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CASE STUDY

Sublethal effects of copper nanoparticles on the histology of gill, liver and kidney of the Caspian roach, *Rutilus rutilus caspicus*

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ABSTRACT: The current study has determined the toxicity effects of copper nanoparticles on the some vital organs such as gill, liver and kidney of Caspian Roach; *Rutillus rutillus caspicus*. For this purpose, 120 fishes were used as experimental samples and exposed to 0.1, 0.2 and 0.5 mg/L of Cu nanoparticles for 21 days, and 30 fishes assumed as the experiment control. The mean water temperature of the aquaria was 22±2 ºC, dissolved oxygen 5.2 mg/L, pH at 7±0.004 and the concentration of calcium carbonate was 270 ppm. On 7, 14 and 21 days after exposing the fishes to copper nanoparticles, three fishes were randomly selected from each aquaria, sacrificed and samples from their gill, liver and kidney were taken and fixed in cold 10 % buffered formalin. Then microscopic sections were prepared and examined by light microscope which showed histological alternations in the gill, liver and kidney tissues. Evaluation of these changes could be useful in estimating the harmful effects of copper nanoparticles. Histological alternation in gills included: hyperplasia, fusion and detachment of secondary lamellae, blood congestion in vascular axis of primary filaments, reduced secondary lamellae length and cellular degeneration. Histological changes in liver included blood congestion in the central veins, cytoplasmic vacuolation of the hepatocytes, cellular degeneration and congestion in the blood sinusoids and necrosis of the hepatocytes. Histological changes in kidneys included glomerular shrinkage, severe degeneration in the tubules cells, interstitial tissue and glomerulus, increase in interstitial tissue cells and macrophages aggregation. The degree of damages was more intensive at higher copper nanoparticles concentrations. The result of the study showed that copper nanoparticles could cause severe damages in the vital tissues of Caspian roach; *Rutillus rutillus caspicus* and have lethal effects for fish.

KEYWORDS: *Caspian Roach; Copper nanoparticle (CuNPs); Gill; Histopathology; Kidney; Liver*

INTRODUCTION

Copper nanoparticles (CuNPs) are among the most used nanomaterials; they are used in textiles, food storage containers, home appliances, paints, and dietary supplements, among others. Because of their exclusive specifications, such as small size, proportion of the surface to high volume ratio, and more activities, much attention has been paid to them (Khanna *et al,*. 2015). The production and use of CuNPs make their release into wastewaters and industrial effluents easy; they eventually spread to the aquatic environment, even though they are hazardous to the aquatic organisms (Mehndiratta *et al.*, 2013; Shaw *et al.*, 2012). The aquatic organisms receive nanoparticles through the gills, digestive tract, olfactory organ, and

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the skin (Griffitte *et al.,* 2007). At the cellular level, nanoparticles enter the cell via endocytosis and then induce toxic effects on the living organisms (Tang *et al.,* 2015). Furthermore, CuNPs could be accumulated in the organism and transferred to higher trophic levels. Human beings get exposed to nanoparticles by industrial products, food items, drug deliveries, and medical applications (Tang *et al.,* 2015); they may suffer from skin inflammations, gastrointestinal injuries, and respiratory disorders (Mehndiratta *et al.*, 2013). Hence, there is a growing concern about the adverse effects of CuNPs on the body system of fish (Wang *et al.,* 2015). Most CuNPs studies focus on the lethal dose (Griffitte *et al.*, 2007; Shaw *et al.,* 2011), accumulation (Zhao *et al.,* 2011; Shaw *et al.,* 2012), stress response (Gomes *et al.,* 2011; Shaw *et al.,* 2011), osmoregulation (Wang *et al.,* 2014; Shaw *et al.,* 2012), pathology (Al-Bairuty *et al.,* 2013), and enzyme activities (Wang *et al.,* 2015). Caspian roach is one of the most economic fish in the Caspian Sea. However, the population of this valuable fish has diminished in recent years because of over-fishing, destruction of its habitat, and environmental pollutants (Hoseini *et al.,* 2012). However, there is limited information on the sub-lethal toxicity of CuNPs in the Caspian roach. This study has been conducted to determine the histopathological effects of CuNPs in the Caspian roach; *Rutillus rutillus caspicus* after a sub-acute exposure to this material. The gills, liver, and kidneys were evaluated for histological changes. The study has been carried out in the Razi laboratory, Science and Research Branch, Islamic Azad University, Tehran, Iran, in 2016.

MATERIALS AND METHODS

Fish preparation and adaptation

To determine the toxicity of Cu nanoparticles in the Caspian roach, 300 fishes were obtained from the Syjeval fish hatchery pond located in the Turkaman Port of Golestan Province, Iran in summer 2016. The samples were stocked in bags containing water and oxygen; they were then transferred to the laboratory. Caspian roaches with a mean body length of 100.1±20 mm and body mass of 30±10 g were chosen. In the laboratory, for adapting fish to the environmental conditions, they were stocked at aquaria with 110 L of water and aerated with the air pump seven days before experiments. After the adaptation period, they were weighed and separated, and a survival test was conducted later with three replications. Ten fish were included in each replication with 1 g/L of density and a constant aeration for a period of 21 days, (photoperiod 12-12 h). The temperature was 22±2 ºC, dissolved oxygen was 5.2 mg/L, pH was at 7±0.004 and the concentration of CaCO3 was 270 ppm. Based on preliminary tests and results, CuNPs 96 h mean that lethal concentrations (96 h LC50) were 1.41±0.24 mg/L in the Siberian sturgeon (Hua *et al.,* 2014), while sub-lethal concentrations of CuNPs (T0=control, T1=0.1 mg/L, T2= 0.2 mg/L, T3= 0.5 mg/L) were used in triplicate to each concentration in a semi-static condition. The control group, without CuNPs, was kept under the same experimental fish conditions.

Nanoparticles used in the experiment

The nano-copper specifications were included as (Cat, no 007440-50-8, Stock, US), particle size in 40 nm, density 8.9 g/Cm^3 and purity ≥ 99.9% (Fig 1 A-C). Copper nanoparticles suspensions (0.05 g/L) were scattered by sonication in bath type sonicators for half an hour (250 w, 40 kHz. 24 ˚C. Wiseclean).

Fish sampling

At the first, second, and third weeks of the experiments, three fish were randomly selected from each aquarium (Total number = 108). For histopathological study, a piece of liver, a gill arch, and a whole kidney were removed and fixed in cold 10% buffered formalin for at least 24 h before processing.

Histology

Histological analysis followed the standard techniques (Roberts, 2001). Briefly, the samples were prepared in ethanol for dehydration. Then, they were cleared with xylene, and finally impregnated with liquid paraffin wax at 58° C and embedded in paraffin blocks. Samples were then sectioned at 6 µm using a Rotary microtome (leica RM2255) and stained by Hematoxylin and Eosin with Microm HMS7. The stained sections were examined by a light microscope (Olympus CX21).

RESULTS AND DISCUSSION

It is necessary to point out that no mortality was observed during the study in treatments and control performances.

Histological changes of gill

There were no abnormalities in the control fish and their gill showed a normal architecture of the lamellae

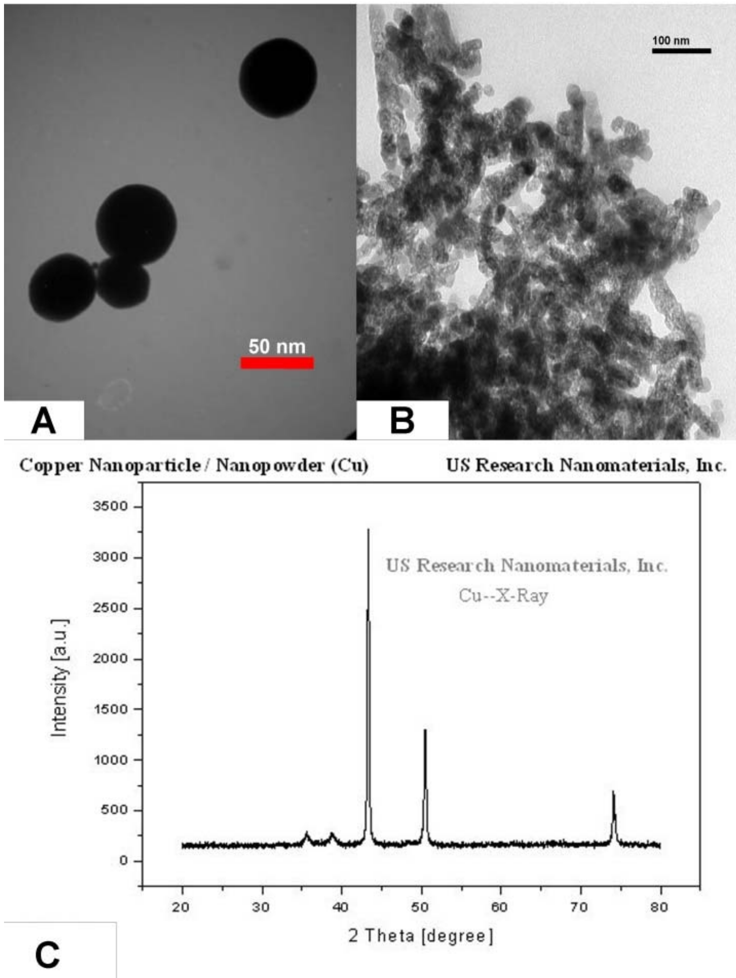

Fig. 1: Specifications of CuNPs used in the experiment: Plate (A) indicate, Transmission Electron Microscopy (TEM) illustration, scale bar=50 nm; Plate (B) indicate, Scanning Electron Microscopy (SEM) illustration, scale bar=100 nm; Plate (C) indicate, size distribution of CuNPs.

(Fig. 2A). Several forms of histopathological changes, a fusion of secondary lamellae, cellular hyperplasia in the primary filaments and telangiectasis were observed in the gill tissues of the fish treated with CuNPs. During the experiment, blood congestion in the primary filaments and epithelial detachment were observed. Enhancement of CuNPs concentrations caused a reduction in secondary lamellae length (Fig. 2 B-E). The examination showed that 0.5 mg/L caused cellular degeneration of gill epithelial tissues (Table 1).

Histological changes of liver

The hepatocytes demonstrated a normal cytoplasm with a large nucleus in the control group (Fig. 3A). When the fish liver was exposed to CuNPs, the results showed some alternations including blood aggregation in the vessels. The rise of CuNPs concentration caused deformation of nuclei, cytoplasmic vacuolation, cellular degeneration, congestion in the blood sinusoids and necrosis of the hepatocytes (Fig. 3B-E). These degenerations were more intensive at higher CuNPs concentrations (Table 2).

Histological changes of kidneys

The histological study showed a typical structure of the kidneys in the control group (Fig. 4A). However, the results showed that the concentration of CuNPs has made some histopathological changes in the kidneys

Table1: Histopathological scores of Caspian roach gill exposed to continuous exposure of CuNPs

Treatments / Lesions	7 day T1	7 day T2	7 day T3	14 day T1	14 day T2	14 day T3	21 day T1	21 day T2	21 day T3
Hyperplasia	+	+	+	+	++	++	+	+++	+++
Fusion	-	-	-	-	+	+	-	++	++
Detachment	-	+	+	-	+	+	-	++	++
Blood congestion in primary and secondary filament	++	++	++	++	++	+++	-	++	+
Secondary lamellae deformation	-	++	+	+	++	++	++	+++	+++
Telangiectasia	-	+	-	+	+	+	++	+++	++

- No significant microscopic changes
\+ Mild changes (10 percent change in 40x objective microscope view)
++ Moderate changes (20 percent change in 40 x objective microscope view)
+++ Severe changes (more than 20 percent change in 40 x objective microscope view); (Dutta *et al.,* 1996)
CuNP concentration was * (T1= 0.1 mg/L, T2=0.2 mg/L and T3= 0.5 mg/L).

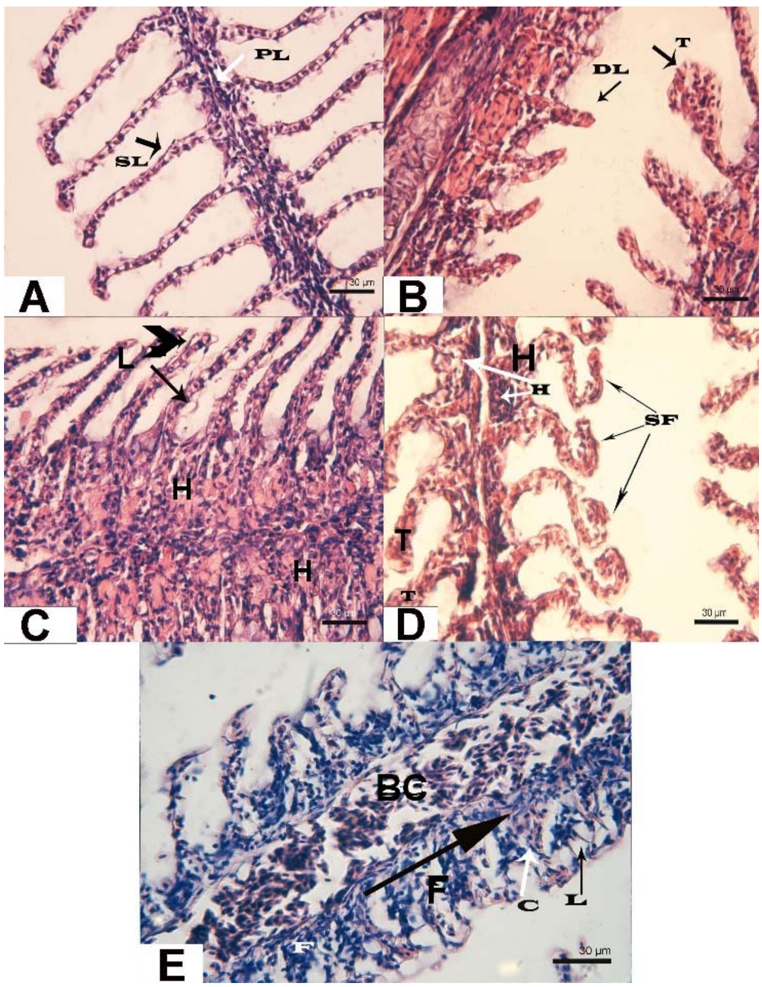

Fig. 2: Micrographs of the gill of Caspian roach (Stained by Hematoxilin and Eosin)
[A] Normal image of the gill, primary lamellae (PL), Secondary lamellae (SL)
[B] Telangiectasis at the secondary lamellae (T), Decreasing in lamellae length (DLa)
[C] Detachment (Da), hyperplasia (H)
[D] Telangiectasis at the secondary lamellae (T), S formation of lamellae (SF), Hyperplasia (H)
[E] Blood congestion in primary lamellae (BC), fusion (Fa), Detachment (Da), Congestion (C)

of treated fish. The abnormalities included glomerular shrinkage, severe degeneration in the tubular cells, interstitial tissue, and glomerulus, an increase of interstitial tissue cells, macrophages aggregation and vacuolation (Fig. 4B-E), (Table 3).

In accordance with the study of Hua *et al.,* (2014), nanoparticles toxicity is size-dependent, where smaller particles have more toxic effects. It is significant that the concentration of CuNPs is increasing every year in aquatic environments, and this concentration may be fatal for aquatic creatures (Ostaszewska *et al*., 2016). CuNPs have destructive effects on lives of fish. The results of histological surveys revealed some abnormalities caused by CuNPs in the vital organs such as the gills, the kidneys, and liver of the Caspian roach, with the most severe histological changes being exhibited in the fish exposed to 0.5 mg/L of CuNPs. Histological lesions of detachment, hyperplasia and telangiectasia were reported earlier due to exposure of ZnO nanoparticles to other fish (Linhua and Lei, 2012), silver nanoparticles (Louei Monfared *et al.,* 2015), TiO2 nanoparticles (Ostaszewska *et al.,* 2016), and other contaminants (Saber, 2012). The abnormalities, such as fusion and shortening of lamellae and hyperplasia,

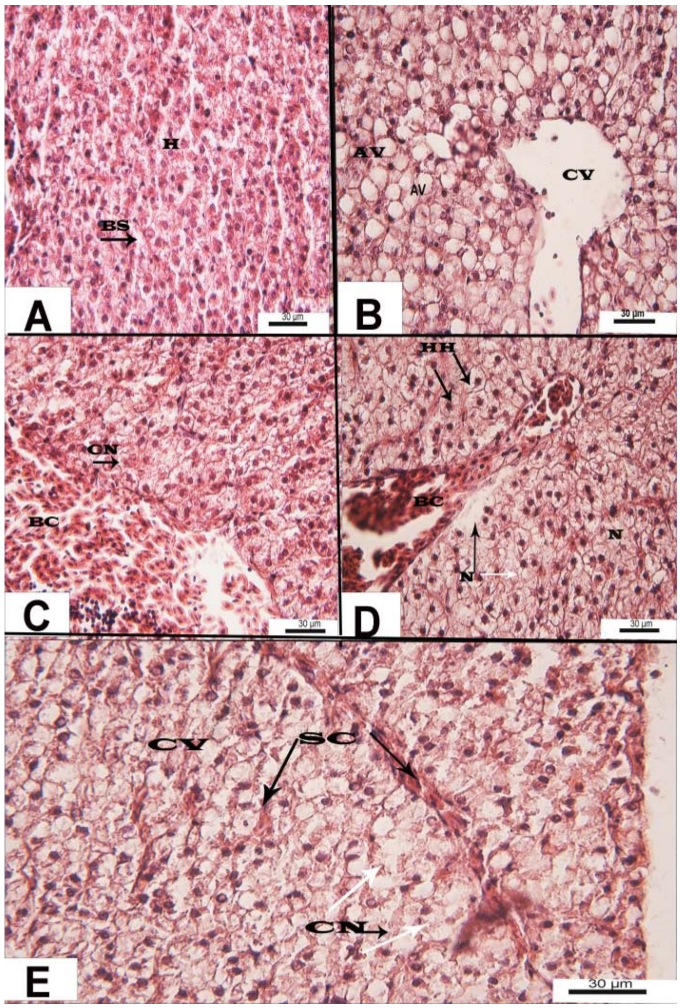

Fig. 3: Micrographs of the liver of Caspian roach (Stained by Hematoxilin and Eosin)
[A] Normal liver tissue, hepatocytes (He), Blood sinusoid (BS)
[B] Acute vacuolation (AV), Vein (Ve)
[C] Necrosis (N), Central vein blood congestion (BC)
[D] Necrosis (N), Blood congestion (BC), Hepatocyte hypertrophy (Hy)
[E] Vacuolation (V), Congestion (C), Necrosis (N)

Table 2: Histopathological scores of Caspian roach liver exposed to continuous exposure of CuNPs

	7 day			14 day			21 day		
Treatments / Lesions	T1	T2	T3	T1	T2	T3	T1	T2	T3
Blood congestion in the central veins	++	++	++	++	++	+++	+	+	-
Vacuolation	-	-	-	-	-	++	+	++	+++
Hypertrophy	-	+	++	+	++	+++	+	+	-
Necrosis	-	-	+	+	+	++	+	+	+++
Blood congestion in the sinusoid	++	++	+++	++	++	+	+	+	-

- No significant microscopic changes
+ Mild changes (10 percent change in 40x objective microscope view)
++ Moderate changes (20 percent change in 40 x objective microscope view)
+++ Severe changes (more than 20 percent change in 40 x objective microscope view); (Dutta *et al.,* 1996)
CuNP concentration was * (T1= 0.1 mg/L, T2=0.2 mg/L and T3= 0.5 mg/L).

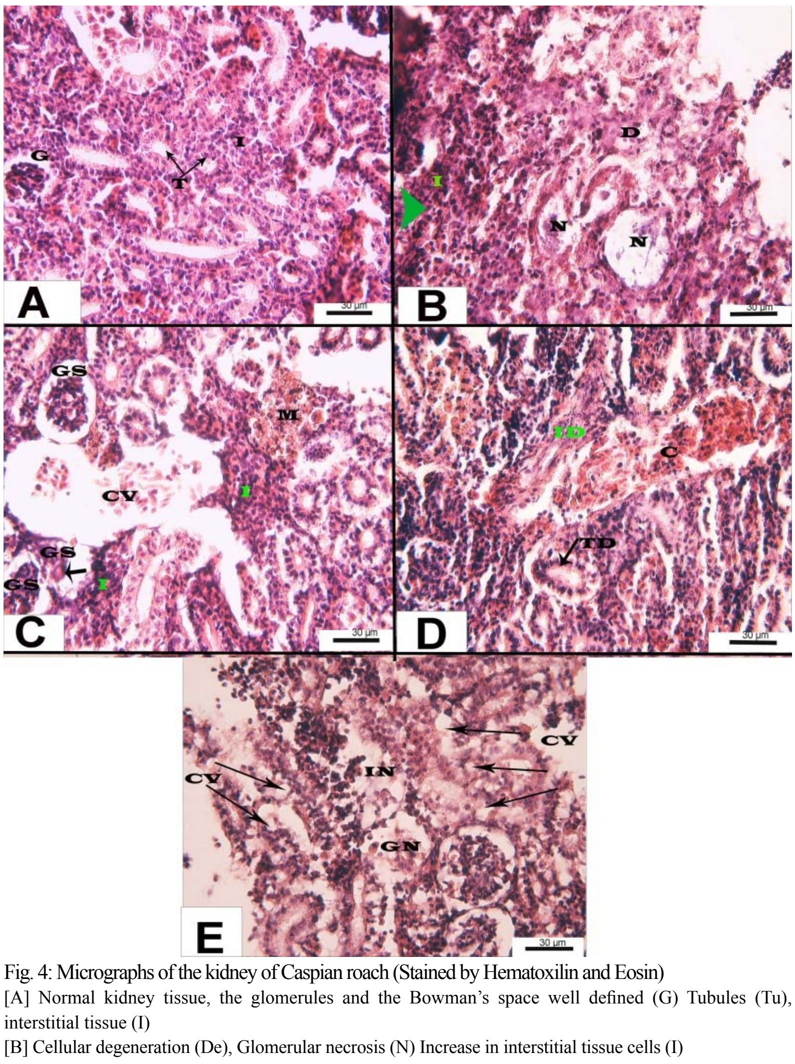

Fig. 4: Micrographs of the kidney of Caspian roach (Stained by Hematoxilin and Eosin)
[A] Normal kidney tissue, the glomerules and the Bowman's space well defined (G) Tubules (Tu), interstitial tissue (I)
[B] Cellular degeneration (De), Glomerular necrosis (N) Increase in interstitial tissue cells (I)
[C] Glomerular shrinkage (GSh), Increase in interstitial tissue cells (I), Macrophages aggregation (M), Central vein (V)
[D] Interstitial tissue degeneration (IDe), Congestion (C), Tubule degeneration (TD)
[E] Cellular vacuolation (CeV), Glomerular necrosis (GN), Interstitial tissue necrosis (IN)

decrease of the relations between gills and environment, which reduces ion and gas exchanges. Bilberg *et al.,* (2010) observed respiratory disorders and decrease in resistance against hypoxic conditions in Eurasian perch, after 24 hours, exposure to silver nanoparticles. The hypoxic conditions resulting from histological changes have been reported in the common carp by Linhua and Lei, (2012), and based on the findings of these authors, these damages have occurred owing to oxidative stresses. In the present study, CuNPs made dilation of capillaries and congestion of erythrocytes. According to Martinez *et al.* (2004), these abnormalities demonstrate injuries to pillar cells and blood vessels and increase of the blood flow in lamellae. Based on the reports of various authors, detachment always occurs because of edema in filaments (Jinyuan *et al.*, 2011). These changes have been observed in fish exposed to various nanometals. This phenomenon can be attributed to a defensive manner, since disruption of lamellae raises the spacing between pollutants and the blood flow (Arellano *et al*., 1999). Telangiectasis is an aggregation of blood cells at the end of secondary lamellae, which is produced due to the injuries occurred on the pillar cells and capillaries by contaminating materials (Hadi *et al.,* 2012). Nanoparticles disturbing the ion transfer and consequently osmotic balance is disturbed (Shaw *et al.,* 2012). According to the findings of Karlsson *et al*. (1985), increases in layers of epithelium cells is due to mitotic divisions in the epithelium of lamellae. Kanthom *et al*. (1995), stated that hyperplasia and fusion in the gills are due to toxicants, and the toxicants change glycoprotein compositions in mucous cells. Hence, they have a negative influence on the adherence of adjacent lamellas (Ferguson, 1989). Changes, such as hyperplasia, with the fusion of some filaments and epithelial detachment is considered as defensive mechanism of the body for increasing the distance between blood and the surrounding environment in such a way that it prevents contaminants from entering the body (Fernandes and Mazon, 2003).

The hepatic histological changes are mostly measured in toxicological research, and considered as a sign of pollution in the environment (Abarghoei *et al*., 2012). The liver can degenerate toxic agents owing to the great potential in the enzymatic system. However, hepatocytes may receive negative effects by a high concentration of contaminants (Bruslé *et al.,* 1996). Histological hepatic changes are varied based on the types of nanoparticles, its concentration, fish species, and time exposed to it besides other items (Shaw *et al*., 2012). Histological changes in liver cells have been reported in the fishes treated with different nanoparticles (Govindasamy and Rahuman, 2012; Al-Bairuty *et al.,* 2013; Ostaszewska *et al.,* 2016). The intensity of these histological damages in the Caspian roach was enhanced by an increase in concentration of nanoparticles. Fish livers treated with 0.5 mg/L of CuNPs demonstrated the vacuolization of hepatocytes and necrosis. The necrosis is one of the most severe cases of tissue damage and final stage of cells' lives. In other hand, most of the cells die because of damage. As a result, tissues cannot perform their functions. Similar observations were reported by other authors (Hao *et al.,* 2009; Albairuty *et al.,* 2013; Ostaszewska *et al.,* 2016). Unnatural reposition of triglycerides and other lipids may make vacuoles in cells and can create histological damage such as necrosis (Kelly and Janz, 2009). Formation of vacuoles is a defence

Table 3: Histopathological scores of Caspian roach kidney exposed to continuous exposure of CuNP

Treatments / Lesions	7 day			14 day			21 day		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
Glomerular shrinkage	-	+	++	+	++	+++	++	++	-
Degeneration	-	-	-	-	+	+	+	++	+++
Increase in interstitial tissue cells	+	+	++	+	+	++	+	+	-
Macrophages aggregation	+	+	++	+	++	+++	+	-	-
Vaculation	-	-	-	-	-	+	+	+	+++
Blood congestion	-	+	++	+	++	+++	-	-	-
Necrosis	-	-	-	-	+	++	+	++	+++

\- No significant microscopic changes
\+ Mild changes (10 percent change in 40x objective microscope view)
++ Moderate changes (20 percent change in 40 x objective microscope view)
+++ Severe changes (more than 20 percent change in 40 x objective microscope view); (Dutta *et al.,* 1996)
CuNP concentration was * (T1= 0.1 mg/L, T2=0.2 mg/L and T3= 0.5 mg/L).

mechanism against harmful compounds (Mollendroff, 1973); it can collect the injurious elements and avoid any disturbance in the biological activities of this organ (Hadi *et al.,* 2012). Findings of this study demonstrated sinusoidal dilatation in the fish livers exposed to 0.1, 0.2, and 0.5 mg/L of copper nanoparticles. Similar changes have been reported by other authors (Gonzalez *et al.,* 2006; Monfared *et al.,* 2013; Govindasamy and Rahuman, 2012; Ostaszewska *et al.,* 2016). Hypertrophy usually means an increase in the size of cells. When a cell is exposed to some materials, they may cause proliferation of the endoplasmic reticulum membrane and hence make the cells grow (Hinton and Laurèn, 1990). Figueiredo-Fernandes *et al.,* (2007) believed that hepatocytes growth could be the result of high accumulation of lipids in cells. However, Braunbeck *et al.,* (1990) reported that deformation of the nucleus is mostly the sign of an increase in cell metabolic activities, whereby the cell tries to omit harmful factors from body surroundings. Sanad *et al.,* (1997) declared that the necrosis of hepatocytes can be due to the prevention of DNA synthesis required for development and maturity of cell by the contaminants.

Similarly, the kidneys are important organs that are influenced by water pollution (Thophon *et al.,* 2003). As the kidneys have a major role in the removal of toxic substances, this research reviewed the histological changes resulting from CuNPs in the kidney structure of the Caspian roach. Histological findings consisted of glomerular shrinkage, blood congestion and increase in the number of inflammatory cells between tubules and interstitial tissues, severe degeneration in the tubules, vacuolization, and macrophages aggregation in interstitial tissues. Degeneration of tubules could be recognized by various signs, such as cellular hypotrophy which created a net-like appearance reflecting the primary stages of degeneration. This initial stage in the deformation process may develop to make hyaline eosinophilic granules inside or outside kidney cells. Re-absorption of plasma protein excreted in urine may cause such granules, reflecting damage in the corpuscle (Takashima and Hibiya, 1995). Xu *et al.,* (2007) found that CuNPs cause an increase in oxidative stress and frustration in the oxidant-antioxidants balance in cells, which may lead to apoptosis induction in glomeruli and cause cytotoxicity in glomerulus. These damages may cause cellular degeneration, haemorrhages and congestion of the kidneys which had been reported earlier by other authors (Abdelhamid and El-Ayouty, 1991; Hadi *et al.,* 2012). Shaw *et al.,* (2011) evaluated that CuNPs affect the cell membrane and prohibit ion transfer. Hence disturbing the fluid transfer system to and out of cells. Therefore, there is an idea that nanoparticles are merged with the cell membrane and a great volume of cellular fluids are filtered. For instance, aggregation of erythrocytes in interstitial tissue owing to the degeneration of endothelium in capillaries may have the same reason.

CONCLUSION

This study concluded that the histopathological evaluation of organs was indispensable for recognition of any damage to tissues by nanoparticles. Among the tissues, the gill, liver and kidneys might be the most sensitive to CuNPs. According to the findings, CuNPs can induce severe necrosis and other tissue injuries to the gill, liver, and kidneys of the Caspian roach. The extent of tissue damage depends on the degree of the CuNPs exposure. This study could help researchers to determine water quality criteria, and it is important to make regulations that forbid companies to spread nanoparticles into aquatic environments and conserve this valuable fish species.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

ABBREVIATIONS

AV	Acute vacuolation
BC	Blood congestion
BS	Blood sinusoid
C	Congestion
ºC	Degree Celsius
$CaCO_3$	Calcium carbonate
CeV	Cellular vacuolation
Cu	Copper
CuNPs	Copper nanoparticles
Da	Detachment
De	Degeneration
DLa	Decreasing in lamellae length
DNA	Deoxyribo nucleic acid
Fa	Fusion
G	Glomerules
g	Gram

g/cm³	Gram per square centimeter
g/L	Gram per liter
GN	Glomerule necrosis
GSh	Glomerular shrinkage
H	Hyperplasia
h	Hour
He	Hepatocytes
Hy	Hepatocyte hypertrophy
I	Interstitial tissue
IDe	Interstitial tissue degeneration
IN	Interstitial tissue necrosis
L	Liter
µm	Micrometer
mm	Millimeter
M	Macrophages aggregate
mg/L	Milligram per liter
N	Necrosis
Nm	Nano meter
PL	Primary lamellae
SEM	Scanning electron microscopy
SF	S formation
SL	Secondary lamellae
T	Telangiectasis
T0	Control group
T1	Fish exposed to 0.1 mg/L CuNPs
T2	Fish exposed to 0.2 mg/L CuNPs
T3	Fish exposed to 0.5 mg/L CuNPs
TEM	Transmission electron microscopy
TDe	Tubule degeneration
TiO2	Titanium dioxide
Tu	Tubules
V	Vacuolation
Ve	Vein
ZnO	Zinc oxide

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