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Chronic Toxicity Assessment of Histological Changes and Micronuclei in Fish *Cyprinus carpio* L. After Exposed to Copper

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Abstract

This study was conducted to assess the histological changes of (gill, liver, kidney and muscle) and The micronucleus test were applied in circulating erythrocytes of in freshwater fish common carp, *Cyprinus carpio* after chronic exposure to copper. For chronic tests, the fish were exposed to different concentrations (0.5, 0.9 and 1.2 mg/L) of copper for 3 and 6 weeks. Control fish were maintained in parallel with the experimental groups. Several histological alterations were observed in the gills, including the epithelium of gill filaments and secondary lamellae, degeneration and congestion of secondary lamellae and short villi. The liver showed dilation in cells hepatic, degenerative and necrosis of hepatocyte cell with mild inflammatory cell and accumulation of cholesterol inside the cell. Regarding in kidney, Renal tissue showed congestion and haemorrhage with certain degeneration and necrosis of renal tubules tissue. In the muscle, showed mild hyalinization of the skeletal muscles fibres with the loss of interstitial fibres in between the muscles fibres and focal degeneration and necrosis with mild inflammatory cell infiltration. Micronucleus test was applied to evaluate the genotoxic effects of heavy metals on *Cyprinus carpio*. Results of micronucleus test showed a progressive increase in the percentage of micronuclei ($P \geq 0.001$) with increases in the intensity of exposure of copper. The obtained results showed that fish common carp, *Cyprinus carpio* erythrocytes are good models for cytotoxicity studies.

Keywords: Copper; common carp (*Cyprinus carpio* L.); gill; liver; kidney; muscle Histological Changes; Micronucleus Test.

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1. Introduction

Heavy metals are considered a major anthropogenic contamination in coastal and marine environments worldwide [1]. The concentration of heavy metals in fish is related to several factors, such as the food habit, foraging behaviour of the organisms, trophic status, source of particular metal, and distance of the organism from the contamination source [2]. It can contribute to a degradation of marine ecosystems by reducing species diversity and abundance and through the accumulation of metals in living organisms and food chains [3]. Copper, also existent naturally in plants and animals as essential micronutrient to perform various physiological and biochemical processes, yet the hyper concentration (even a little bit more than needed) causes a serious threat to life. These pollutants in the aquatic environment can pose adverse effects on growth, physiology, and reproduction and survival risk of aquatic organisms especially on fish [4].

Fish have been found to be good indicators of trace metal contamination in aquatic systems [5]. Fish have a tendency to bioaccumulation heavy metals and human beings can be at serious risk through contamination of the food chain [6]. Common carp (*Cyprinus carpio* L. 1758) is one of the most important fishes in farm culture and due to being economical and because of its delicious meat, this fish has a special importance in many countries [7]. Generally, common carp considered is one of the major consumers as a food for Iraqi people and broadly used in the estimation of genotoxicity studies.

Histological changes have been widely used as biomarkers in the evaluation of the health of fish exposed to contaminants, both in the laboratory and field studies. Gills are the first target of waterborne pollutants due to the constant contact with the external environment, as well as the main place for copper uptake [8]. The fish liver is a vital organ concerned with basic metabolism and is the major organ of accumulation, biotransformation and excretion of contaminants in fish [9]. The liver is particularly susceptible to damage from a variety of toxicants. One of the most important functions of the liver is to clean pollutants from the blood so it is considered as an indicator of aquatic environmental pollution [10]. Kidney a vital organ of the body and proper kidney functioning is important to maintain the homeostasis. Kidney is not only involved in removal of wastes from blood but it is also responsible for selective reabsorption, which helps in maintaining volume and pH of blood and body fluids, erythropoiesis and help in regulating blood pressure by producing the enzyme rennin [11].

The micronucleus test (MN) is one of the most employed cytogenetic techniques for genotoxicity assessment and has been widely applied in peripheral blood of teleostean fishes in studies of field (in situ) and bioassays [12]. In addition, changes in the normal morphology of the nuclei are also considered indicators of genotoxic damage [13].

The first objective is to study the histological alterations of gill, liver, kidney and muscle of common carp (*Cyprinus carpio*). The second objective of this study is to assess the micronucleus test of a freshwater fish, *Cyprinus carpio* exposed to sublethal concentrations of copper (Cu).

2. Material and Methods

2.1 Samples of study

Fish *Cyprinus carpio* were obtained hatchery incubators (the city of Madain, south of Baghdad) at a weight of 34.72±9.90 gm in the body (Figure 1). Fish were acclimatized in dechlorinated tap water in glass aquaria (70×40×40 cm) for 10 days to laboratory conditions. Water was renewed every day and a 12-12 h photoperiod was maintained during the acclimatization and test periods. The fish were fed with a commercial food twice daily.

Figure 1: Al- Madain Hatcheries in south of Baghdad (Google earth 2015).

2.2 Chronic toxicity test

Three groups of fishes were used in each aquarium and subjected to 0.5, 0.9 and 1.2 mg/L. and left for 3 and 6 weeks. Water was refreshed every 48 hr. to remove any wastes and provide oxygen. Fishes were fed once per day [14].

2.3 Histological changes

At the end of exposure period 3 and 6 weeks, fish were taken from each replicate tank. Tissues like gill, liver, kidney and muscles were isolated from normal and experimental fish. The samples were initially fixed in a 10% formalin buffer for 24 hours and then processed and embedded in paraffin for block (56-58 °C) preparation. The sections were cut at 5-6 micron and stained in Heamatoxylin and Eosin [15]. The slides were examined under a light microscope and photographed for histological effects. All sections were examined and photographed using a built-in camera.

2.4 Micronucleus Test (MN)

Blood was collected from heart puncture in a heparinized syringe and thin smear on precleaned slides was

made. Blood was smeared directly onto one clean glass slide, air dried, and then fixed in absolute ethanol for 20 min. In the lab, each slide was stained with (0.01%) Acridine orange staining solution for 4 to 5 minutes [16]. Micronucleus was identified and scored microscopically in the total of 1,000 erythrocytes from each slide [12]. The smears were inspected using a Nikon D5100 microscope (at 2000 × magnification) connected with Nikon Coolpix digital camera and a computer equipped with an image analysis system. The MN frequency (%) was calculated as:

$$\text{MN \%} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells scored}} \times 100$$

2.5 Statistical analysis

Means ± standard deviation (SD) were calculated for each experimental group. All experiments were repeated three replicates. Data were analysed with SPSS statistical analyzed software. Differences among the results were considered to be statistically significant when the P value was $P \leq 0.05$ and $P \leq 0.01$.

3. Results

3.1 Chronic exposure

Three series of exposure were conducted after we calculated safe concentrations for long-term exposure to copper (Table 1). In series 1, fish specimens exposed to 0.5 mg/L of copper, while in other series experiments studied fish placed in 0.9 and 1.2 mg/L respectively. In this exposure, no mortality was observed at all periods of exposure for each tested concentrations.

Table 1: Safe concentrations values of exposure to copper

Element	Sc, when x=2	Sc, when x=3
copper	0.93	0.86

3.2 Histological changes

Exposure of the fish common carp *Cyprinus carpio* to the sublethal concentration of copper 0.5 mg/L, 0.9 mg/L and 1.2 mg/L separately for during period 3 and 6 weeks that led into several alterations in the histological of the gill, liver, kidney and muscle. The results are shown in tables 2,3,4 and 5 Figures 2, 3, 4,5,6,7 and 8.

3.2.1 Histological changes in gill

The histological changes in the gill of carp fish *Cyprinus carpio* L. during a period of 3 weeks and 6 weeks.

Nuclear degenerative changes in parenchyma cells with necrosis was reported in *Cyprinus carpio* due to heavy metals [17]. Hyperplasia in the gill epithelium of freshwater fish species induced by trace metals was reported by Figueiredo-Fernandes [18]. However, this work has found for the first time the different levels of gill epithelium proliferation and areas of a severely damaged histological structure of gills. The results appear in table 2 and shown in Figure (2 and 3).

Table 2: Histological changes in gill of *C. carpio* after chronic exposure to (0.5, 0.9, 1.2 mg/L) of copper

Con. **mg/L**	**Exposure period of gills**	
	3 Weeks	**6 Weeks**
control	Normal structure of branching of the blood vessel and cartilage, The gill sections normal fish consists of gill arch, gill septum, and gill lamellae.	
0.5	Showing certain degeneration and congestion of gills short of villi.	Normal structure appearance with shortness of villi.
0.9	Showing certain degeneration and congestion of gills short of villi.	Normal structure appearance with shortness of villi.
1.2	Showing certain atrophy and congestion of gills short of villi.	Normal structure appearance with shortness of villi.

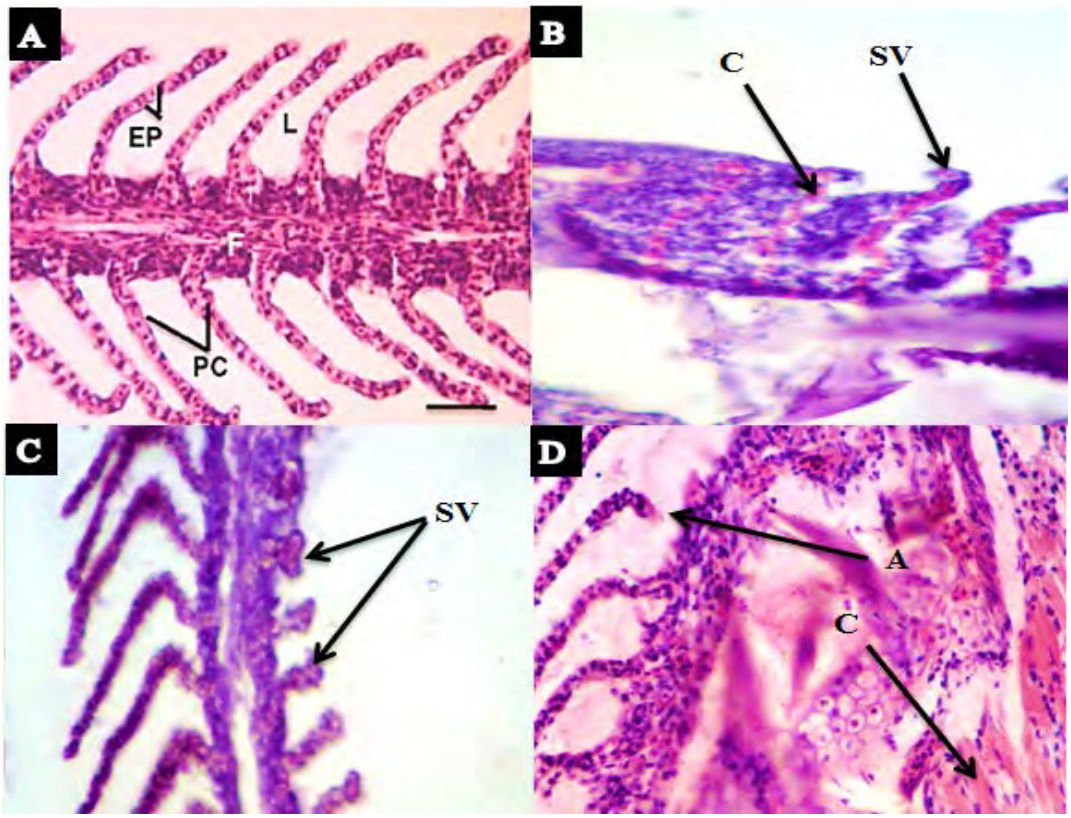

Figure 2: Histological changes of gill during 3 weeks [A] normal [B] At a concentration of 0.5 mg Cu/L ; [C] At a concentration of 0.9 mg Cu/L ; [D] At a concentration of 1.2 mg Cu/L.

[A] It is clear that there was no any abnormal changes in control sample and showing primary filament (F) secondary lamellae(L), pillar cell (PC) epithelial cell (EP); While, [B] At a concentration of 0.5 mg/L during 3 weeks showing presence of congestion (C) with short villi (SV); [C] At a concentration of 0.9 mg/L during 3 weeks showing short villi (SV); [D] At a concentration of 1.2 mg/L during 3 weeks showing atrophy (A) and congestion (C). H&E; 400x.

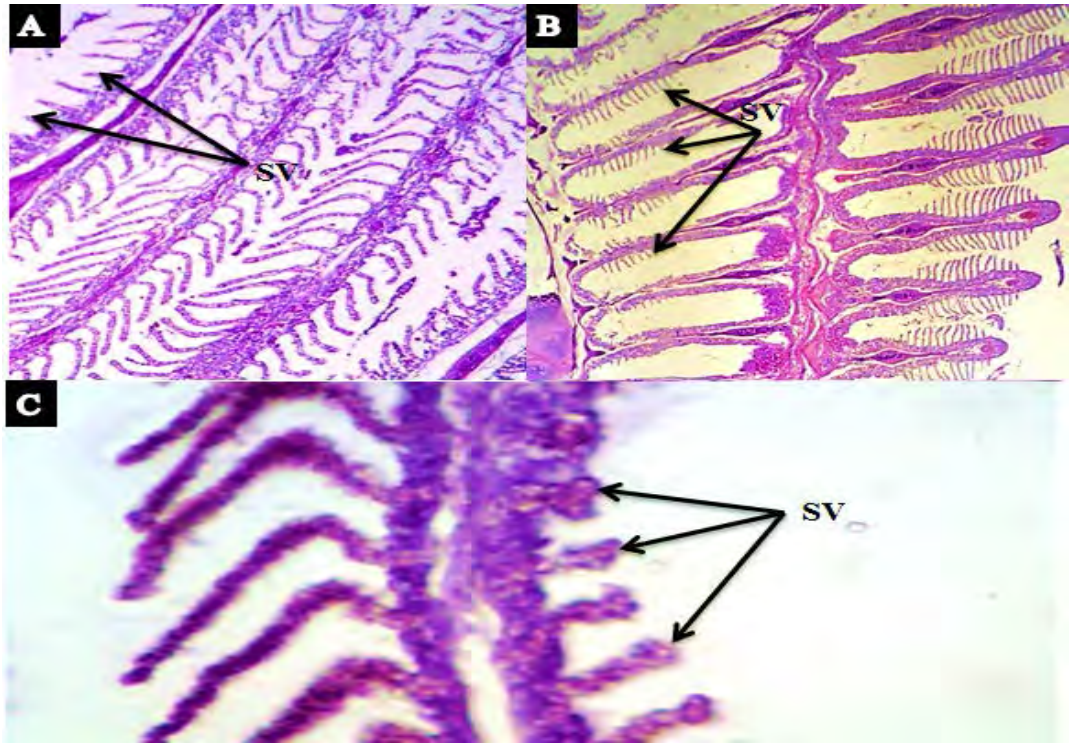

Figure 3: Histological changes of gill during 6 weeks [A] At a concentration of 0.5 mg Cu/L [B] At a concentration of 0.9 mg Cu/L [C] At a concentration of 1.2 mg Cu/L.

[A] In case of exposed to 0.5 mg Cu/L, fish sample had showing physiology changes such as short villi (SV); furthermore, [B] showing elongation of the villi (EV); while, [B&C] At a concentrations of 0.9 mg/L and 1.2 mg/L during 6 weeks showing short villi (SV). H&E; 200x.

3.2.2 Histological changes in liver

The histological changes in the liver of carp fish *Cyprinus carpio* L. during a period of 3 weeks and 6 weeks. Histological biomarkers of toxicity in fish organs are the useful indicator of environmental pollution [19]. Degeneration and necrosis of hepatocytes may be due to the cumulative effect of the metals and increase in their concentration in the liver. The histology showed that copper caused some alterations of the liver parenchyma, like vacuolization and necrosis. The liver histological changes observed were more evident in fish exposed to high copper concentrations. These alterations are often associated with a degenerative-necrotic condition [20]. Moreover, it was also reported by several studies that chronic copper accumulation in the liver of fish causes hepatocyte lysis, cirrhosis and ultimately death [21]. The results appear in table 3 and shown in Figure (4 and 5).

Table 3: Histological changes in liver of *C. carpio* after chronic exposure to (0.5, 0.9, 1.2 mg/L) of copper

Con. mg/L	Exposure period of liver	
	3 Weeks	**6 Weeks**
control	Normal structure appearance of hepatic tissue with the presence of cholesterol inside the hepatocyte presence of bile duct with the blood vessel.	
0.5	Showing degenerative and necrosis of hepatocyte cell with mild inflammatory cell infiltration and accumulation of cholesterol inside the cell.	Normal structure appearance of hepatic tissue with apoptosis of cells.
0.9	Showing degenerative and necrosis of hepatocyte cell with mild inflammatory cell infiltration and accumulation of cholesterol inside the cell.	Normal structure appearance of hepatic tissue with apoptosis of cells.
1.2	Showing degenerative and necrosis of hepatocyte cell with mild inflammatory cell infiltration and accumulation of cholesterol inside the cell.	Normal structure appearance of hepatic tissue with apoptosis of cells.

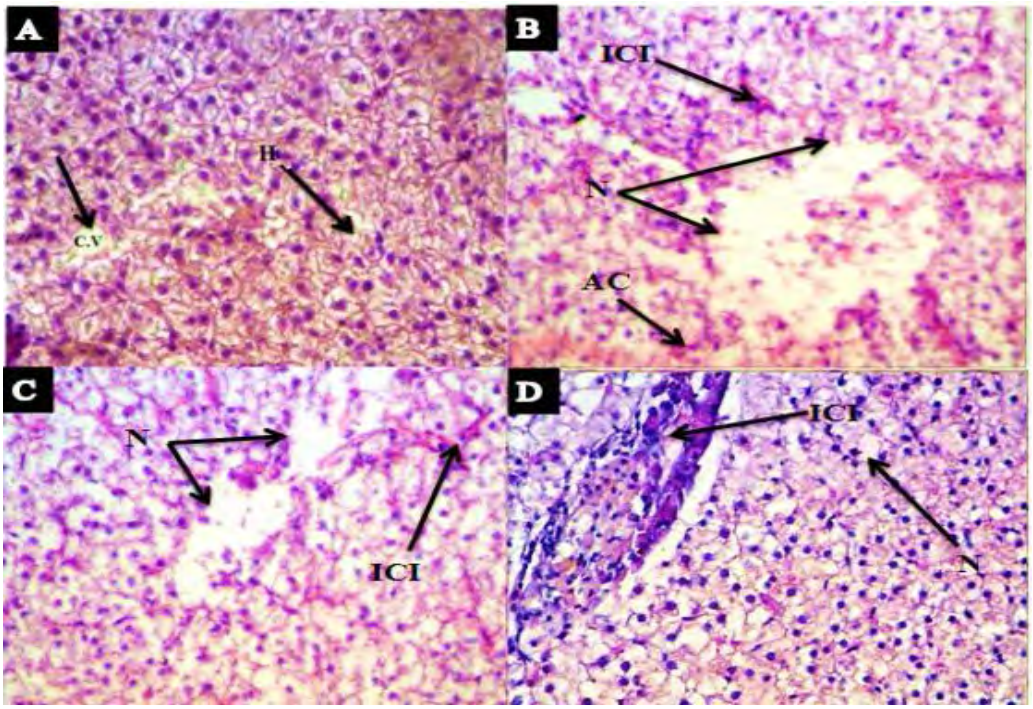

Figure 4: Histological changes of liver during 3 weeks [A] normal [B] At a concentration of 0.5 mg Cu/L ; [C] At a concentration of 0.9 mg Cu/L ; [D] At a concentration of 1.2 mg Cu/L.

[A] control liver showing normal histology of hepatocytes (H) with central vein (CV); While, [B] At a concentration of 0.5 mg/L during 3 weeks showing presence of necrosis (N) with mild inflammatory cell infiltration (ICI) and accumulation of cholesterol (AC) inside the cell ; [C&D] At a concentrations of 0.9 mg/L and 1.2 mg/L during 3 weeks showing necrosis (N) with mild inflammatory cell infiltration (ICI) H&E; 200x.

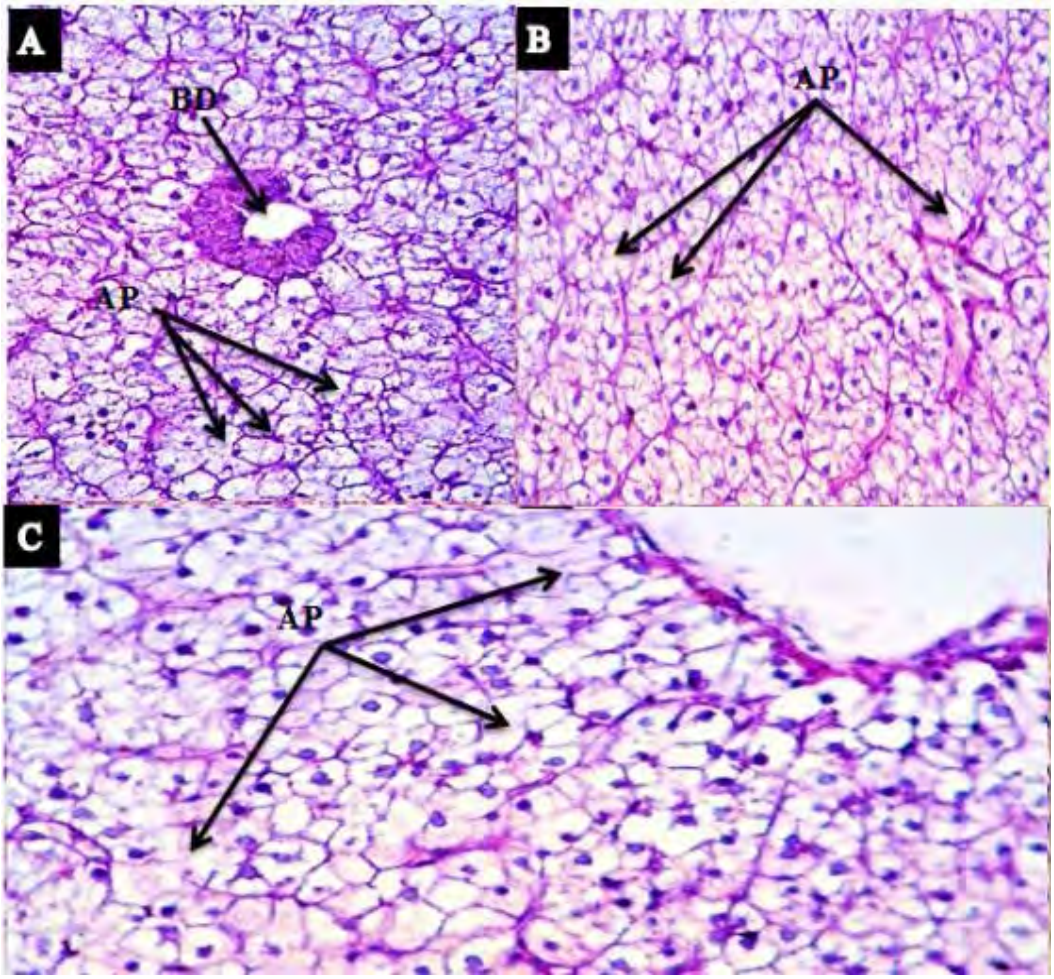

Figure 5: Histological changes of liver during 6 weeks [A] At a concentration of 0.5 mg Cu/L [B] At a concentration of 0.9 mg Cu/L [C] At a concentration of 1.2 mg Cu/L.

[A] In case of exposed to 0.5 mg Cu/L, fish sample had showing physiology changes presence of bile duct (BD) and Apoptotic cells (AP); furthermore, [B] showing elongation of the villi (EV); while, [B&C] At a concentrations of 0.9 mg/L and 1.2 mg/L during 6 weeks showing presence Apoptotic cells (AP). H&E; 200x.

3.2.3 Histological changes in Kidney

The histological changes in the kidney of carp fish *Cyprinus carpio* L. during a period of 3 weeks and 6 weeks. The kidney is one of the first organs to be affected by contaminants in the water [22] Because the important role of the kidney in the excretion of harmful materials. Disturbance of living processes at the molecular and subcellular levels of biological organization by xenobiotic can lead to cell injury, resulting in degenerative and neoplastic diseases in target organs [23].

The present study proved the occurrence of several histological alterations in the kidney resulting from copper toxicity, which the results appear in table 4 and shown in Figure (6 and 7).

Table 4: Histological changes in kidney of *C. carpio* after chronic exposure to (0.5, 0.9, 1.2 mg/L) of copper

Con. mg/L	Exposure period of kidney	
	3 Weeks	**6 Weeks**
control	Normal structure appearance of renal tissue showing glomeruli with renal tubules.	
0.5	Renal tissue showing congestion and haemorrhage with certain degeneration and necrosis of renal tubules tissue.	Showing certain degeneration and hyalinization of renal tubules with accumulation of hemosiderin pigment due to haemorrhage.
0.9	Renal tissue showing congestion and haemorrhage with certain degeneration and necrosis of renal tubules tissue.	Showing certain degeneration and hyalinization of renal tubules with accumulation of hemosiderin pigment due to haemorrhage.
1.2	Renal tissue showing congestion and haemorrhage with certain degeneration and necrosis of renal tubules tissue.	Showing certain degeneration and hyalinization of renal tubules with accumulation of hemosiderin pigment due to haemorrhage.

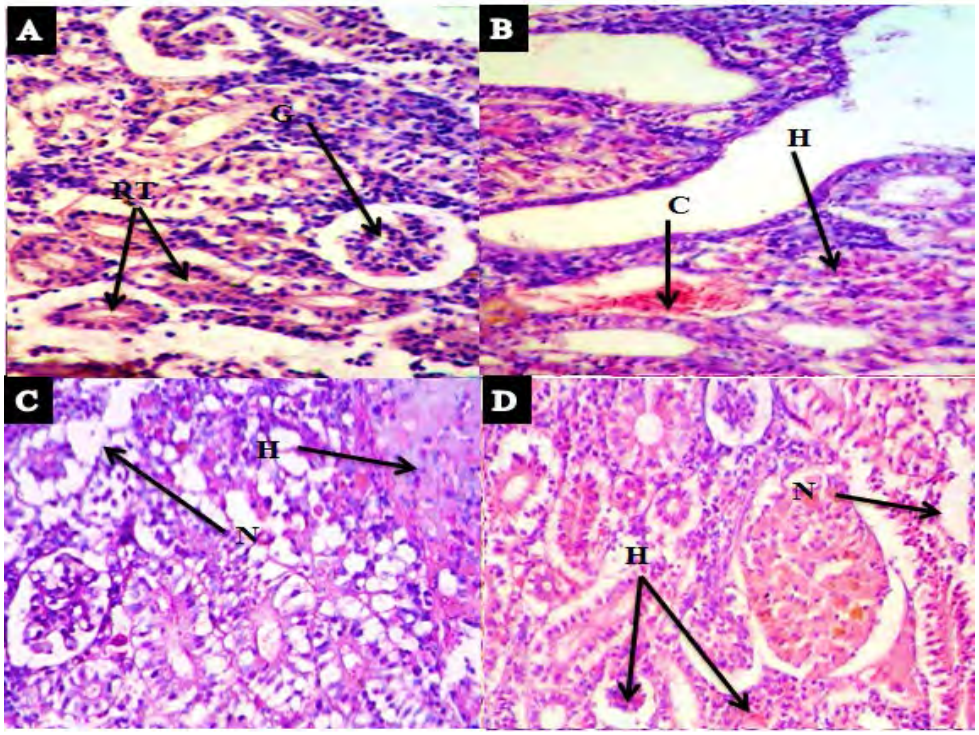

Figure 6: Histological changes of kidney during 3 weeks [A] normal [B] At a concentration of 0.5 mg Cu/L ; [C] At a concentration of 0.9 mg Cu/L ; [D] At a concentration of 1.2 mg Cu/L.

[A] control kidney showing normal histology of glomeruli (G) with renal tubules (RT); While, [B] At a concentration of 0.5 mg/L during 3 weeks showing presence of congestion (C) and hemorrhage (H); [C&D] At a concentrations of 0.9 mg/L and 1.2 mg/L during 3 weeks showing necrosis (N) with hemorrhage (H). H&E; 400x.

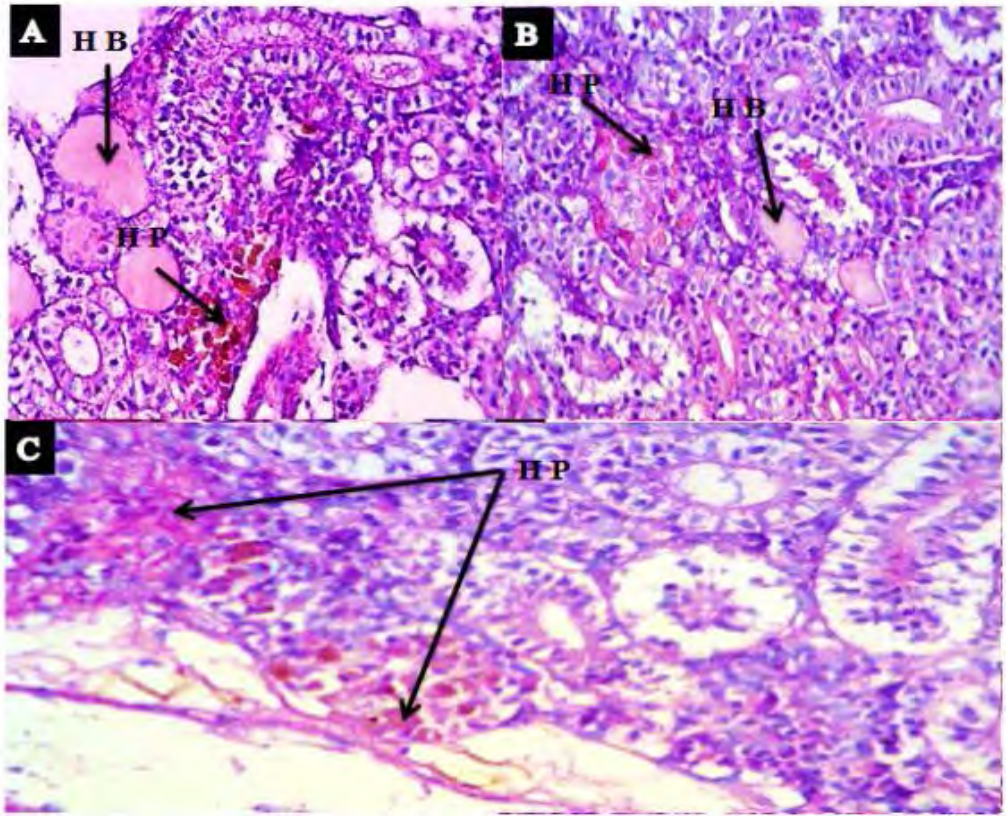

Figure 7: Histological changes of kidney during 6 weeks [A] At a concentration of 0.5 mg Cu/L [B] At a concentration of 0.9 mg Cu/L [C] At a concentration of 1.2 mg Cu/L.

[A] In case of exposed to 0.5 mg Cu/L, fish sample had showing presence of hyaline bodies (HB) inside of renal tubules with accumulation of hemosiderin pigment (HP) due to hemorrhage (H); [B&C] At a concentrations of 0.9 mg/L and 1.2 mg/L during 6 weeks showing necrosis (N) with hyaline bodies (HB) and hemosiderin pigment (HP). H&E. 400x.

3.2.4 Histological changes in muscle

The histological changes in the muscles of carp fish *Cyprinus carpio* L. during a period of 3 weeks and 6 weeks. Muscles tissues also come in close contact with pollutants dissolved in water. Hence, reactions in the ultrastructure of the muscle were spontaneous. Separation of muscle bundles was an interesting observation [24]. Patnaik *et al.,* (2011) have found in their study on the muscle of *Cyprinus carpio* that fish showed marked thickening and separation of muscle bundles with severe intramuscular oedema more pronounced in sublethal treatment of cadmium. The results appear in table 5 and shown in Figure (8).

Table 5: Histological changes in muscles of *C. carpio* after chronic exposure to (0.5, 0.9, 1.2 mg/L) of copper

Con. mg/L	Exposure period of muscles	
	3 Weeks	6 Weeks
control	A section of muscle showing normal muscle fibers with structure.	
0.5	Showing mild hyalinization of the skeletal muscles fibres with the loss of interstitial fibres in between the muscles fibres and focal degeneration and necrosis with mild inflammatory cell infiltration.	Normal structure appearance of muscles fibres tissue.
0.9	Showing mild hyalinization of the skeletal muscles fibres with the loss of interstitial fibres in between the muscles fibres and focal degeneration and necrosis with mild inflammatory cell infiltration.	Normal structure appearance of muscles fibres tissue.
1.2	Showing mild hyalinization of the skeletal muscles fibres with the loss of interstitial fibres in between the muscles fibres and focal degeneration and necrosis with mild inflammatory cell infiltration.	Normal structure appearance of muscles fibres tissue.

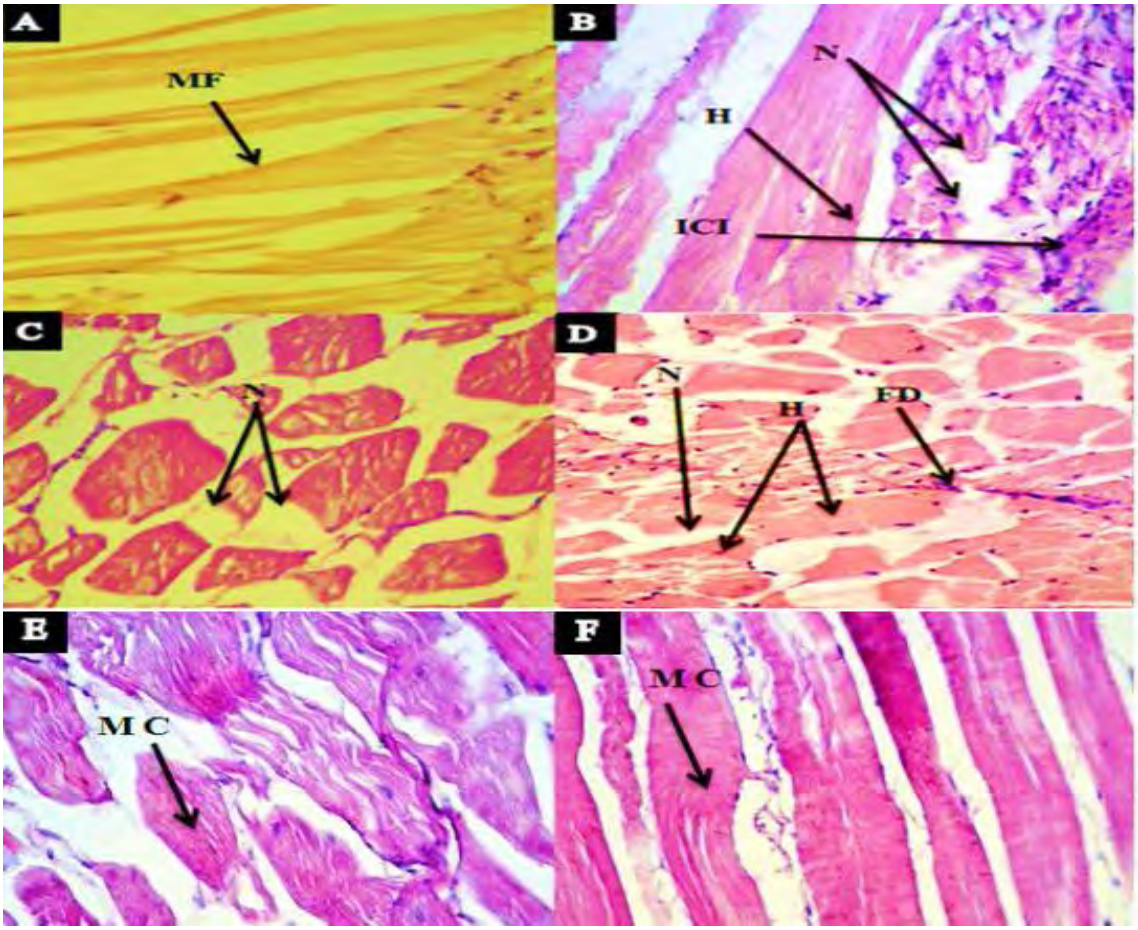

Figure 8: Histological changes of muscles during 3 [A] normal [B] At a concentration of 0.5 mg Cu/L ; [C] At a concentration of 0.9 mg Cu/L ; [D] At a concentration of 1.2 mg Cu/L. ; [E&F] At a concentrations of 0.5, 0.9 and 1.2 mg/L during 6 weeks.

[A] Section of mussels showing normal mussels fibers (MF) with structure; While, [B] At a concentration of 0.5 mg/L during 3 weeks showing presence of hyalinization (H) and necrosis (N) with mild inflammatory cell infiltration (ICI) ; [C] At a concentration of 0.9 mg/L during 3 weeks showing necrosis (N); [D] At a concentration of 1.2 mg/L during 3 weeks showing presence of hyalinization (H), focal degeneration (FD) and necrosis (N).While, [E&F] At the concentrations of 0.5, 0.9 and 1.2 mg/L during 6 weeks showing Normal structure appearance of muscles fibres tissue. H&E; 200x.

3.3 Micronucleus Test (MN)

The current work has found that the highest mean value of micronuclei per blood cell was 21.0±2.160 in 1 cell at concentration of 1.2 ppm while the lowest mean number was 0.33±0.471 at 0.5 ppm metal concentration in 3 cell. [25] observed that copper and cadmium had increased the micronucleus and binucleus frequencies in cells of gill and liver tissues of three fish species; Common carp, Prussian carp and Peppered cory, while in most cases no significant increase was found in peripheral blood erythrocytes. Results of micronucleus test of *Cyprinus carpio* exposed to repeated different concentration of copper period three weeks and six weeks shown in the table (6 and 7) and figures (9, 10 and 11).

Table 6: Mean ± standard deviation of the number of nuclei per blood cell in fishes subjected to different copper concentrations for three and six weeks.

Con.	Mean Copper ± SD							
	3 weeks				6 weeks			
	0	1	2	3	0	1	2	3
0.5	986.7±1.25	11.0±0.816	2.0±0.816	0.33±0.471	971.7±2.055	12.67±0.471	10.33±1.247	5.33±0.471
0.9	984.3±1.25	11.3±0.471	3.0±0.816	1.33±0.471	965.7±3.091	12.67±2.055	14.33±1.247	7.33±0.471
1.2	981.3±1.25	13.7±2.054	2.67±0.471	2.33±0.471	953.3±2.624	21.0±2.160	15.67±0.471	10.0±0.0

Table 7: Mean ± standard deviation of the number of nuclei per blood cell in control fish sample.

Con.	Mean control ± SD			
	0	1	2	3
control	991.7±1.247	7.33±1.247	1.0±0.816	0.0±0.0

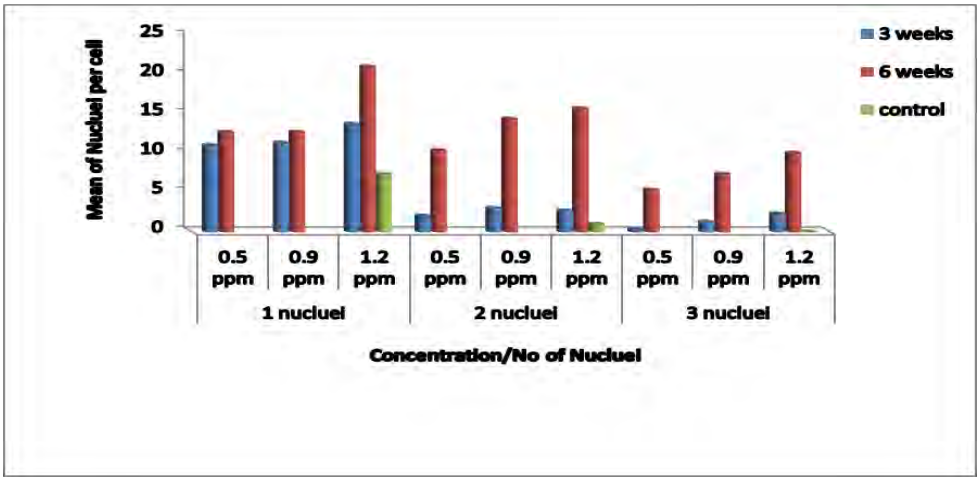

Figure 9: The mean number of micronuclei per cell of fish samples exposed to 3 different Copper concentrations for 3 and 6 weeks.

Analysis of variance of these data reveals significant effects ($P \leq 0.001$) of all number of micronuclei, copper concentrations and the period of exposure. The least significant values of each of these variables are confirming such differences between these examined variables.

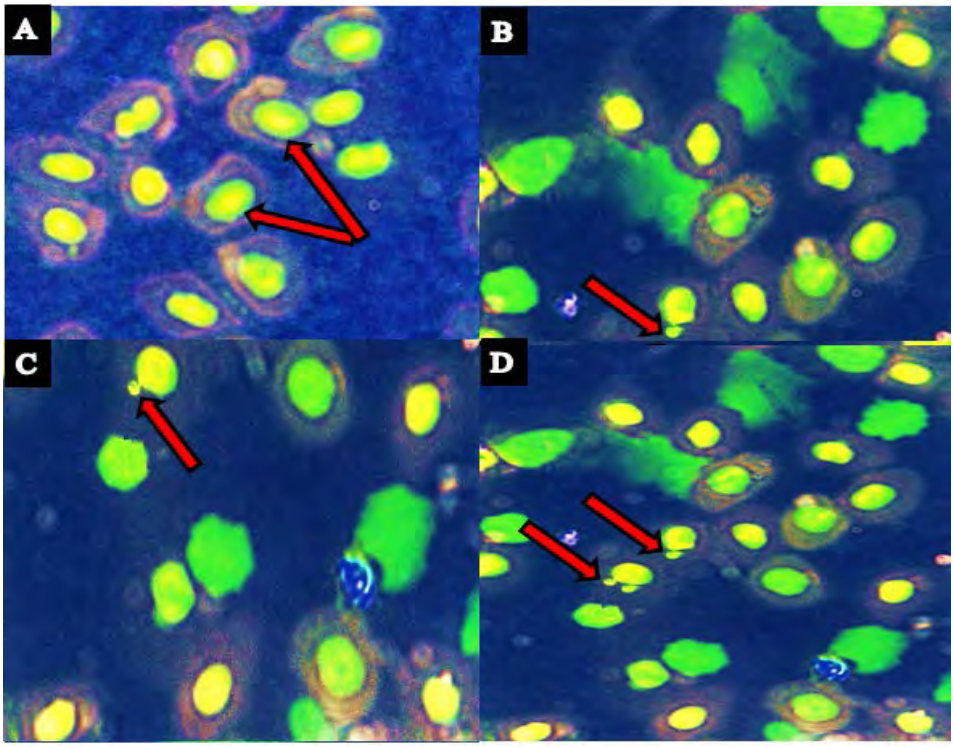

Figure 10: Photomicrographs of Micronucleus (MN) test exposed to different concentration of copper for 3 weeks [A] showing normal erythrocytes; [B] At a concentration of 0.5 mg/L during 3 weeks showing presence of 1 micronucleus (MN) in erythrocytes; [C] At a concentration of 0.9 mg/L during 3 weeks showing presence of 1 micronucleus (MN) in erythrocytes ; [D] At a concentration of 1.2 mg/L during 3 weeks showing of 1 and 2 micronucleus (MN) in erythrocytes. 1000x.

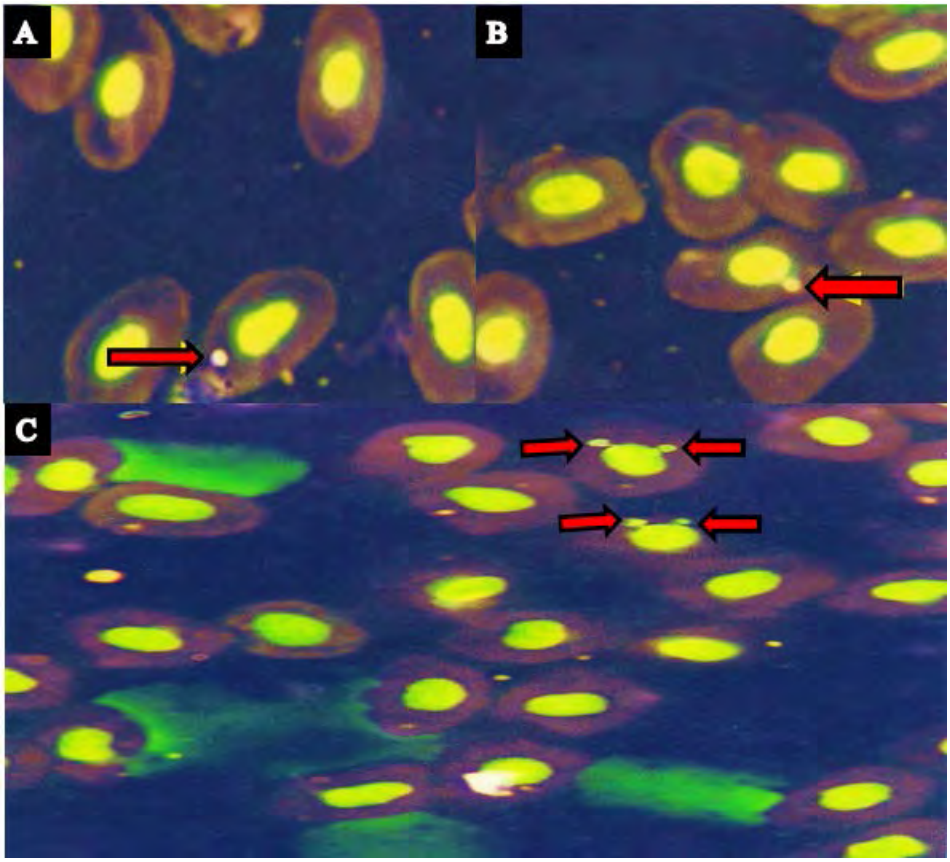

Figure 11: Photomicrographs of Micronucleus (MN) test exposed to different concentration of copper for 6 weeks [A] At a concentration of 0.5 mg/L during 6 weeks showing presence of 1 micronucleus (MN) in erythrocytes; [B] At a concentration of 0.9 mg/L during 6 weeks showing presence of 1 micronucleus (MN) in erythrocytes; [C] At a concentration of 1.2 mg/L during 6 weeks showing of 2 micronucleus (MN) in erythrocytes. 1000x.

4. Conclusions

The current study showed that the examined copper have profound effects of *Cyprinus carpio* represented by noticeable changes in histological parameters. It can be concluded that gill, liver, kidney and muscle alterations as a result of heavy metal exposition of fish may serve as a sensitive biomarker for the toxicity of sublethal concentrations of metals as well as other pollutants. The result indicates that the heavy metal contamination definitely affects the aquatic life of the freshwater fish. Hence, a scientific method detoxification and close monitoring of metal pollution of the river is essential to improving the life of these economically important fishes in any stressed environmental conditions. The results of micronucleus test (MN) tests may be considered as bio-indicator which describes the environmental pollution and risk assessment in a short period.

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