 100 µl 0.9 % NaCl and 50 µl DMEM/MTT (1mg/1ml) was added, after 3 hours at 37°C, the medium was removed and the violet crystals were dissolved with 100 % methanol (50 µl /well),after shaking horizontally, plates were read by ELISA reader at 492 nm.

C) Evaluation of the different concentrations of the best anti-adhesion agents.

For the purpose of detection the best concentration of the best anti-adhesion substances (Starch and $CaCl_2$), different concentrations (0.5, 1, 1.5 and 2 %) of the two substances were tested for their activity as anti-adhesion agents through mixing with two ml of the freshly prepared bacterial culture then added to HEp-2 cells.

Investigation of the adhesion, invasion and anti-adhesion of *Staphylococcus epidermidis* using Scanning Electron Microscope (SEM) was performed as the following: two ml fresh culture of *Staph. Epidermidis* were added to 1 ml of human epithelial cell culture, incubated at 37°C for three hours then centrifuged. The bacterial pellet was fixed and examined by (SEM) for observation of cell adherence. For the purpose of investigation of cell invasion, the cells were incubated for six hours then investigated by (SEM).

Anti- adhesion of *Staphylococcus epidermidis* was investigated through suspension of the bacterial culture in 1 ml of the best concentration (Starch and $CaCl_2$) of the most anti-adhesive effect of the tested substances then investigated by (SEM).

3. Results

The work in this study included three standard *staphylococcal* strains obtained; *Staph. aureus* ATCC 25923, *Staph. aureus* ATCC 6538, *Staph. aureus* NCTC 6571 and twenty five bacterial isolates isolated from different human infected tissues which were identified as *Staph. epidermids* from pus and *E. coli* from stool.

1) Determination of Adhesion of freshly and overnight prepared bacterial cultures on (HEp-2):

The results in figure (1) explained that, the maximum value of the bacterial adherence was recorded in the case of fresh cultures in *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* and *E. coli* while *Staph. aureus* NCTC 6571 only showed more adherence in the case of overnight culture rather than fresh culture.

2) Effect of plant extracts as anti-adhesion agents on HEp-2 cells.

Trigonella fosnum, *Eucalyptus globules* and *Nigella sativa* were tested for their anti-adhesion activity. It was found that all plants affect on adhesion of all strains. The effect changed from strain to another one. The results indicated that the maximum effect was on ***E. coli, Staph. aureus NCTC 6571, Staph. epidermidis*** and ***Staph. aureus ATCC 25923*** respectively with *Nigella sativa* while ***Staph. aureus ATCC 6538*** only gave the best result with *Eucalyptus globules*.

So *Trigonella fosnum* was found to have the minimum effect as anti-adhesion agent.

Results of anti-adhesion activities of the tested plant extracts were represented in figure (2).

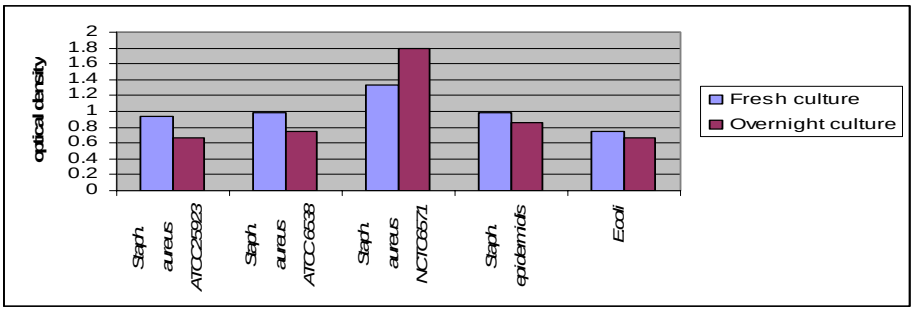

Figure (1): Adhesion of freshly and overnight prepared bacterial culture on (HEp-2) cells showing values of optical density at 492 nm of different tested bacterial strains as an indicator to the degree of adhesion in fresh and overnight cultures.

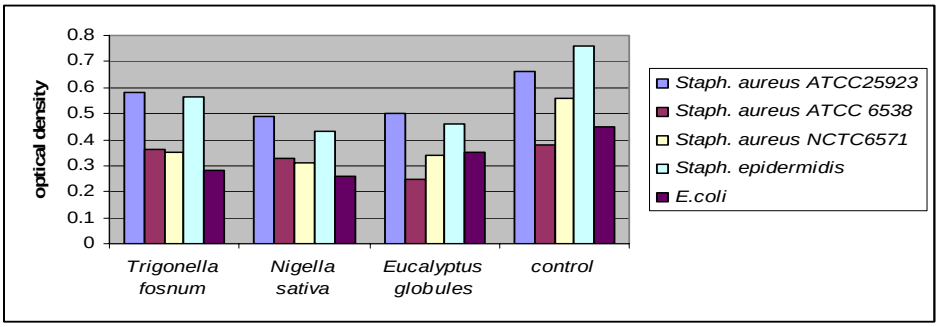

Figure (2): Effect of plant extracts as anti-adhesion agents on HEp-2 cells showing values of optical density at 492 nm of different tested bacterial strains as an indicator of the degree of adhesion in the presence of different plant extracts in comparison to a control (free from plant extracts).

3) Effect of monosaccharide as anti-adhesion agents on HEp-2 cells.

Different monosaccharide sugars including (glucose, fructose, xylose, arabinose and galactose) were tested for their activity as anti-adhesion compounds on HEp-2.

It was found that all monosaccharide affect on adhesion of all strains. The effect changed from strain to another one. The maximum effect on all strains was with glucose except ***Staph. aureus ATCC 25923*** which recorded maximum effect with galactose in comparison with control which contained no sugars.

Results of anti-adhesion activities of the tested monosaccharide were represented in figure (3).

4) Effect of disaccharide as anti-adhesion agents on HEp-2 cells.

Different disaccharide sugars including (sucrose, maltose and lactose) were tested for their activity as anti-adhesion on HEp-2 cells.

Finally it was found that the maximum effect on all strains were with sucrose except ***Staph. aureus ATCC 25923*** which recorded maximum effect with lactose in comparison with control .

Results of anti-adhesion activities of the tested disaccharide were represented in figure (4).

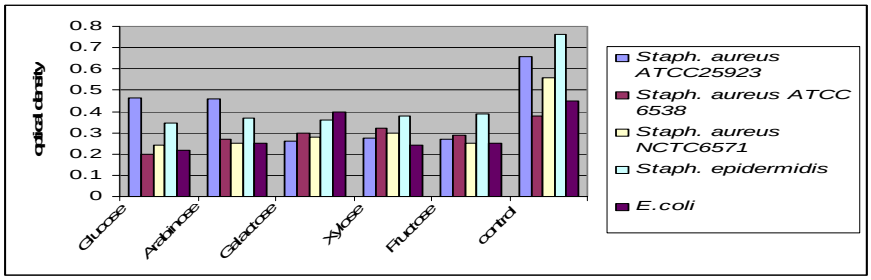

Figure (3): Effect of monosaccharide as anti-adhesion agents on HEp-2 cells showing values of optical density at 492 nm of different tested bacterial strains as an indicator of the degree of adhesion in the presence of different monosaccharide in comparison to a control (free from monosaccharide).

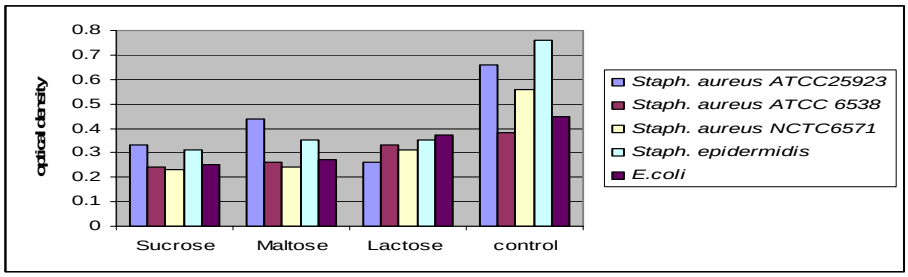

Figure (4): Effect of disaccharide as anti-adhesion agents on HEp-2 cells showing values of optical density at 492 nm of different tested bacterial strains as an indicator of the degree of adhesion in the presence of different disaccharide in comparison to a control (free from disaccharide).

5) Effect of polysaccharide as anti-adhesion agents on HEp-2 cells.

Also Different polysaccharide sugars including (starch and cellulose) were tested for their anti-adhesion activity on HEp-2 cells.

It was found that the maximum effect on all strains ***Staph. aureus ATCC 25923***, ***Staph. aureus ATCC 6538***, ***Staph. aureus NCTC 6571, Staph. epidermidis*** and ***E. coli*** were with starch which gave best result rather than cellulose in comparison with control which contained no sugars.

Results of anti-adhesion activities of the tested polysaccharide were represented in figure (5).

6) Evaluation of inorganic compounds on HEp-2 cells as anti-adhesion agents.

Different inorganic compounds including (NaCl & $CaCl_2$) were tested for their activity as anti-adhesion agents.

It was found that the maximum anti-adhesion of the tested inorganic compounds was recorded in $CaCl_2$ with **Staph. *aureus ATCC 25923***, ***Staph. aureus ATCC 6538*, Staph. *aureus NCTC 6571 and Staph. epidermidis***, while NaCl was found to give the maximum anti-adhesion result with only ***E. coli*** on HEP-2 cells in comparison with control which contained no sugars.

Results of anti-adhesion activities of the tested inorganic compounds were represented in figure (6).

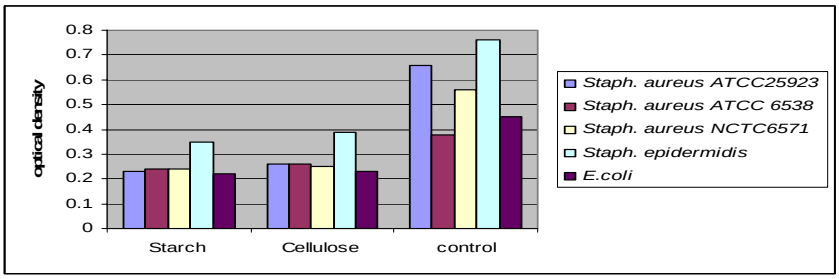

Figure (5): Effect of polysaccharide as anti-adhesion agents on HEp-2 cells showing values of optical density at 492 nm of different tested bacterial strains as an indicator of the degree of adhesion in the presence of different polysaccharide in comparison to a control (free from polysaccharide).

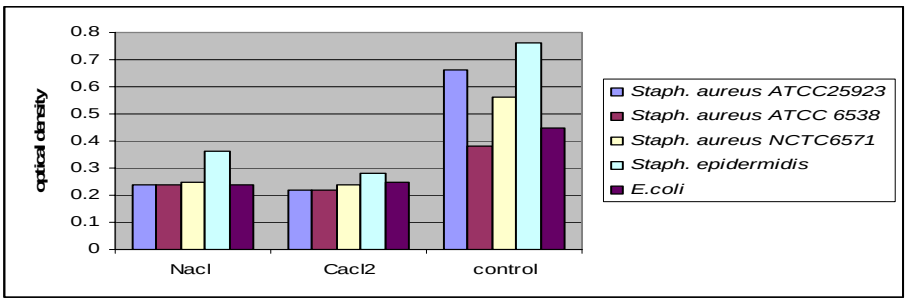

Figure (6): Effect of inorganic compounds as anti-adhesion agents on HEp-2 cells showing values of optical density at 492 nm of different tested bacterial strains as an indicator of the degree of adhesion in the presence of different inorganic compounds in comparison to a control (free from inorganic compounds).

7) Effect of different starch concentrations on HEp-2 cells.

Different concentrations of starch (0.5, 1, 1.5 and 2 %) were tested as anti-adhesion agents.

It was found that the maximum anti-adhesion was found by increasing the concentration of starch, so the maximum anti-adhesion of starch was recorded at 2 % for all strains followed by 1.5 %, 1 gm and then 0.5 %.

So by increasing concentration the effect increases on all strains under study.

Results of anti-adhesion activities of starch were represented in figure (6).

8) Effect of different $CaCl_2$ concentrations on HEp-2.

Different concentrations of $CaCl_2$ (0.5, 1, 1.5 and 2 %) were tested as anti-adhesion agents on HEp-2 cells.

It was found that the maximum anti-adhesion was found by increasing the concentration of $CaCl_2$, so the maximum anti-adhesion of $CaCl_2$ was recorded at 2 % for all strains followed by 1.5 %, 1 % and then 0.5 %.

So by increasing concentration the effect increases on all strains under study.

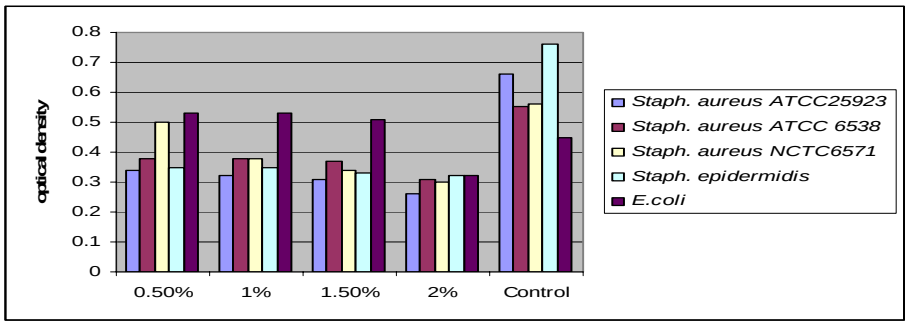

Figure (7): Effect of different starch concentrations on HEp-2 cells showing values of optical density at 492 nm of different tested bacterial strains as an indicator of the degree of adhesion in the presence of different concentrations of starch in comparison to a control (free from starch).

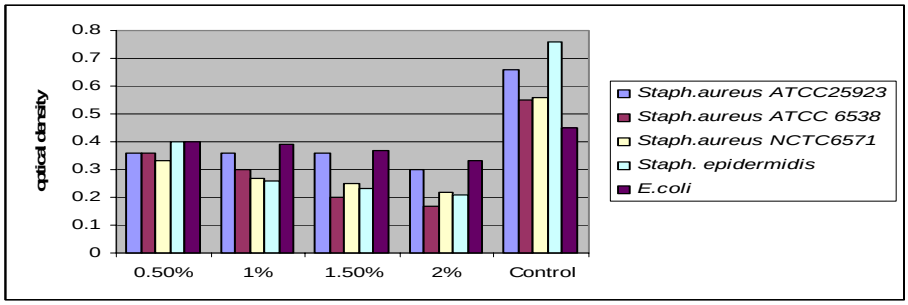

Figure (8): Effect of different $CaCl_2$concentrations on HEp-2 cells showing values of optical density at 492 nm of different tested bacterial strains as an indicator of the degree of adhesion in the presence of different concentrations of $CaCl_2$in comparison to a control (free from $CaCl_2$).

Investigation of adhesion, invasion and anti-adhesion of *Staph. epidermidis* by scanning electron microscope.

For the purpose of investigate cell adhesion the cells were further incubated for three hours then investigated by (SEM), and also incubated for six hours to investigate cell invasion while anti-adhesion of *Staphylococcus epidermidis* was investigated through suspension of the bacterial culture in 1 ml of the 2 % starch then investigated by (SEM). Results of Adhesion, invasion and anti-adhesion of *Staphylococcus epidermidis* by scanning electron microscope was illustrated in plates No. 1, 2, 3&4.

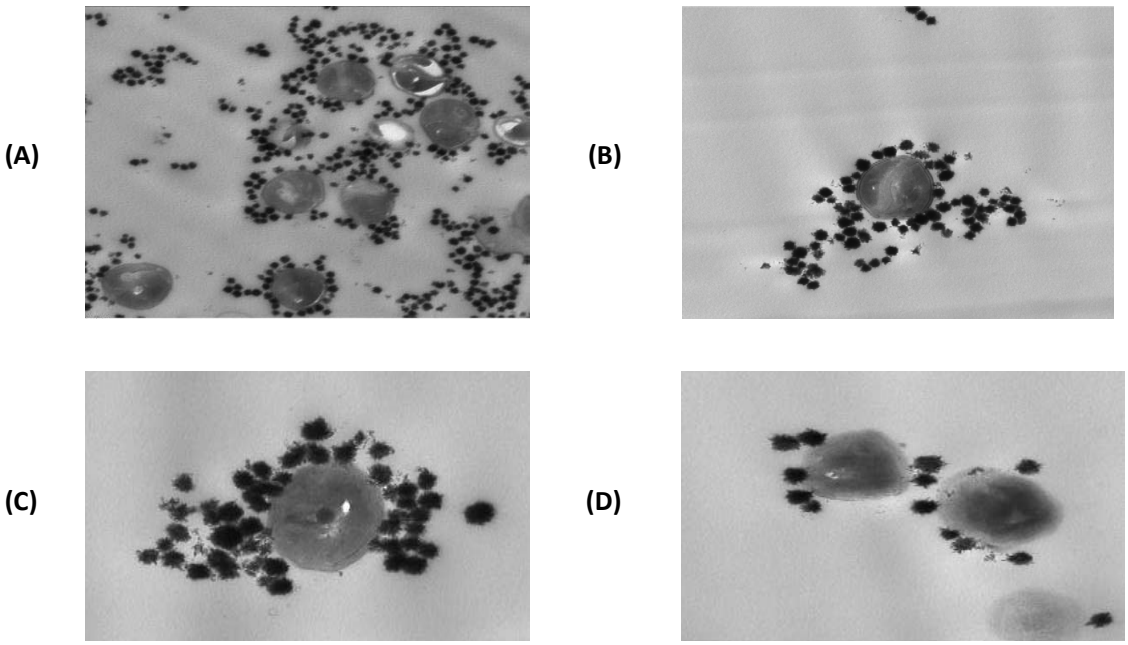

Plate no. 1: Scanning electron microscope photographs showing attachment of *Staph. epidermidis* cells to HEp-2 cells at different magnifications A(X 6000), B(X 8000), C&D(15000)

The previous photos (A, B, C & D) showed bacterial cells *Staph. epidermidis* and human epithelial cells in the first stage of microbial infection (adhesion)and the arrow showed the place of attachment.

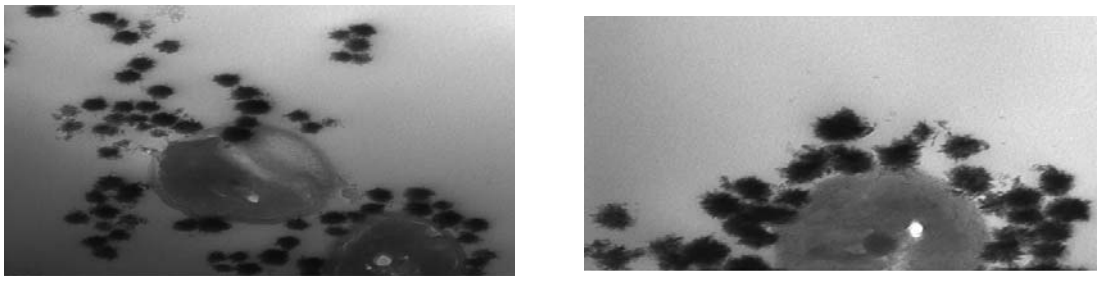

Plate no. 2: Scanning electron microscope photographs showing *Staph. epidermidis* cells invade to HEp-2 cells at magnification x 15.000

The previous plate showed *Staph. epidermidis* and human epithelial cells in the stage of invasion and the arrow showed the place of invasion.

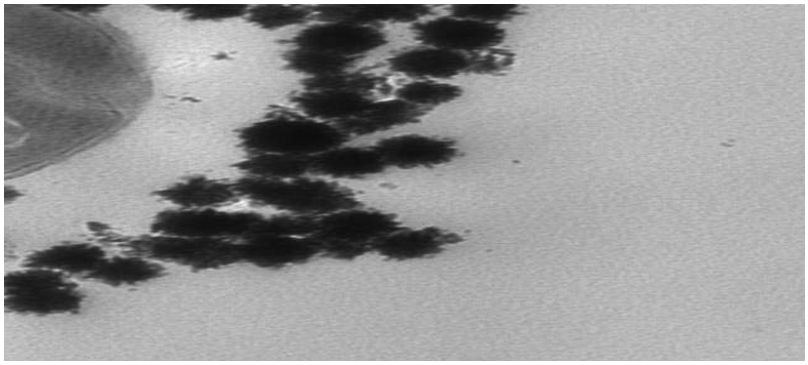

Plate No. (3): Scanning electron microscope photograph showing prevention of adhesion of *Staph. epidermidis* to HEp-2 by starch used as antiadhesion agents magnification was (× 20.000)

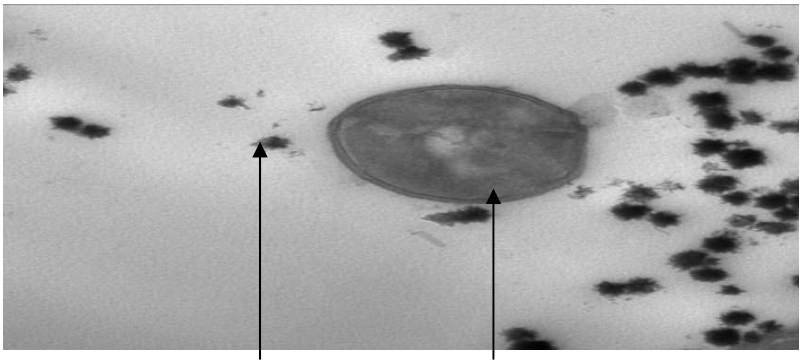

Plate No. (4): Scanning electron microscope photograph showing prevention of adhesion of *Staph. epidermidis* to HEp-2 by $CaCl_2$ used as anti adhesion agents magnification was (× 20.000)

Plate showed ***Staph. epidermidis*** away from human epithelial cells by the action of **$CaCl_2$** as anti-adhesion

4. Discussion

The strategy for using adhesion analogs to prevent infections is based on the assumption that the isolated adhesion molecule, or an active synthetic or recombinant fragment, binds to the receptor and competitively blocks adhesion of the bacteria (**Ofek *et al.*, 2003a**).

Bacterial adhesion to epithelial cells is a key step in infections, allowing subsequent colonization, invasion and internalization of pathogens into tissues. Antiadhesive agents are therefore potential prophylactic tools against bacterial infections. The range of anti-adhesive compounds is largely confined to carbohydrate analogues (**Aneta and Herbert, 2010**).

This study have been aimed at providing a basis for the developments of methods showing new means of controlling the emergence and adhesion of pathogenic *Staphylococcus* strains, through screening of different substances (plant extracts, organic and inorganic compounds) as control agents to prevent their colonization. (**Ehsanollah *et al.*, 2009**) reported that one of the key steps in controlling nosocomial infectious by MRSA could be through preventing their colonization.

Adhesion of pathogenic organisms to host tissues is the prerequisite for the initiation of the majority of infectious diseases. Five bacterial strains (*Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC 6538, Staphylococcus aureus NCTC 6571, Staphylococcus epidermidis & Escherichia coli*) were tested for their adherence activity through adding of freshly prepared and overnight incubated cultures to the HEp-2 in order to determine the optimum state for the bacterial adherence; it was found that the freshly prepared culture recorded maximum values in comparison with values of overnight incubated cultures, This may be return to the cell wall of fresh culture as it have higher water content (**Soto and Hultgren, 1999**). At the same time the maximum value of the bacterial adherence was recorded in the case of the strain of *Staph. Aureus NCTC 6571*, followed by *Staph. epidermidis*. Similar results were obtained by **Blickwede *et al.*, 2000.**

During the infectious process, inflammatory stimuli activate vascular endothelial cells to express adhesion molecules and chemokines that physically engage circulating leukocytes. A coordinated sequence of adhesion and locomotion steps, including (i)

leukocyte rolling, (ii) cell activation, (iii) firm cell adhesion, and (iv) transendothelial migration, requires that adhesion receptors on leukocytes and endothelial cells are up-regulated and activated (**Springer, 1994**).

There is evidence that the receptor analogs work as agents for anti-adhesion therapy would be practical primarily against pathogens that bind to animal cells via carbohydrate-specific adhesins (i.e. lectins). In these cases, the receptor analogs are saccharides that are structurally similar to receptors for the adhesion and therefore, act by competitive inhibition. It was less than three decades ago that mannose was first shown to be a receptor for enterobacteria (**Ofek *et al.*, 1977**). Since then, the sugar specificities of many bacteria have been determined, leading to the development of receptor-like carbohydrates, which inhibit the adhesion of pathogens to host cells and tissues (**Ofek *et al.*, 2003a**).

Although plant lectins are well represented in the human diet and many of these lectins are well characterized (**Nachbar and Oppenheim, 1980; Liener, 1986; Cowan, 1999**). Their application to anti-adhesion therapy is very limited. Theoretically, these lectins could interact with animal cell surface saccharides to block adhesion mediated by lectin-carrying bacteria and they may enhance clearance of bacteria from the host (**Slifkin and Doyle, 1990**).

Because of their ready availability, plant materials possessing anti-adhesin activities are attractive candidates for antibacterial agents. There is, however, a relative paucity of information regarding the anti-adhesive properties of most plant materials. It was found the maximum anti-adhesion activity of the tested plant extracts was recorded in the case of *Trigonella fosnum* extract, followed by the extract of *Eucalyptus globules* then *Nigella sativa*; compared with control values**.Ehsanollah et al., 2009 ,** reported that, the oil based di-herbal extract formulated in this study shows a very good antimicrobial, anti-adhesive and anti-invasive activity against MRSA.

Thus, it appears only reasonable that much attention has been paid to carbohydrates as anti-adhesive agents of potential medicinal value that block the surface adhesions. Bearing in mind the well-known capabilities of proanthocyanidins to interact with macromolecules, including carbohydrates and proteins, members of this class of compounds may be another group of promising anti-adhesive compounds (**Aneta and Herbert, 2010**).

It was found the maximum anti-adhesion activity of the tested monosaccharides sugars was recorded in the case of glucose, followed by fructose and arabinose in comparison with control values. Sucrose and glucose are dietary sugars commonly consumed in the western world and the results reported here imply that diets rich in these sugars may facilitate adhesion and colonisation of the oral mucosa (**Samaranayake *et al.*, 1980; Samaranayake and MacFarlane, 1980**) and these findings have been confirmed by (**McCourtie and Douglas 1981**) Blocking or inhibition of these lectins by suitable carbohydrates or their analogs for the prevention and treatment of microbial diseases is the aim of anti-adhesion therapy of such diseases (**Kahane and Ofek, 1996; Zopf and Roth, 1996;Karlsson, 1998; Kelly and Younson, 2000; Sharon and Ofek, 2001; Ofek *et al.*, 2003**), while the maximum anti-adhesion activity of the tested disaccharides sugars was recorded in the case of sucrose, followed by maltose and lactose in comparison with control values. Also the maximum anti-adhesion activity of the tested polysaccharide sugars was recorded in the case of starch, followed by cellulose in comparison with control values.

Very significantly, lectin-inhibitory saccharides have been shown to protect mice, rabbits, calves and monkeys against experimental infection by lectin-carrying bacteria (**Nathan, 2006**).

Different inorganic compounds including (NaCl & $CaCl_2$) were tested for their activity as anti-adhesion agents.

It was found the maximum anti-adhesion activity of the tested inorganic compounds was recorded in the case of $CaCl_2$, followed by NaCl in comparison with control values.

But it was revealed that calcium ions significantly increased the binding of tested lactobacilli to IPEC-J2 cells; and therefore, added calcium may be useful in enhancing the adhesion of normally weakly adhesive probiotic cultures. The increase of adhesion in the presence of Ca^{2+} is due to the creation of additional Ca^{2+} mediated bonds which shield more hidden length (**Nadja *et al.*, 2007**)

Different concentrations of starch were tested for their activity as anti-adhesion agents. And the maximum anti-adhesion activity of the tested concentrations was recorded.

These results imply that exogenous or endogenous carbon sources may affect the oral and vaginal carriage of *C. albicans*, by modifying their adhesive properties.

Different concentrations of $CaCl_2$ were tested for their activity as anti-adhesion agents. And the maximum anti-adhesion activity of the tested concentrations was recorded.

It has thus far been impractical to use analogs of adhesions for anti-adhesion therapy, because they are typically macromolecules that are not readily available and because they must be employed at relatively high concentrations. In addition, careful consideration must be given to their toxicity and immunogenicity. Nevertheless, modern proteomics and recombinant biotechnology have permitted the development of

unique types of relatively small peptides for anti-adhesion therapy, as reported by (**Kelly *et al*., 1999**).

Many extracts not only has antibacterial activity which will be very useful in the treatment, but also has anti-adhesive and anti-invasive property that adds the value of the extract to be a colonization inhibitor, hence the extract could be used for treatment and prophylaxis (**Ehsanollah *et al*., 2009**).

Investigation the adhesion, invasion and anti-adhesion of *Staphylococcus epidermidis* were carried out, investigation the adhesion of *Staphylococcus epidermidis*, was carried out after incubation.

Also investigation of cell invasion was carried out from six hours incubated bacterial culture. While anti-adhesion of *Staphylococcus epidermidis* was investigated through suspension of the bacterial culture.

Surface modification is an effective way to decrease bacterial adhesion. In this study, we prepared surfaces with different wettability on titanium surface based on TiO_2 nanotube to examine the effect of bacterial adhesion. Observed by SEM and contact angle measurements, the different surfaces have different characteristics (**Tang *et al*., 2011**).

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Histological and Biochemical Effects of Diazinon on Liver and Kidney of Rabbits

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Abstract: The present study was carried out to investigate the effect of diazinon on histological and biochemical aspect of liver and kidney of rabbit. Diazinon induced blood vessel congestion, leucocytic infiltrations in the liver parenchyma in addition to cytoplasmic vacuolation, fatty degeneration and pyknotic nuclei in the hepatocytes. On the other hand, renal damage was observed in the kidneys of treated rabbits. Renal tissues showed hypertrophied glomeruli, destructive of its lining epithelia. Renal blood vessels were congested and the inter-tubular spaces were filled with red blood cells. Biochemical investigation proved that treatment with diazinon for 4 weeks induced a significant increase in ALT, AST, creatinine and blood urea. Finally, the investigators concluded that diazinon toxicity induced hepatocellular and renal damage.

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Key Words: Diazinon, liver, kidney, rabbit, histology and biochemistry.

1. Introduction

Pesticides are synthetic organic compounds that are deliberately introduced into the environment to control selected organisms. Contact with organophosphorus pesticides is an important health problem for agricultural workers (Hurtig *et al*., 2003). Some of these pesticides are highly toxic for mammals (Abdel-Salam and Ford, 1987; El-Shenawy *et al*., 2009). Organophosphorus insecticides are used throughout the world for control of agricultural and domestic insect pests. Diazinon [phosphoric acid, O, O-diethyl O (2-isopropyl-6-methyl-4-pyridinyl)] phosphorothioate is an organophosphorus insecticide widely used in agricultural practice throughout the world to control flies, lice, and other insect pests of ornamental plants and food crops (Johnson and Hanstbarger, 1966). Due to extensive use of diazinon, its residues have been detected in foodstuffs designed for human consumption (Johnson and Manske, 1977). The toxic effects of diazinon on animals were studied by some investigators (Abdou and ElMazoudy, 2010; Shah and Iqbal, 2010). Ceron *et al*. (1996) reported that diazinon inhibits acetylcholinesterase activity and other organic functions. Diazinon was also found to lead to alterations in blood factors, plasma testosterone and glucose levels in male rats (Alahyary *et al*., 2008). Oral administration of diazinon to mice resulted in decrease in splenic T-dependent antibody response to DNP-ficoll and a dramatic thymus atrophy (Kump *et al*., 1996). Other studies have indicated that diazinon has the capacity to disrupt reproductive function in animals (Rodriguez and Bustos-Obregn, 2000 and Yehia et *al*., 2007). Gokcimen *et al*. (2007) reported that diazinon induced histopathological changes in liver and pancreas of rats. Diazinon treatment induced hematological changes (Kalender *et al*., 2006) as well as hepatotoxicity (Kalender *et al*., 2005) in rats. The present work aims to investigate the histological and biochemical effects of diazinon on liver and kidney of rabbits.

Materials and Methods

Animals and treatment

15 Male New Zealand white rabbits weighing 1.8-2 kg were housed in the laboratory at controlled light and temperature. They were provided with rabbit chow and fresh water. Animals were divided equally into 3 groups:

Group 1. Animals of this group were considered as controls.

Group 2. Animals of this group were given diazinon in drinking water at a dose level of 20 mg/kg body weight, every 48 hrs for 2 weeks.

Group 3. Animals of this group were given diazinon in drinking water at a dose level of 20 mg/kg body weight, every 48 hrs for 4 weeks. Diazinon was applied as commercial emulsifiable concentrate formulation containing 60% active ingredient, then, it was further diluted in distilled water to obtain the desired concentration.

Histological examination

The treated animals and their controls were killed, quickly dissected and their liver and kidney were removed, sliced and fixed in Bouin's fluid. After 24 h, tissues were rinsed three times in 70% ethanol, dehydrated using a graded ethanol series and then embedded in paraffin wax. Paraffin sections were cut

into 5 micrometers thick slices and stained with haematoxylin and eosin and examined under light microscope.

Biochemical assays

Blood was collected from controls and treated animals after 2and 4 weeks of treatment. For biochemical study sera were obtained by centrifugation then creatinine and urea were determined using Henry's methods (1974). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically according to Reitman and Frankel (1957).

3. Results

Histological results:

Figure 1 showed histological structure of liver in control rabbit. The liver is divided into hepatic lobules formed of radially arranged strands of hepatocytes that extend from the central vein to periphery of the lobule. The hepatocytes strands are separated from each other by blood sinusoids that are lined with the endothelial cells and Kupffer cells. Treating animals with diazinon caused several histopathological alterations. After 2 weeks of treatment (group 2), the blood vessels were congested and the sinusoidal spaces were filled with blood (Fig. 2). The hepatocytes plates were disrupted and the cells were swollen and vacuolated (Fig. 3). Focal inflammatory cells infiltration was abundant (Fig.4). These histopathological alterations were severed in animals examined after 4 weeks (group 3). The blood vessels, central and portal veins were severely congested and bile ducts were degenerated (Fig. 5). The sinusoidal spaces were dilated and filled with red blood cells. The hepatocytes appeared with cytoplasmic vacuolation and pyknotic nuclei (Fig. 6). Some signs of fatty degeneration were observed (Fig. 7).

Histological examination of the kidney of control rabbit revealed normal histological features, illustrated in figure (8). The administration of diazinon caused many histological damage to the renal cortex. Examination of the kidney sections of animals after treatment with diazinon for 2 weeks (group 2), showed that renal blood vessels were congested (Fig. 9). Most of renal tubules were damaged and lost their characteristic appearance and their lining epithelial cells were destructed. The glomeruli were hypertrophied (Fig. 10). After 4 weeks (group 3), the renal tubules were severely damaged and their cells showed cytoplasmic vacuolation and some glomeruli were atrophied (Fig. 11). Blood capillaries in between the degenerated tubules were congested (Fig. 12).

Biochemical results

Treating animals with diazinon induced an increase in ALT and AST. This increase became significant ($P<0.05$) after 4 weeks of treatment (Figs.13&14). Similarly, creatinine and blood urea increased significantly ($P<0.05$) in the rabbits determined after 4 weeks of treatment with diazinon (Figs.15&16).

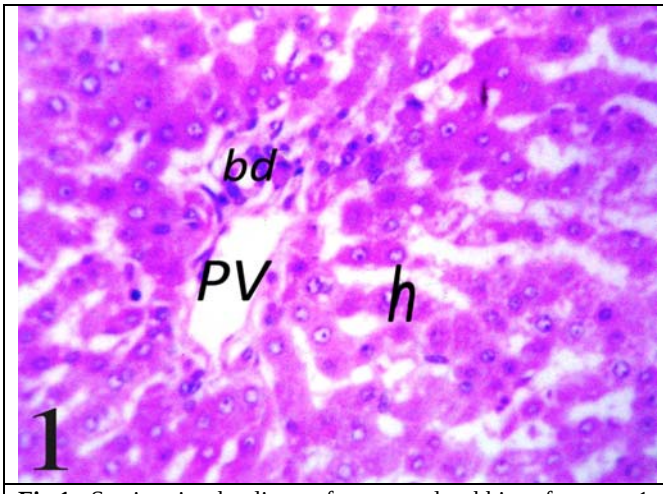

Fig.1. Section in the liver of a control rabbit, of group 1, showing portal vein (PV) and bile duct (bd) in the portal area and surrounding hepatocytes (h), (X 200).

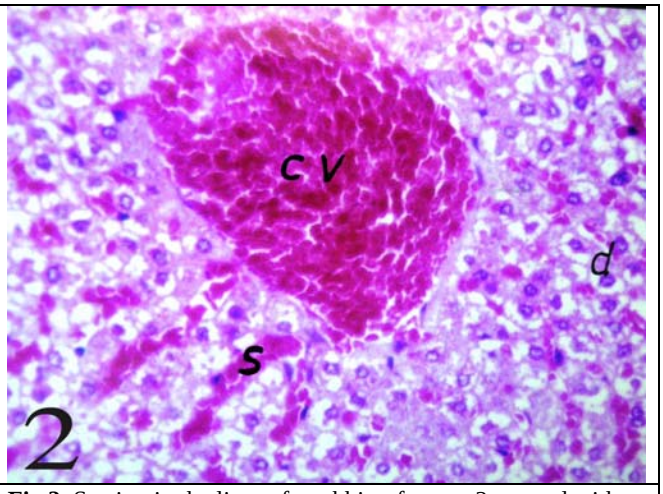

Fig.2. Section in the liver of a rabbit, of group 2, treated with diazinon for 2 weeks showing severe congestion in central vein (CV) and sinusoids (S) with degeneration in the hepatocytes (d), (X 200).

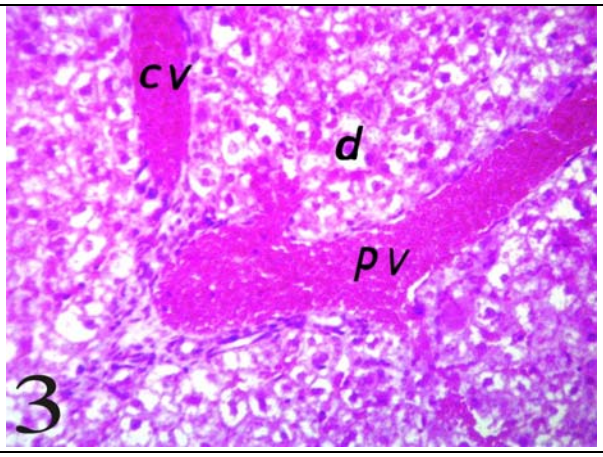

Fig.3. Section in the liver of a rabbit, of group 2, treated with diazinon for 2 weeks showing congestion of central (CV) and portal (PV) veins. The degenerated hepatocytes (d) showed cytoplasmic vacuolation, (X 200).

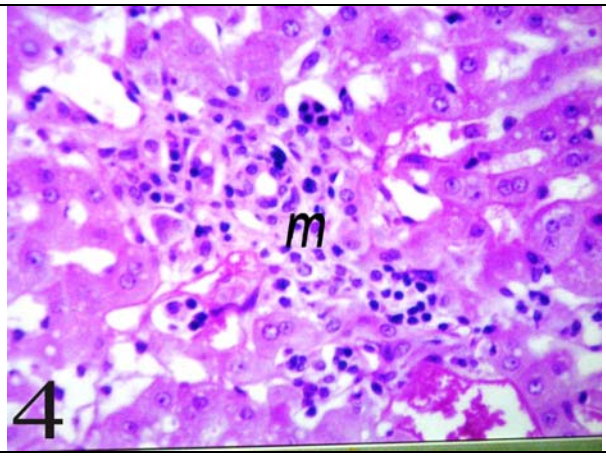

Fig.4. Section in the liver of a rabbit, of group 2, treated with diazinon for 2 weeks showing focal inflammatory cells infiltration (m) in hepatic parenchyma, (X 200).

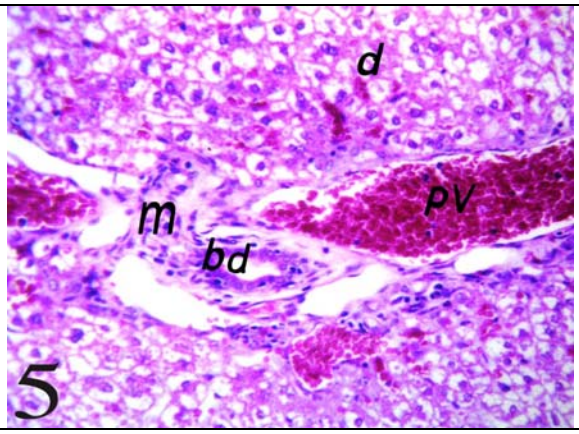

Fig.5. Section in the liver of a rabbit, of group 3, treated with diazinon for 4 weeks showing degenerated hepatocytes (d), congestion in portal vein (PV), inflammatory cells infiltration (m) and degenerated bile duct (bd), (X 120).

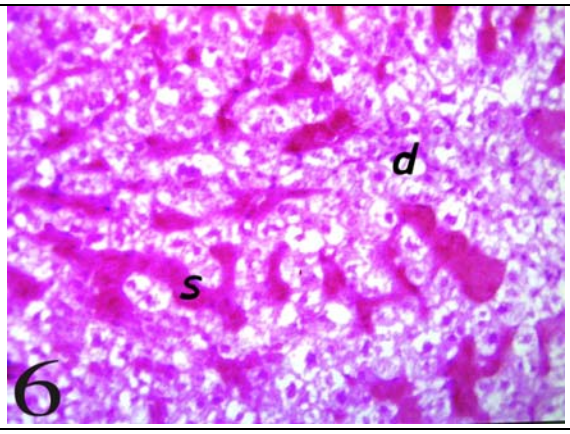

Fig.6. Section in the liver of a rabbit, of group 3, treated with diazinon for 4 weeks showing degenerated hepatocytes (d) and severe congestion in the sinusoids (S) between them, (X 120).

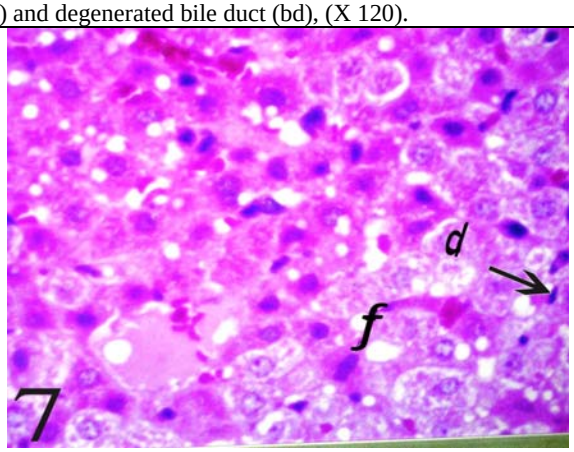

Fig.7. Section in the liver of a rabbit, of group 3, treated with diazinon for 4 weeks showing degenerated hepatocytes (d), fatty degeneration (f) and activated Kupffer cells (arrow), (X 200).

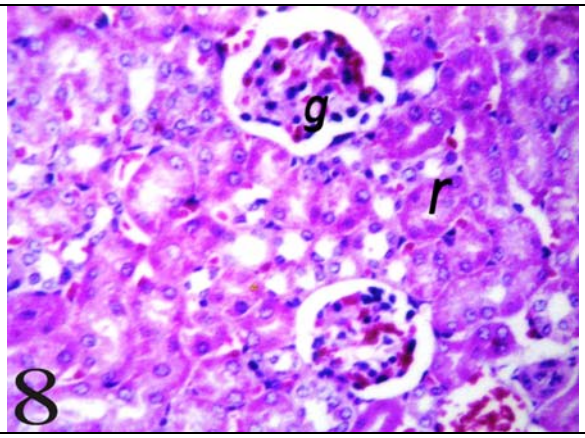

Fig.8. Section in the kidney of a control rabbit showing normal renal tubules (r) and glomerulus (g), (X 200).

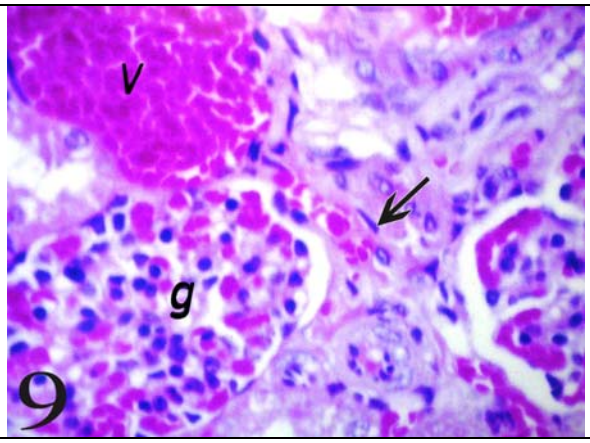

Fig.9. Section in the kidney of a rabbit, of group 2, treated with diazinon for 2 weeks showing enlarged and congested renal vein (V), congested glomerulus (g), and intertubular fibrosis (arrow), (X 200).

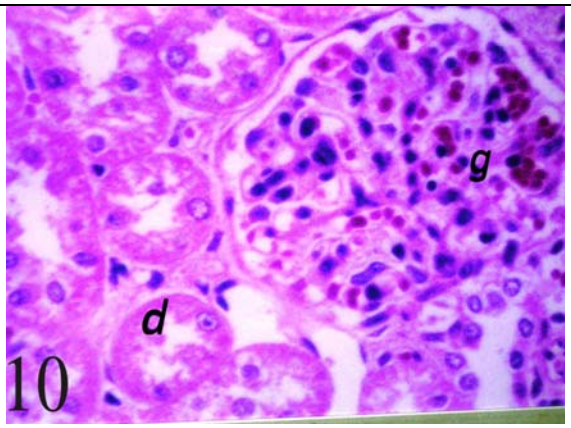

Fig.10. Section in the kidney of a rabbit, of group 2, treated with diazinon for 2 weeks showing hypertrophy, proliferation and swelling in the lining endothelium of the glomerulus tuft (g) with degeneration in the lining epithelial cells of renal tubules (d), (X 200).

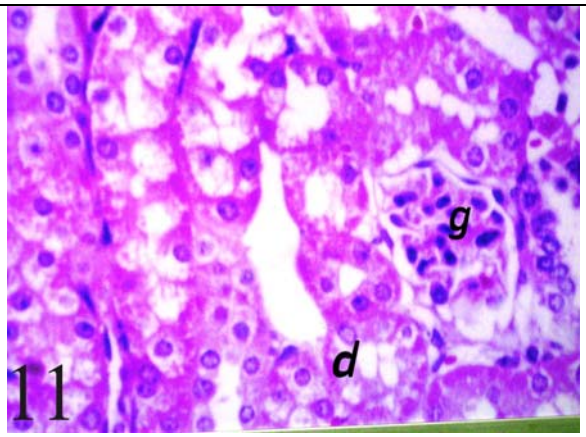

Fig.11. Section in the kidney of a rabbit, of group 3, treated with diazinon for 4 weeks showing atrophy of a glomerulus (g) with degeneration in the lining epithelial cells of renal tubules (d), (X 200).

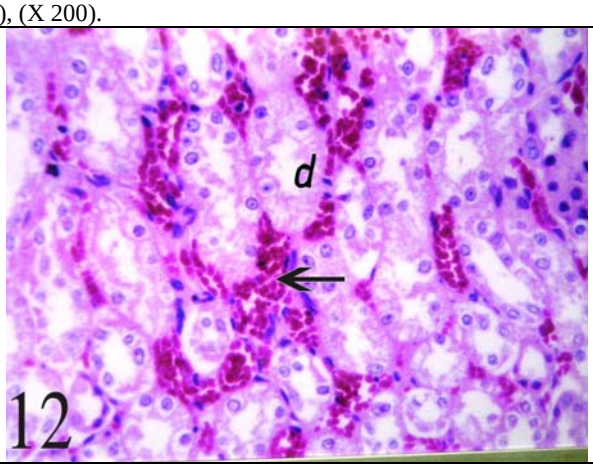

Fig.12. Section in the kidney of a rabbit, of group 3, treated with diazinon for 4 weeks showing congestion in blood capillaries (arrow) in between the degenerated renal tubules (d), (X200).

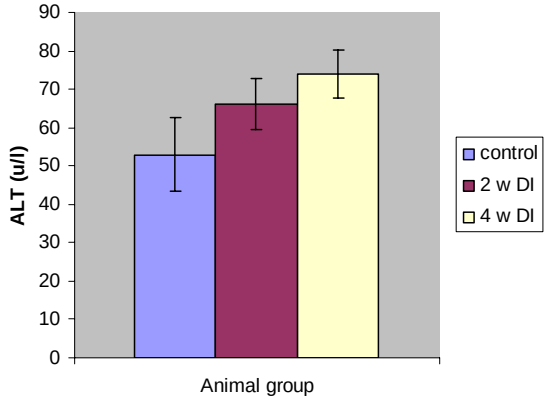

Fig.13. Effect of treatment with diazinon (DI) on ALT.

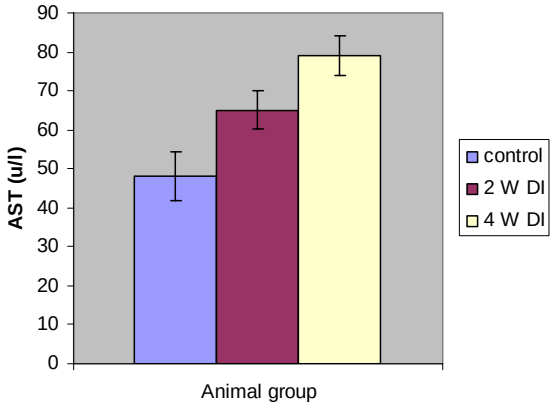

Fig.14. Effect of treatment with diazinon (DI) on AST.

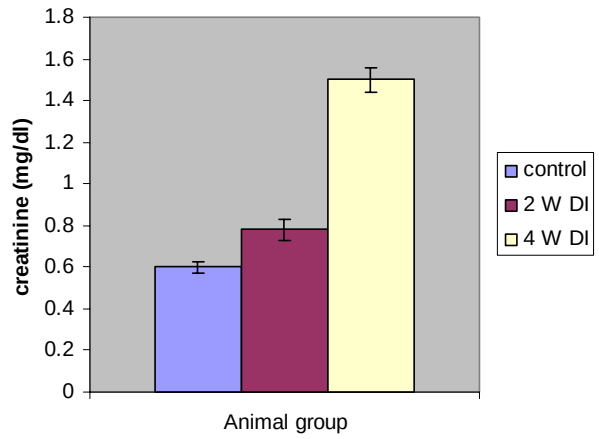

Fig.15. Effect of treatment with diazinon (DI) on creatinine.

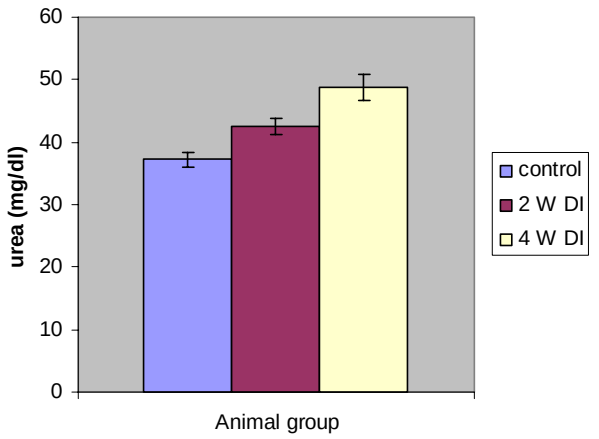

Fig.16. Effect of treatment with diazinon (DI) on serum urea.

4. Discussion

Results of the present work indicated that diazinon induced histopathological alterations in the liver of rabbit. The liver showed congestion of veins, leucocytic infiltrations, cytoplasmic vacuolation of the hepatocytes and fatty degeneration. Similarly, El-Shenawy *et al*. (2009) reported that intoxicated mice with diazinon resulted in hydropic degeneration, necrosis and focal microvesicular steatosis in liver. Jacqueson *et al.* (1977) and Anthony *et al*. (1986) observed that the liver of male Wistar rats chronically treated with sublethal doses of diazinon sustain a form of hepatic injury characterized by cellular lipid accumulation. Abdel-Salam and Ford (1987) showed that diazinon induced liver and kidney damage in ruminant live stock. They added that this hepatotoxic effect was enhanced by carbon tetrachloride, while the renal toxic effect was enhanced by mercuric chloride. Hyperplasia of hepatocytes, necrosis, lymphocytic infiltrations and steatosis were observed in rats treated with 1/20 LC_{50} of diazinon (Hassan *et al*., 2007). Cytoplasmic vacuolations were observed in hepatocytes of diazinon-treated animals. It is considered as a form of cell injury which is most frequent in parenchymal cells of liver with wide network of internal membranes that are concerned with ions pumping (Mori, 1987). Cytoplasmic vacuoles develop due to accumulation of ions and water in cytosol and rapidly pass through leaky membranes of cell organelles. Massive accumulation of fluids in the vacuoles may finally lead to cell lysis (Gores *et al*., 1990).

AST and ALT showed a significant increase in rabbits given diazinon. In agreement with these results, Ahmed (2006) who found elevation of transaminases (AST, ALT) in rats treated with 1/30 LD_{50} diazinon for 3 weeks. Kalender *et al.* (2005) recorded an elevation in ALT, AST, ALP, total cholesterol, and triglyceride levels in rats treated with diazinon. Transaminases were considered to be a more sensitive measure in evaluating liver function and damage (Sherlock, 1981). Hatoff and Hardison (1980) reported that elevations in serum levels of these enzymes were mostly attributed to acute hepatocellular damage or extrahepatic obstruction, or both.

The present results showed that diazinon treatment led to degeneration of renal tubules, hypertrophy of glomeruli and leucocytic infiltrations. These results indicated that diazinon metabolites caused toxicity in renal system; and the immune system makes a good role for defending against foreign particles. The effect of diazinon on kidney was studied in different animals. Oral administration of diazinon for 2 months to male albino rats showed degeneration of the renal tubules (Hassan *et al*., 2007). El-Shenawy *et al*. (2009) reported that exposing mice to diazinon caused degeneration of renal tubules, atrophy of glomeruli and interstitial inflammatory cells infiltrations.

The present results revealed also a significant increase in serum creatinine and urea in response to diazinon toxicity. Similar results were obtained by El-Shenawy *et al*. (2009) in mice. Diazinon (1/30 LD_{50}) markedly decreased serum urea but did not affect creatinine level (Ahmed, 2006). The increase in creatinine recorded in this work might be due to impaired kidney function by the used fungicide. This view was supported by Kluwe (1981) who indicated that an elevation of creatinine level in the blood is an indicative of impaired kidney function.

Toxicity of diazinon is realized through the inhibition of enzyme acetylcholinesterase whose biological role is the termination of impulse transmissions at cholinergic synapses within the nervous system by rapid hydrolysis of the neurotransmitter -acetylcholine (Schumacher *et al*., 1986). Other studies reported that oxidative stress plays

an important role in the toxicity of diazinon. Malondialdehyde is an indicator of lipid peroxidation, free radical generation and oxidative stress. Catalase and superoxide dismutase are antioxidant enzymes. Diazinon was found to increase malondialdehyde level and decrease antioxidant enzymes in rat erythrocytes (Sutcu *et al*., 2007). Treatment of rats with diazinon significantly enhances renal lipid peroxidation which is accompanied by a decrease in the activities of renal antioxidant enzymes (e.g. catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, glutathione S-transferase) and a depletion in the level of glutathione reduced (Shah and Iqbal, 2010). Abdou and ElMazoudy (2010) reported that diazionninduced significant increases in the level of serum malondialdehyde and the activity of lactate dehydrogenase in female rats. Treatment with diazinon induced significant increase in lipid peroxidation and decreased total antioxidant capacity in rat liver and muscle (Amirkabirian *et al*., 2007). The observed hepatotoxicity and nephrotoxicity in the present work may be attributed to oxidative stress generated by diazinon.

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