Toxic effects of mercuric chloride on DNA damage, hematological parameters and histopathological changes in common (*carp Cyprinuscarpio)*

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Summary

The present study aimed at evaluating the effects of mercuric chloride ($HgCl_2$) at different levels of biological organization in common carp *Cyprinuscarpio* following exposure for 30 days to a range of environmentally levels of mercuric chloride (0.01 and 0.02 mg L^{-1}) and recovery for 3 weeks. Prior to evaluation of genetic damage (determined in erythrocytes using comet assay), enzymatic activity (ALT and AST), hematological parameters and histopathological examination of gill. The maximum tolerated concentration was also determined which was found to be 1.63 µg l-1 above which complete mortality over the exposure period was observed. The results indicated that there was a strong induction for DNA damage at high level of Hg. Hematological indices indicated a significant ($P \leq 0.05$) increase in Hb, RBCs and Hct value in Hg treatment groups compared to control group after 15 and 30 days of exposure. Histopathological examination showed distinct abnormalities in secondary lamellae of gill including epithelial lifting, fusion of the secondary lamellae and necrosis. The present findings suggest that exposure to a low concentration (0.01mg L^1) of inorganic mercury can cause significant changes in DNA, hematological parameters and also could cause histopathological changes. Therefore, estimation of these indices could provide a useful indicator for monitoring water bodies pollution.

Keywords: *Cyprinus carpio,* DNA damage, Mercuric chloride, Hematological parameters.

Introduction

The increasing pollution of aquatic ecosystems with thousands of anthropogenic and natural chemicals is becoming the major environmental threat facing human and environmental health (1). In addition to anthropogenic chemicals and radio nuclides, in recent years, significant attention has been paid to the problems of environment contamination by toxic pollutants including heavy metals which are ubiquitous in polluted aquatic environment (2). Heavy metals are continually released into the aquatic environment from natural processes such as volcanic activity, weathering of rocks and industrial processes. Many of these metals occur naturally in the environment and are essential for normal metabolism of the aquatic organisms. However, agriculture and industrial wastes have elevated the natural levels of such metals in the aquatic environment. Among various water pollutants, heavy metals pose a great threat to fishes. They still pose immense health hazards to aquatic ecosystem (1 and 2). Mercury receives the largest attention in view of its high toxicity at relatively low concentrations and a long biological half-life resulting in a cumulative effect (3). Mercury is distributed throughout the environment from both natural sources and human activities. Methyl mercury is the main form of organic mercury found in the environment and is the form that accumulates in both fish and human tissues (3 and 4).

In general, two complex global cycles are involved in the environmental transport and distribution of mercury. One is global in scope and involves the atmospheric circulation of elemental mercury vapor from sources on land to the oceans. The second cycle is local in scope and depends upon the methylation of inorganic mercury mainly from anthropogenic sources. Therefore, environmental levels of methyl mercury depend upon the balance between bacterial methylation and demethylation (4).

Once in the aquatic environment, metallic mercury is readily transformed to organic methyl mercury through biological processes that include sulfate reducing bacteria and some fungi (3 and 5), greatly increasing its

bioavailability to aquatic biota. Fish mainly accumulate mercury through dietary pathways (5) Piscivorous fish species normally accumulate mercury at faster rates than similarly sized omnivorous, planktivorous, or benthivorous species. The 96-h LC_{50} vary between 33 and 400 µg L^{-1} for freshwater fish and are higher for seawater fish (6). A wide variety of physiological, reproductive and biochemical abnormalities have been reported in fish exposed to sub lethal concentrations of mercury. Mercury at low concentrations represents a major hazard to microorganisms (7 and 8). Inorganic mercury has been reported to produce harmful effects at 5 µg/l in fish tissue. Organomercury compounds can exert the same effect at concentrations 10 times lower. The organic forms of mercury are generally more toxic to aquatic organisms and birds than the inorganic forms (3). Given that, toxicological effects caused by mercury in fish are still unclear and therefore this work aimed at determining the Median lethal concentration (LC50) of mercuric chloride ($HgCl_2$), in common carp *(Cyprinuscarpio*) and to determine damage to the DNA (using Comet assay as a biomarker of DNA damage), and to determine the potential influences on haematological parameters (red blood cells (RBCs), white blood cells (WBCs) count, haematocrit (Hct) value and hemoglobin (Hb concentration). Also, it aimed at examining the effects of $HgCl_2$ exposure on histopathological changes in the gill tissue.

Materials and Methods

Experimental design: *C. carpio* L., (weighing 125-150 g, length 12 cm; n = 200) were obtained from a local fish farm (Also wera Fish Farm, Iraq) and transported to the aquarium facilities. After 2 weeks acclimation and on-growing, 100 fish were used for determination of LC_{50}. The LC_{50} value was obtained by the probit analysis method based on observed percentage of test animals surviving at concentrations that were lethal to more than half and less than of the test subjects (9). A total of ninety fish were reared in aerated glass tanks (80 L capacity). They were divided randomly into 3treated groups of 30 fish each. Each further randomized into three replicate experiments (10 fish $tank^{-1}$). The fish in treatment 1 and 2 were treated with 0.01, 0.02 mg L^{-1} of mercuric chloride, respectively for 30 days, after that tap water for 3weeks (recovery period). The 3rd treated group was exposed to tap water only and it served as the control. The water in the replicate experiments was changed every day to maintain the toxicants concentration. The water temperature, pH and dissolved oxygen (DO) concentration were measured daily (22±.4 ºC, 7.25±3.6 pH and 6.12±1.9 mg L-1 DO). Fish within different treatment groups were fed twice daily at a rate of 2% of average body mass during experimental period.

Biological sampling and analysis: Two fish per tank (n = 6) were netted randomly after15 and 30 days and also, after recovery period (i.e. after 21 days), and quickly anaesthetized in a buffered solution of clove oil (eugenol; 25-50 mg L^{-1} water for 10 min). Fresh blood samples (200µl) were immediately obtained from the caudal vein for analysis by single cell electrophoresis (Comet assay) and for the determination of haematocrit%, haemoglobin concentration, leucocytes and red blood cell counts. All hematological parameters were measured according to standard methods as earlier described by (10).The remaining blood per fish was centrifuged at 10,500 xg for 10 min; collected supernatant was subjected to a further 1 min spinning at 10,500 xg. The blood serum samples were stored at - 20ºC for analysis of enzymes activity (ALTand AST) as earlier described by (11).

Single cell gel electrophoresis/Comet assay: Comet assay was conducted to determine DNA damage. Viability of the cells was evaluated by trypan blue exclusion method and the sample showing 85% were processed for the comet assay. The comet assay was performed as described by (12) Briefly, frosted end microscope slides were coated with 1.5% normal melting point (NMP) agarose and allowed to air dry. Erythrocytes were pelleted in a micro centrifuge tubes and suspended in 170 lL molten 0.75% low melting point (LMP) agarose. This was then applied as two drops (85 lL) to the precoated slides. Cover glasses were placed over each drop and gels were allowed to set at 4ºC for 1 h. When gels had solidified, cover glasses were gently removed and slides were

immersed in cold (4ºC) lysing solution (pH 10,1 h). After cell lysis, the slides were placed in electrophoresis unit containing freshly prepared electrophoresis buffer (pH < 13) to unwind DNA for 15 min. Electrophoresis was performed under 25 V and300 mA for 20 min. Lysis, DNA unwinding and electrophoresis were performed at 4ºC, After that, the slides were washed in neutralization buffer (pH 7.4, 10 min), before the final wash in distilled water. Finally, to visualise Comets, ethidium bromide stain (40 lL; 0.2%) was applied to each gel. Scoring was conducted using fluorescence microscopy at magnification 100 x (Leica DMR) using Comet 5.0 image analysis software (Kinetic Imaging, Ltd., UK). Comet scores for 100 cells from each slide (50 cells gel^{-1}). Tail DNA (%) was chosen as a reliable measure of single-strand DNA breaks/alkali labile sites (13).

Histological assessment of the gill tissues (six fish experimental group^{-1}) was conducted after 15 and 30 days and at the end of recovery periodusing light microscopy as described by (14). Briefly, tissue samples were fixed in 10% neutral buffer formalin, dehydrated in serial grades of ethyl, alcohol and cleared by xylol, embedded in paraffin wax. Sections (3-5 µm thick) were stained with Haematoxyline and Eosin (H&E) and then examined microscopically for recording the hisopathological changes.

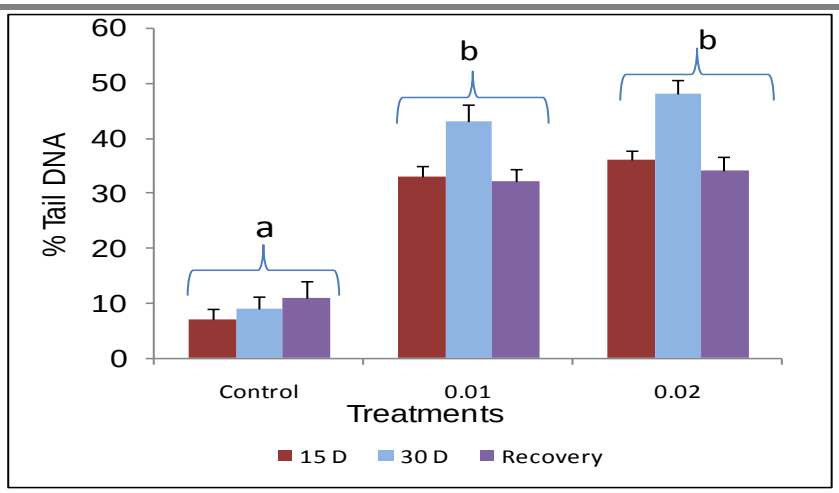
Statistical analysis was performed using Statgraphics v5.1 software (StatSoft, USA). All data were presented as mean ± standard error (S.E.) and analysed using one way analysis of variance (ANOVA) or Kruskal Wallis test, followed by multiple range tests. P values < 0.05 were considered significant.

Results and Discussion

Determination of single cell gel electrophoresis by Comet assay: All $HgCl_2$ treated groups showed a statistically significant ($P \leq 0.05$) increase in average tail length values compared to control group. The DNA damage ranged between 33-48% in treated fish. The obtained values differed significantly from the mean control values. As the concentration of $HgCl_2$ increased and the exposure period increased the percentage of DNA in tail also significantly ($P \leq 0.05$) increased between different levels. For the recovery stage there were significant decreases in all treated groups in comparison with control groups (Figure, 1). These results indicated that DNA depended on concentration of Hg and exposure period. The comet assay under alkaline conditions (pH13) (15) was able to detect DNA damage (i.e. single strand breakage). The damage grade in all metal treated groups were significantly ($P<0.05$) different compared to control group.

The DNA damage (% tail DNA) increased as the exposure concentrations of different levels of HgCl2 increased and also with increased exposure times. These results are in agreement with (16) who also recorded DNA damage in *Therponjaru* in cells lines (kidney, gill, and erythrocyte). Copper exposure also causes an increase in the DNA damage as observed in erythrocytes from the gilthead bream, *Sparusaurata*, and in haemocytes of the bivalve *Scapharcainaequivalvis* (17). Bucio et al. (18) reported that comet assay studies carried out on mercury compounds indicate that low concentrations (5 M) of mercury chloride produce DNA damage in a human hepatic cell line and that this compound produces DNA damage by a non apoptotic mechanism in a human cell line with monocytic characteristic (19).

It is well known that the increased generation of ROS results in DNA damage and apoptosis (20). The pathway for Hg compounds genotoxicity is very complex. Heavy metals react directly with DNA as metals are positively charged ions and easily complex with DNA by reacting with negatively charged nucleophilic sites which cause mutagenesis (21). Therefore, this result suggests that DNA damage in *C. carpio* could potentially be used as a biomarker for detecting genotoxicants in water bodies.



Figure, 1: Induction of DNA single strands breaks (represented as percentage tail DNA) in *C. carpio*erythrocytes following 15, 30 days exposure to $HgCl_2$ (0.01, 0.02 mg L^{-1}) and recovery for 21 days. Values are mean ± S.E. Different letters indicate significant different at P≤ 0.05, (n = 6).

Enzymatic activities: In this study, at 15 and 30 days, compared with controls, ALT, AST, were increased significantly (P≤0.05). Serum ALT and AST activities were used in diagnosis of damage fish tissues (i.e. gill, muscle, liver) (22). Determinations of transaminases (AST and ALT) have been useful in the diagnosisof liver and kidney diseases in fish (23). These enzymes of *C. carpio* increased in responseto mercury exposures when compared to control after15 and 30 days (Table, 1 A & B). After recovery period, the activities indicated no significant differences (P>0.05) in both levels for the activities of these enzymes in fish exposed to Hg (Table, 1 C). There are numerous studies in this serum activity of fish such as *Sparusaurata* (24) and *Cyprinuscarpio* (25). The researchers concluded that necrosis or disease of livercaused to leak age of this enzyme into blood stream and that might be responsible for the increase of thisenzyme in blood.

Table, 1: Changes in enzymes activity (ALT and AST) of *C. carpio* (A) after 15 days exposure to $HgCl_2$ (B) after 30 days, (C) after 21 days recovery period.

A

Concentration (mg L^{-1})	ALT (U ml)	AST (Uml)
0.00	14.33±1.20[a]	126.0±2.64 [a]
0.01	18.00±0.01[b]	121.1±0.57 [b]
0.02	29.66±0.66 [c]	97.00±1.00[c]

B

Concentration (mg L^{-1})	ALT (U ml)	AST (U ml)
0.00	16.66±0.88[a]	125.3±0.58[a]
0.01	20.32±0.87[b]	131.6±0.77[b]
0.02	26.66±0.65 [a]	111.6±1.10[c]

C

Concentration (mg L^{-1})	ALT (U ml)	AST (U ml)
0.00	15.13±1.45[a]	122±2.64[a]
0.01	13.40±1.25[a]	126±0.77[a]
0.02	15.33±1.32[a]	122±0.24[a]

Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significantly different at *P* ≤ 0.05; (n=6).

Hematological parameters: Hematological parameters are RBCs and WBCs a count, Hb content and haematocrit value of *C. carpio*for 15 and 30 days periods for different levels of $HgCl_2$ are presented in (Table, 2 A-C). There were significant increases (P≤0.05) in both periods (i.e. 15 and 30 days) for RBCs, Hb and Hct at two level of $HgCl_2$ compared to control

group. No significant differences for WBCs count were observed after 15 days at two levels of Hg in comparison to control group. However, a significant ($P<0.05$) increase for WBCs count was recorded after 30 days of exposure to Hg at two levels compared to control group. After 21 days (i.e. recovery period) there were no significant differences ($P>0.05$) in both levels for RBCs, WBCs, Hb and Hct at two levels of $HgCl_2$ compared to control group. According to (26) the study of the haematological indices is frequently utilized for the detection of physiopathological changes in different stress conditions such as exposure to heavy metals. Therefore, hematological variables are environmentally sensitive and allow the most rapid detection of changes in fish (27).The erythrocytosis observed in this study was also reported (28) in rainbow trout and carp exposed to copper. Similarly, (26) recorded erythrocytosis and increase in Hb content and Hct value in *Oreochromismossambicus* after short time exposure to Cu (0.16 mg L^{-1}). It is also in line with (29) who reported increase in Hb content and RBCs count but decreased haematocrit in the dogfish after 24h exposure to 25 Cdµg L^{-1}.

Table, 2: Changes in haematological parameters of *C. carpio* (A) after 15 days exposure to $HgCl_2$ (B) after 30 days and (C) after 21 days recovery period.

A

Concentrations mg L^{-1}	Hb (g dl^{-1})	RBC $\times10^6$ mm^{-3}	WBC $\times10^3$ mm^{-3}	Hct (%)
0.00	**7.33±0.62^a**	**1.86±0.06^a**	**13.03 ± 1.32^a**	**28.00±1.14^a**
0.01	**10.55±0.23^b**	**2.32±0.05^b**	**13.09 ± 0.06^a**	**36.00±1.14^b**
0.02	**9.10±0.42^b**	**2.74±0.08^b**	**13.02 ± 0.03^a**	**34.00±1.41^b**

B

0.00	**6.70±0.07^a**	**1.95±2.24^a**	**13.16 ± 1.42^a**	**29.00±3.24^a**
0.01	**9.30±2.25^b**	**2.56±0.08^b**	**13.05 ± 0.05^a**	**38.00±1.14^b**
0.02	**9.95±1.65^b**	**2.74±2.82^b**	**15.08 ± 0.08^a**	**33.00±2.82^b**

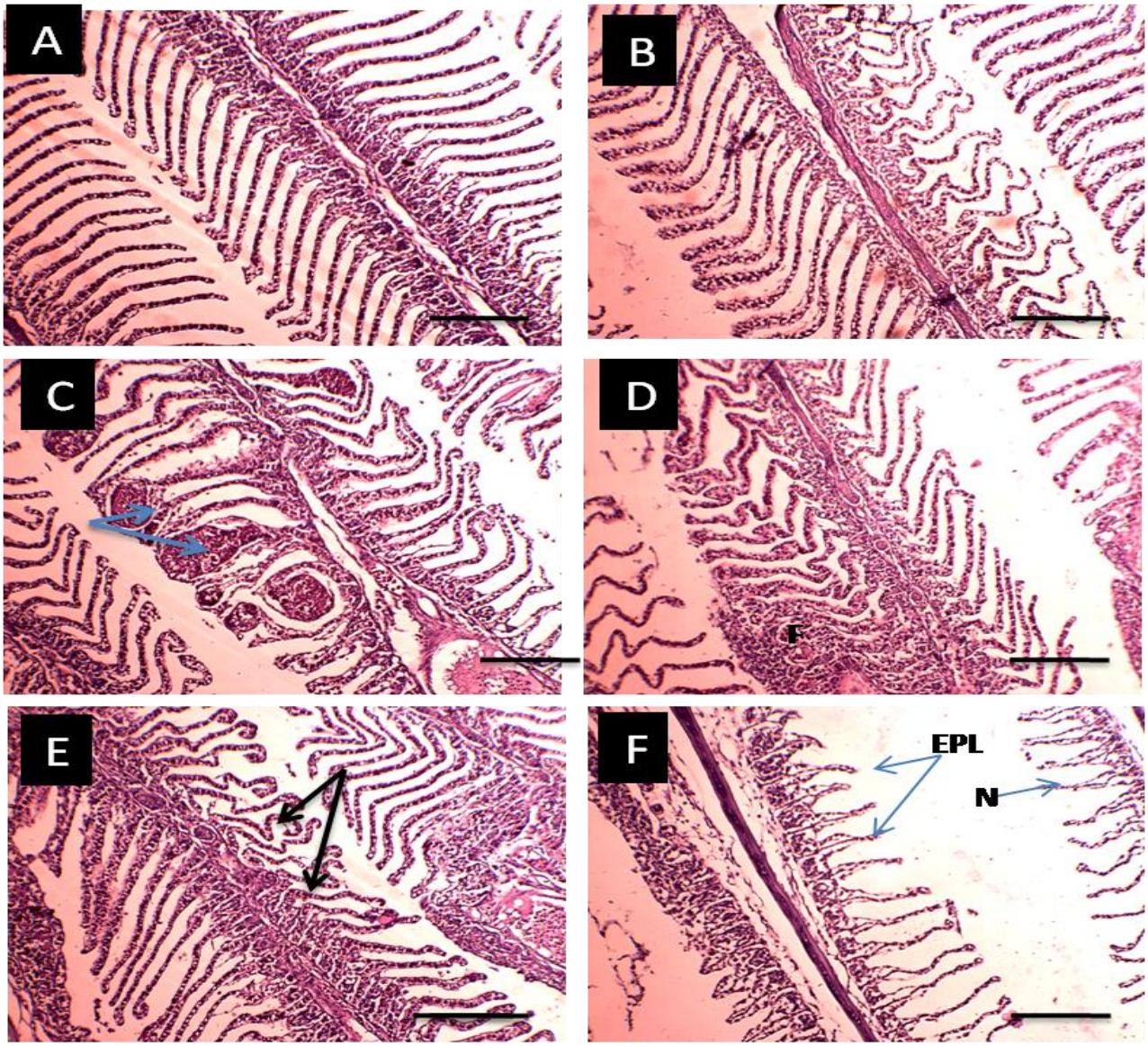
C

0.00	**6.70±0.08^a**	**1.70±2.24^a**	**13.09 ± 1.20^a**	**30.00±2.16^a**
0.01	**6.20±2.05^a**	**1.84±0.08^a**	**13.08 ± 0.03^a**	**32.00±2.08^a**
0.02	**7.65±1.90^a**	**1.95±2.82^a**	**13.03 ± 0.06^a**	**31.00±2.55^a**

Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significantly different at $P \leq 0.05$; (n=6).

Histopathological changes: The gill morphology in the control group showed typical structures in which lamellae were lined by epithelial cells (Figure, 2A). After 30 days (Figure, 2B) exposure to 0.01 mg $L^{-1}HgCl_2$, minimum changes were observed in secondary lamellaewhich appeared to be curved (clubbing). However, 0.02 mg $L^{-1}HgCl_2$ after 30 days showed pronounced changes such as,congestion of blood vessels throughout the entire lamellae (aneurysm) (Figure, 2C), fusion of the secondary lamellae (Figure, 2D and E), clubbing of the ends of the secondary lamellae lifting up of the epithelium and necrosis (Figure, 2F). Gill damage of fish exposed to high levels of organic and inorganic Hg was previously described by (30). Similar results had also, been recorded by (31) whereas the gills of the experimental fish exposed to cadmium and mercury (10, 20 and 30 days). The results are also in agreement with the works of (32) who have reported for *Boleopthalmusdumieric exposed* to sublethal concentration of cadmium. Alterations described above in this study (lamellar fusion and epithelial necrosis with blood emergence) may be causes of respiratory and osmoregulatory disorders. Although, these changes can be used as biomarker to assess the environmental pollution in this vital organ. However, these changes are not specific to Hg and may also be observed with some other contaminants or as a chronic response to bacterial or parasitic infections (8, 31 and 33). In addition, gill damage in tissues provides a sensitive but not

specific indication of the general quality of water in a contaminated environment (31). These types of damage seem reversible even at acute dosages and cannot be seen as indicators of long-term chronic exposure. Therefore, the sealterations may play a general defense role against contamination rather than having an irreversible toxic effect (3 and 33). In conclusion, the results of this study showed that sub lethal concentrations of $HgCl_2$ affect the hematological parameters of *C. carpio* and suggests that the assessment of these parameters and other biochemical tests could be a useful tool in monitoring environmental pollution. Also, this study confirms that DNA damage and histopathological of *C. carpio*gills were affected by exposure to mercury. This phenomenon confirmed those reported in other fish species under a wide range of exposure conditions. Therefore, the histological changes on fish is a noteworthy and promising tool to understand the extent to which changes in the structural organization are occurring in the tissues due to environmental pollution.



Figure, 2: Light micrograph showing histological structures of the gill of *C. carpio* from Hg exposures stained with H&E at 3-5 µm thickness. (A) Control gill showing normal structure (B) 0.01 mg L^{-1} $HgCl_2$ showing epithelial clubbing (C-F) 0.02 mg L^{-1} $HgCl_2$showing aneurysm, of the secondary lamellae (blue arrow); fusion of the secondary lamellae (F); epithelial lifting (EPL) and necrosis (N): Scale bars: 50µm.

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التاثيرت السميه لكلوريد الزئبق على مستوى تحطم الحامض النووي والمعايير الدميه والتغيرات النسجيه المرضيه في اسماك الكارب العادي *Cyprinuscarpio*

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الخلاصه

ان التعرض للزئبق ، حتى بكميات صغيرة ، يشكل خطرا كبيرا على الانسان والبيئيه المائية، يعد الزئبق من الملوثات ذات الصلة الكبيره في البيئة المائية. هدفت الدراسه إلى تقييم آثار كلوريد الزئبق في مستويات مختلفة من التنظيم البيولوجي في سمكة الكارب العادي *Cyprinuscarpio* بعد التعرض المزمن لمدة 30 يوما الى مجموعة من مستويات بيئيه لكلوريد الزئبق (0,01 و 0,02 ملغم لكل لتر). تم تقييم الضرر الوراثي (في الكريات الحمراء) والمعايير الدميه والفحص النسجي في اعضاء محددة (الكلى و الخياشيم). أشارت النتائج إلى أن هناك ارتفاع عالي في مستوى تحطم الحامض النووي (DNA) في المجموعة المعرضه لتركيز عالي من كلوريد الزئبق, كما أشارت الصوره الدميه زيادة كبيرة ($P\leq0.05$) في عدد كريات الدم البيضاء WBC وفي عد خلايا الدم البيض في مجاميع كلوريد الزئبق مقارنة بمجموعه السيطره . وأظهر الفحص النسجي تغيرات واضحة في الصفائح الثانويه للخياشم تضمنت تضخم ، وتضخم وفرط تنسج ونخر وانفصال في طبقه الخلايا الظهاريه epithelial lifting. تشير نتائج الدراسه الحالية أن التعرض لتركيز منخفض (0.1 ملغم/ لتر) من الزئبق غير العضوي يمكن أن تسبب تغييرات كبيرة وتلف في الحمض النووي وتغيرات في الصوره الدمية فضلاعن تغييرات مرضية نسجيه في سمكة الكارب العاديلذلك ان تقدير هذه المؤشرات يمكن ان يعد مؤشرا مفيدا للتلوث في المسطحات المائية.

الكلمات المفتاحية: اسماك الكارب العادي، حامض نووي، كلوريد الزئبقيك، معايير دمية.