PATHOGENETIC EFFECTS OF MERCURY CHLORIDE IN WHITE RATS

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ABSTRACT

The study was performed on twelve white rats of approximately of the same body weight (200-220 gms) divided equally in to 3 groups ; The first group(T1) was received mercury chloride (1mg/kg B.W intraperitoneally once daily for 30 days).

While the second group (T2) was received mercury chloride (1.5mg/kg B.W intraperitoneally once daily for 30 days).Third group was received only0.2ml of Distilled water as control group.

At the end of experiment, the animals were sacrificed and small pieces of livers had been collected for genetic experiment. Also small specimens (2cm³) were taken from livers and kidneys to histopathology. The genetic experiment was showed that the T1 group demonstrated non-significant increase in p53 mRNA gene expression levels as compared with the control group, while the group T2 showed significant increase (p<0.05) in p53 mRNA gene expression level as compared with the control group. The microscopic examination of histopathological sections of livers and kidneys of (T2) group was showed severe pathological changes characterized by vaculation and necrosis of hepatocyte and marked atrophy of glomeruli, degenerative changes of epithelial layer of renal tubules with cast formation, hemorrhage and congestion. While (T1) group was showed less pathological changes in livers characterized by loss of radially arrangement of hepatocyte and dilatation of sinusoids. In kidneys there was atrophy of glomeruli, hemorrhage and congestion.

INTRODUCTION

Mercury is a serious ecological and manufacturing pollutant which causes acute changes in the body tissues of both humans and animals (1, 2) .water, soil, air and fish protein (as food sources) which contaminated with mercury act as the main source for animal toxicity(3).

Inorganic mercury complex come in water by various routs and afford a process of methylation (4). Organic mercury is absorbed from the lungs, gastrointestinal (GI) tract, and through the skin due to its high lipid solubility. The exposures to mercury may lead to a variety of adverse health effects including: neurological, renal, respiratory, immune, dermatological, reproductive, and developmental abnormalities(5). Furthermore, mutagenicity and teratogenicity of mercury has been reported in fish, birds and mammals(6).

Mercurous and mercuric ions make their toxicological action mainly by molecular reaction for example mercuric ions have high affiliation to sulfhydryl groups prescent particularity in the thiol containing molecules as GSH, cysteine, and metallothionein (MT)(7). However, the connection affinity of mercury to oxygen and nitrogen atoms is comparatively so depressed when contrasted to sulfur.(8). Mercury influences antioxidant mechanisms in the cell lead to cell degeneration, lack of membrane

safety and then cellular necrosis (9). Some data propose that mercury induced nuclear and genetic changes, such as a decrease in DNA synthesis and damages DNA, also some literatures suggest that Mercury treatment induces DNA single-strand breaks at low concentrations in mammalian cells (10). as well as changes in RNA and protein synthesis and cause apoptosis (11)

Apoptosis or programmed cell death is important mode of cell death occur physiologically during embryogenesis until old in multicellular organism (12). This wounderful process is responsible for cell death in development, normal tissue rotation, and as well as calculation for numerous cell deaths after exposure to cytotoxic compounds(13).Control of apoptosis is very complicated using each apoptotic and antiapoptotic factors like p53 gene(14). Some researchers have proposed that mercury could alter the level of pro-apoptotic protein p53 and caspase 3(15).

Histopathologically the liver is a major site of metabolism for mercury and it can accumulate in the liver, resulting in severe hepatic damages (16).

The aim of the study

The study was demonstrated to know the genetic and histopathological effects of Mercury Chloride in white rats.

MATERIALS AND METHODS

Experimental animals:

Twelve white rats (200-220gm) were obtained from animal house of Vet.Med. Collage of AL- Qadisiya University and prior to use the animals were acclimatized for 7 days at 12hrs. light/dark cycle. The animals were housed in plastic cages in an air-conditioned room with temperature maintained at 25±2 C. Rats were given food pellets and water ad libitum. Rats were divided randomly into three groups (4 rats each) and were treated for 30 day.

Chemicals:

Mercury chloride is a heavy metal obtained from central laboratory in AL-Qadisiya University. Mercury chloride(BDH chemical Ltd(England)). The rats administered 1.5mg/kg B.W and 1.0 mg/kg B.W (17) as chronic doses.

Experimental design:

Twelve white rats, both sexes were randomly divided into 3 groups (4 rats each) and were treated as following:

1st group was injected with (1mg/kg B.W) intraperitoneally .

2nd group was injected with (1.5mg/kg B.W) intraperitoneally.

3rd group was injected with (0.2ml) distal water as control group.

Tissue samples

The rats sacrificed and the liver tissues were dislocated by sterile scissor then directly dipped in liquid nitrogen (-196Cº), and then put in epindroff tubes contained DEPC water and sent to polymerase chain Reaction unit for gene expression of p53 mRNA by qTR- PCR and then 10% formalin fixed, small pieces (2cm3) were taken from livers and kidneys of all groups for histopathology.

Primers

Primers were designed using the primer3 plus (Primers sequences are listed in Table (1).

Table 1:The Primers sequences

Primer		Sequence	Amplicon	Gen Bank code
P53	F	ATCCTATCCGGTCAGTTGTTGG	143bp	NM_030989.3
	R	AATGCAGACAGGCTTTGCAG		
β-actin	F	CTAGGCACCAGGGTGTGATG	85bp	NM_031144.3
	R	GTCAGGATGCCTCTCTTGCTC		

Total RNA extraction

Total RNA were extracted from liver tissue by using (Accuzol® reagent kit. Bioneer. Korea) and done according to company instructions as follow; 200mg liver tissue was placed in eppendorf tube contained 1.5 ml DEPC and 1 ml of Accuzol reagent was added and homogenized by micropestle and the tubes shaken vigorously for 1minute. Then, 200μl chloroform added to each tube and shaken vigorously for 15 seconds. Then the mixture was incubated on ice for 5 minutes, and then centrifuged at 12000 rpm, 4C°, for 15 minutes. The supernatant transferred into a new eppendorf tube, and 500μl isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. Then, centrifuged at 12000 rpm, 4C° for 10 minutes. The supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 12000 rpm, 4C° for 5 minutes. The supernatant was discarded and the RNA pellet was left to air to dry. Finally 50μl DEPC water was added to each sample to dissolve the RNA pellet, and then the extracted RNA sample was kept at -20. The extracted total RNA was assessed and measurement by Nanodrop spectrophotometer (THERMO. USA).

DNase I Treatment

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by Promega company, USA instructions as follow :

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Mix	Volume
Total RNA 100ng/µl	10µl
DNase I enzyme	1µl
10X buffer	4µl
DEPC water	5µl
Total	20µl

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl 25mM EDTA was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

cDNA synthesis

DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided from Bioneer company, Korea and done according to company instructions as the following :

RT master mix	Volume
Total RNA 100ng/µl	10µl
Random Hexamer primer	1µl
DEPC water	9µl
Total	20µl

This RT PreMix was placed in AccuPower RocketScript RT PreMix tubes that contains lyophilized Reverse transcription enzyme at form. Then dissolved completely by vortex and briefly spinning down.

The RNA converted into cDNA in thermocycler under the following thermocycler condition as the following table:

Table (2): The thermocycler steps to convert RNA to cDNA.

Step	Temperature	Time
cDNA synthesis (RT step)	50 °C	1 hour
Heat inactivation	95 °C	5 minutes

Quantitative Real-Time PCR (qPCR)

qPCR was performed for quantification of P53 mRNA transcript levels whereas, relative gene expression analysis was carried out by using   ($2^{-\Delta\Delta CT}$ Livak method) (18) . The qPCR reaction was done on a Real-Time PCR system (BioRad. USA) by using SYBER Green dye qPCR master mix that used in detection and amplification of P53 target gene and β-actin housekeeping gene for normalization of gene expression as following table(3):

qPCR master mix	volume
cDNA template (10ng)	5μL
Forward primer (10pmol)	1 μL
Reverse primer(10pmol)	1 μL
2X green star master mix	25 μL
DEPC water	18 μL
Total	50 μL

After that, qPCR master mix reaction component that mentioned above placed in qPCR white tube strips and mixed by (Exispin vortex centrifuge, Bioneer. Korea) for 3 minutes, than the strips placed in Miniopticon Real-Time PCR system BioRad. USA as following thermocycler conditions table (4):

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing\ Extension Detection(scan)	60 °C	30 sec	
Melting	60-95°C	0.5 sec	1

Data analysis of qRT-pCR:

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The data results of qRT-PCR for target (p53) and housekeeping gene (β- actin) were analyzed by the relative quantification gene expression levels (fold changes) livak method described by (18).

In this method, one of the experimental samples is the calibrator such as (control sample) each of the normalized target values (CT values) is divided by the calibrator normalized target values to generate the relative expression levels.

After that, the ΔCT method with a Reference gene was used as following equation:

ΔCT(calibtrator)=CT (ref, calibrator)-CT (target, calibrator)

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT(test)=CT (ref, test)-CT (target, test)

ΔΔCT(test)=CT (ref, test)-CT (calibrator).

Finally, fold change of relative gene expression was calculated by following equation=2- ΔΔCT

RESULTS AND DISCUSSION

Genetic experiment (Gene expression of P53 gene in liver):

1-Cycle Time (CT) values of the target genes and housekeeping genes:

Threshold cycle in which the cycle time (CT) reverse proportioning with quantity of copies from mRNA of target gene.

The following figure showed the significant differences between the number of amplification cycles in the treatment and control group for the target and housekeeping genes.

The amplification plot of p53 gene in the qRT-PCR showed significant differences between the number of amplification cycles in the(T2)group and control group and no significant differences between the number of amplification cycle in the (T1) group and control group as in figure(1).
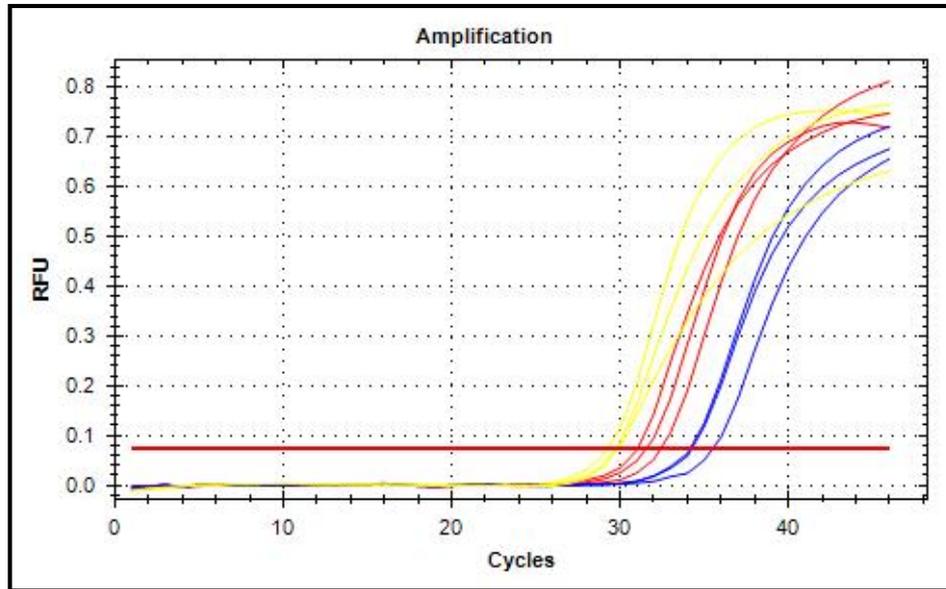
Figure (1): Real-Time PCR amplification plot of p53 gene in liver tissue samples of rat that treated with mercury. Where, red plot: T1 group, T2 group: yellow plot, and C group: blue plot.

2-Relative Quantification:

The calculation of gene expression of p53 gene and β actin was performed by (2-ΔΔct Livak method) depending of housekeeping gene (β actin gene) and the results analyzed by qRT-PCR CT for target gene by using of CT of housekeeping gene for each treatment and control group to complete the normalization process in the gene expression. On the other hand the gene expression results as the following. The T1 group (which injected with mercury chloride (1mg/kg B.W)) showed p53 mRNA levels no significant increase in the levels of gene expression for the treatment group compared with control group (8.006±0.781) in 30 days exposure in comparison with the control group (927±0) but the group T2(which injected with (1.5mg/kg B.W)) for 30 days. The p53 mRNA level demonstrated significant increase ($p<0.05$) (21.439±5.567) comparison with control group (927±0) as in table (5) and fig (2)

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**Table (5):Gene Expression Analysis of p53 Gene in liver tissue**.

Treatment	CT P53	CT B-actin	ΔCT test	ΔCT control	ΔΔCT	Fold change	mean
T1	31.17	30.36	0.808	3.809	-3.001	8.005	8.006
T1	30.92	30.34	0.583	3.809	-3.226	9.358	
T1	31.35	30.27	1.076	3.809	-2.734	6.651	
T1	31.16	30.36	0.807	3.809	-3.002	8.011	
T2	30.46	30.10	0.357	3.809	-3.452	10.947	21.439
T2	29.35	30.44	-1.093	3.809	-4.903	29.913	
T2	29.57	30.31	-0.743	3.809	-4.552	23.456	
T2	29.66	30.27	-0.612	3.809	-4.421	21.421	
C	34.27	30.21	4.057	3.809	0.247	0.842	0.927
C	34.46	30.65	3.807	3.809	-0.003	1.002	
C	34.35	30.45	3.905	3.809	0.095	0.936	
C	34.36	30.45	3.916	3.809	0.107	0.928	

T1: Injected with mercury chloride(1mg/kg b.w)

T2: Injected with mercury chloride (1.5mg/kg b.w)

C: control group

The figure of relative gene expression of p53 show significant differences ($p < 0.05$) between treatment (T2) and control group and no significant increase between treatment (T1) and control group as the following:
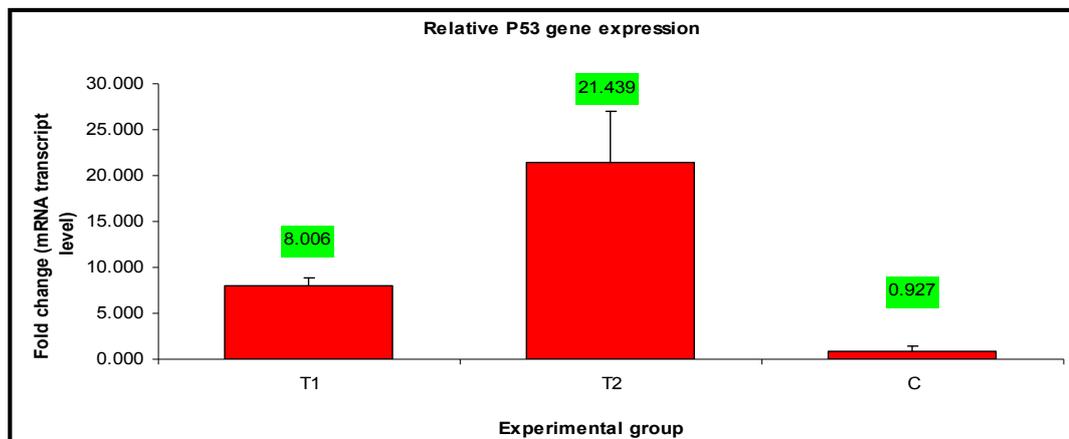
Figure(2): Relative mRNA level of p53 in the liver of rats injected with mercury (1mg/kg B.W) and (1.5mg/kg B.W) for 30 days were determined by quantitative RT-PCR.

Both inorganic and organic mercury decrease cell growth and cell proliferation (19). The cell cycle is usually divided to four phases: mitosis (M) phase, DNA synthesis (S) phase, gap between M and S (G1) and gap between S and M (G2). Two major checkpoints in the cell cycle regulate the fate of the cell. The checkpoint in the G1 phase determines if the cell should stay in G1 phase, go to G0 phase, undergo apoptosis, or go to the S phase. In the S phase, cells start to synthesize new DNA, and the DNA content increases until it becomes tetraploid, which is in the G2 phase. The other checkpoint is in the G2 phase, which determines if the cell is ready for mitotic division. Once a cell starts mitotic division, the tetraploid content returns to diploid. Theoretically, the ratio of the cells in different phases, G1: S: G2 is quiet stable for a given cell under given conditions (20).

Mercuric chloride, specifically blocked the S phase (21), due to decreased DNA replication, therefore, the percentage of cells in the G1 phase decreased with increased percentage of the S phase cells (22).p53 is a transcription factor which function as regulator of cell cycle progression and apoptotic process(23, 24).

This factor is up regulated in response to various cellular stresses and can direct cell to undergo apoptosis(25,26).Our results show there is marked disturbance in important gene (p53) particularly in T2 group which received mercury (1.5mg/kg B.W). The quantitive RT-PCR explained that there is up-regulation or over expression and significant increase ($p<0.05$) of p53 mRNA levels in comparison with control group. In contrast, in T1gruop show non-significant increase of p53 in comparison with the control group.

From these results, we demonstrated the important role of mercury chloride to induce necrosis and apoptosis . This evidence is agreed with (27) who demonstrated that Mercury induces cell death in various cell lines by apoptosis or necrosis due to alteration of apoptosis regulators such as p53 and caspase-3

The p53 protein binds to DNA in the nucleus and transcriptional up- regulation of p53 protein dependant target gene. One of the most important p53 functions is its ability to activate apoptosis when the DNA repair fails by stimulates a wide network of signals.(28).

Also p53 induces apoptosis through its role in control transcription of many pro-apoptotic gene like BAX, APAF-1, Fas-L, caspase-6, caspase-10(29).also agreed with (30) who reported that Mercury seems to be associated in alteration of some regulators level of pro- apoptotic protein p53 . Previous research has documented that mercury is cytotoxic. Its biochemical damage at the cellular level includes DNA damage, and

inhibition of DNA and RNA synthesis (31). Mercury also causes alterations in protein structure, alterations in calcium transport, along with the inhibition of glucose transport and enzyme function.

Histopathology:
1- Liver:

Examination of liver sections of mercury chloride- exposed rats showed that the liver show loss of readily arrangement of hepatic architecture also there is congestion of central vein in (T1) group which treated with mercury chloride (1mg/kg B.W) as in fig.(3 and 4). Also there was degeneration, vaculation of hepatocyte and necrosis in (T2) group which treated with mercury chloride (1.5mg/kg B.W) as in fig.(5).

2- kidney:

Examination of kidneys section of mercury chloride –exposed rats showed atrophy of glomeruli and severe congestion and hemorrhage in (T1) group as in fig.(6).also there was degeneration and destruction of epithelial cells which line the renal convoluted tubules which showed clear dilated and cast in (T2) group as in fig.(7 and 8)
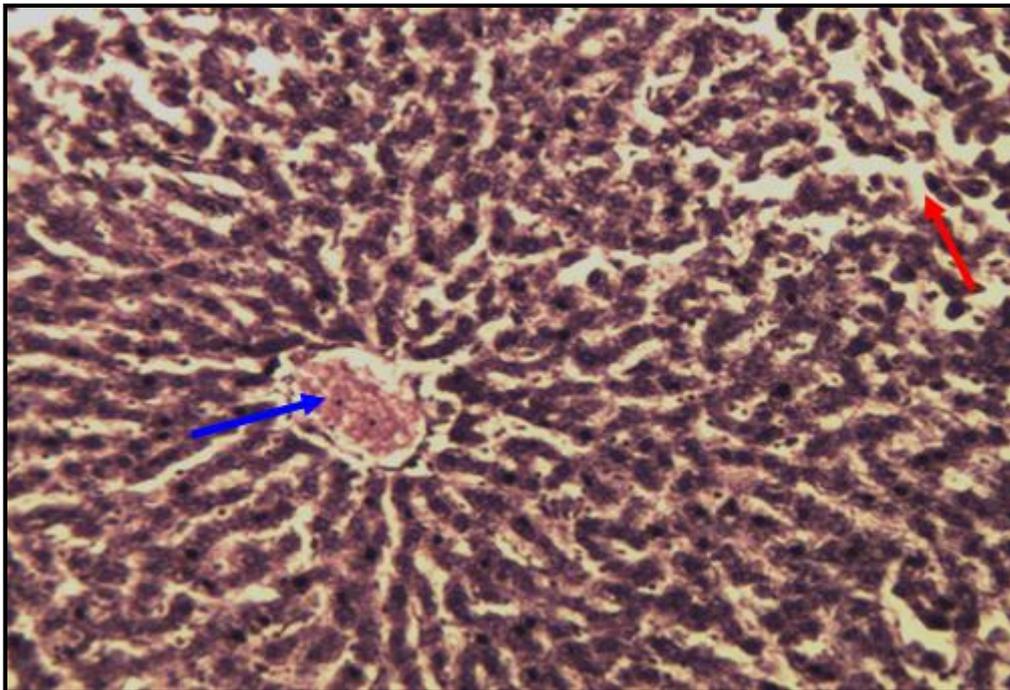

Figure (5): Histological section of liver in rats treated with mercury chloride (1.5mg/kgB.W) show degeneration and vacuolation of hepatocytes (red arrow) and necrosis (blue arrow). 40XH&E
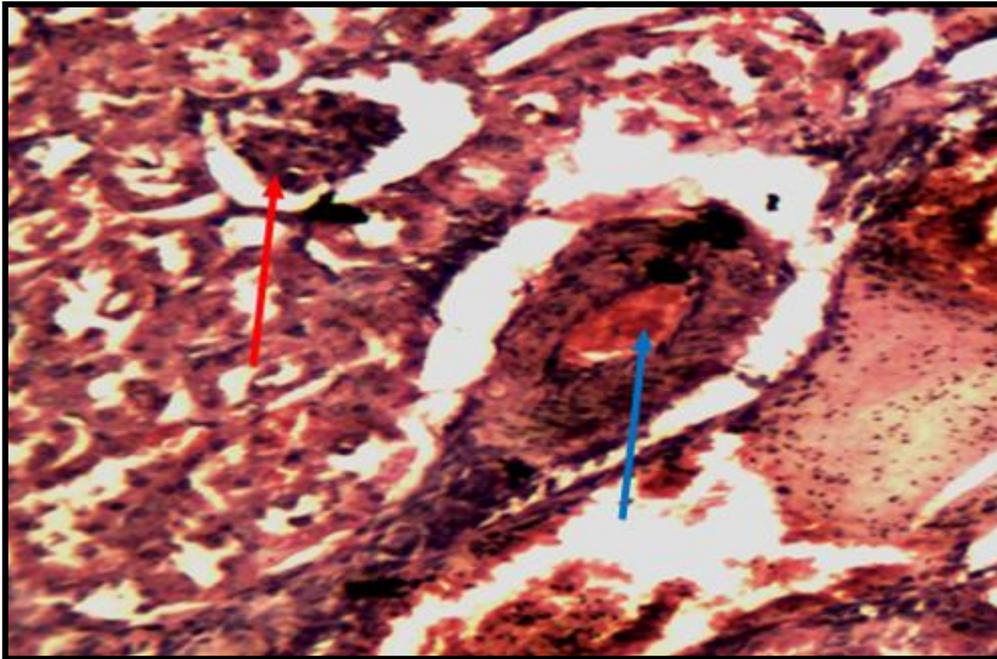
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Figure (6): Histological section of kidney in rats treated with mercury chloride (1.5mg/kgB.W) show atrophy of glomeruli (red arrow) and severe congestion and hemorrhage (blue arrow) 10H&E.
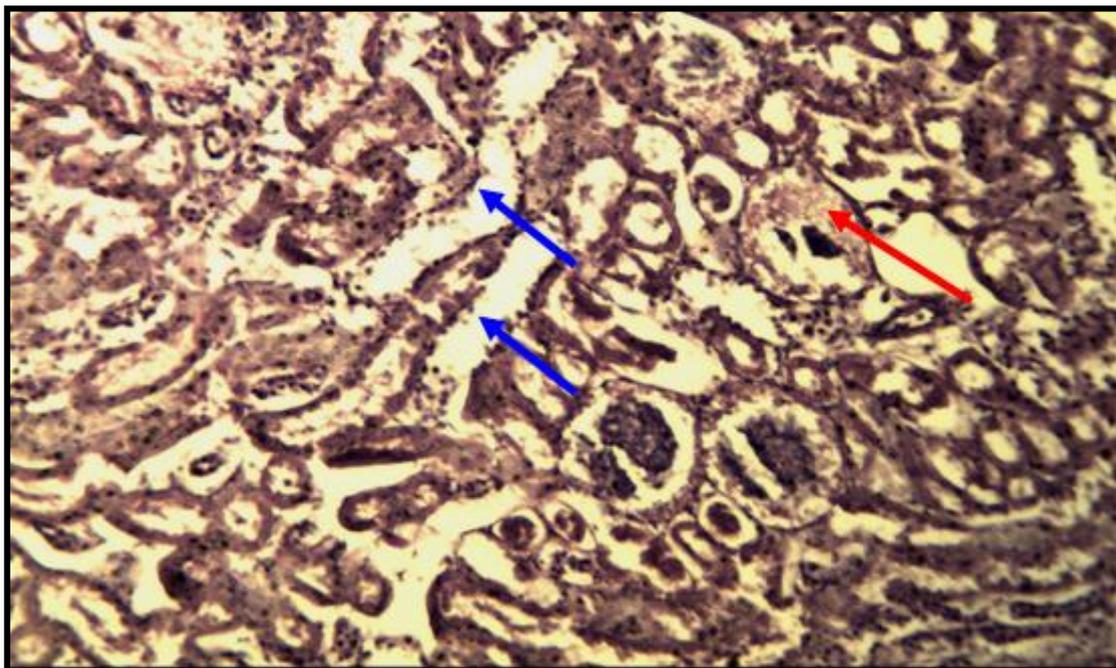

Fig (7) Histological section of kidney in rats treated with mercury chloride (1.5mg/kgB.W) show marked atrophy of glomeruli (red arrow), also there is degeneration and destruction of epithelial cells which lying of renal convoluted tubules which showed clear dilated (blue arrow) 10H&E
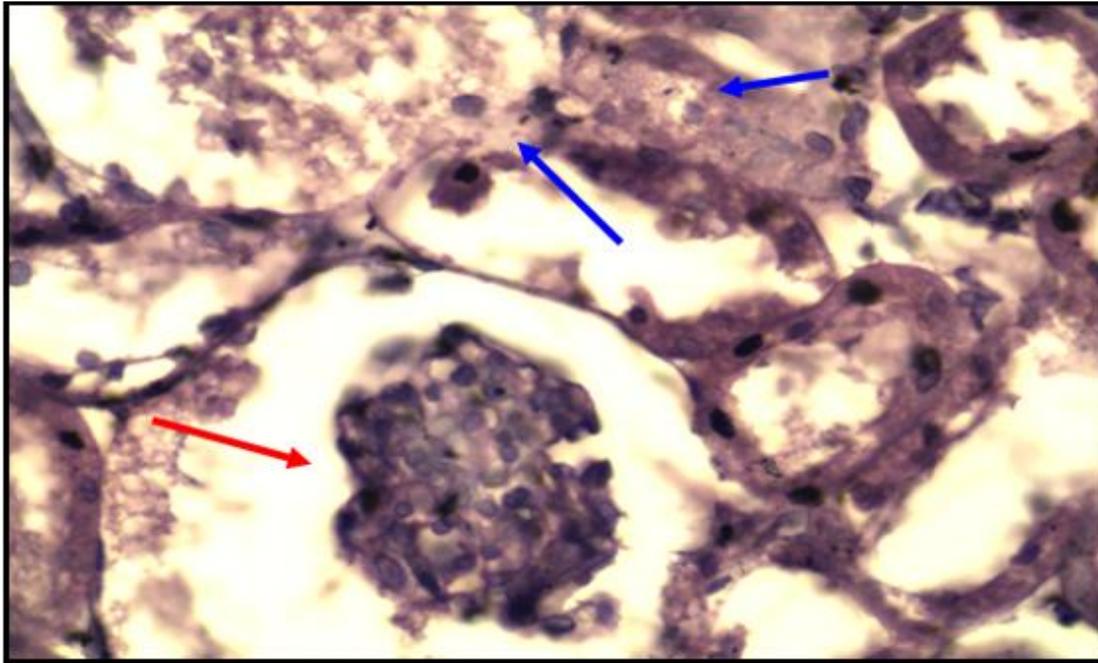
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Figure (8): Histological section of kidney in rats treated with mercury chloride (1.5mg/kgB.W) show marked atrophy of glomeruli (red arrow), also there is degeneration and destruction of epithelial cells which lying of renal convoluted tubules which with cast formation (blue arrow) 10H&E.

The previous results show severe histopathological changes in the liver characterized by degeneration, vaculation and necrosis of hepatocyte. These result agreed with (32) who revealed that mercuric chloride caused histopathological and ultra structural lesions in the liver evidenced by periportal fatty degeneration and cell necrosis The toxic effect of mercury chloride is due to its ability to adhere or to form link with cell enzymes of the respiratory chain and proteins .Also our result show marked atrophy of glomeruli, degeneration of epithelial layer of renal tubules with cast formation and these result agreed with (33) who reported that The interaction of mercury with protein sulfhydryl groups is thought to play an important role in nephrotoxicity induced by mercury at cellular analysis.

Changes in function and structure of mitochondrial morphology very early event which follow mercuric chloride administration, which suggests that mitochondrial dysfunction and oxidative stress have an important role in mercury induced renal toxicity (34)

Also there was hemorrhage and congestion due to endothelial damage by ROS-reactive oxygen species could be the main source for producing large scale hemorrhages in kidney (33)

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التأثيرات المرضية الجينية لكلوريد الزئبق في الجرذان البيضاء

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الخلاصة

أجريت الدراسة على (12) جرذ ابيض وزن 200-220 غم مقسمة بالتساوي الى ثلاث مجاميع على النحو التالي: المجموعة الاولى اعطيت كلوريد الزئبق بجرعة 1 ملغم/ كغم من وزن الجسم داخل البريتون يوميا لمدة 30 يوم بينما اعطيت المجموعة الثانية كلوريد الزئبق بجرعة 1،5 ملغم/ كغم من وزن الجسم داخل البريتون يوميا لمدة 30 يوم.المجموعة الثالثة اعتبرت كمجموعة سيطرة وأعطيت 2و0 مل من المحلول الدارئ المتعادل مره واحده يوميا لمدة 30 يوم داخل البريتون. تم قتل جميع الحيوانات وتم اخذ قطع صغيرة من اكباد الحيوانات للتجربة الجينية ، كما تم اخذ قطع صغيرة(2سم) من الكبد والكلى للتقطيع النسيجي. اظهرت الدراسة الجينية ان المجموعة الاولى اظهرت زياد غير معنوية في مستويات التعبير الجيني للحمض النووي الرايبوزومي المراسل للجين P53 مقارنة مع مجموعة السيطرة، كذلك تسبب كلوريد الزئبق في زيادة معنوية (P<0.05) في مستويات التعبير الجيني للحمض النووي الرايبوزومي المراسل للجين P53 للمجموعة الثانية بالمقارنة مع مجموعة السيطرة. اظهر الفحص ألمجهري للتقطيع النسيجي للكبد والكلى في المجموعة الثانية بتغيرات نسيجية شديدة تمثلت بالتفجي والتنخر لخلايا الكبد وضموربالكبيبات وتغيرات تنكسية شديده للظهارة المبطنة للنبيبات الكلوية.بينما اظهرت المجموعة الاولى تغيرات طفيفة تمثلت بفقدان الترتيب الشعاعي للخلايا الكبدية مع توسع بالجيبانيات وضمور طفيف للكبيبات الكلوية مع وجود نزف واحتقان بالنسيج الكلوي.

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