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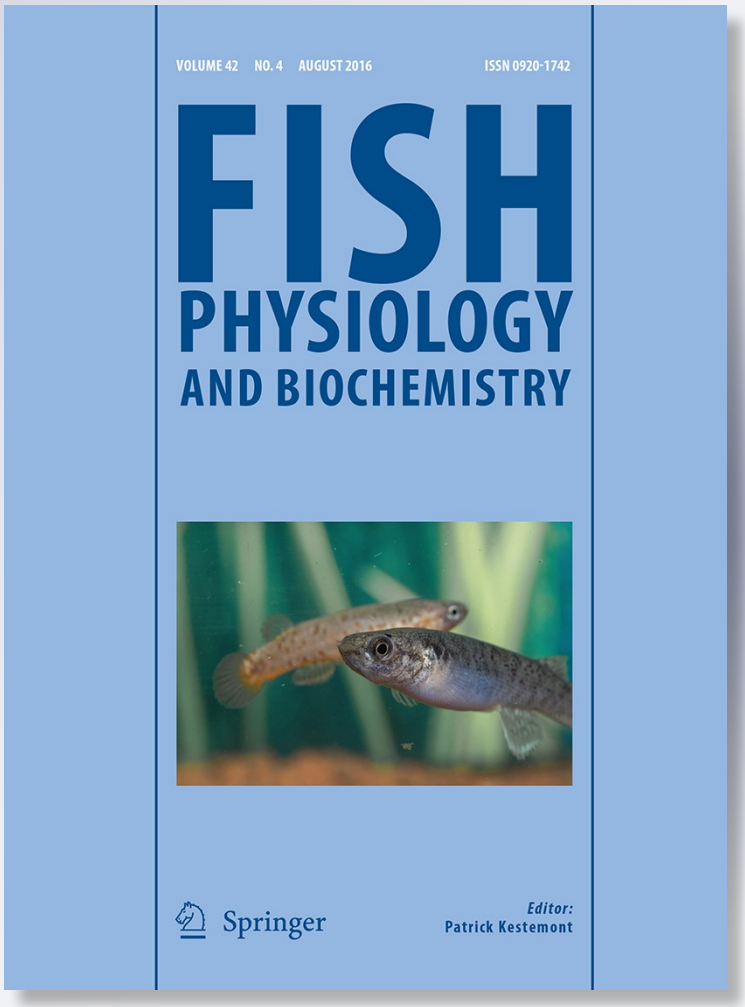

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Toxicity evaluation of copper oxide bulk and nanoparticles in Nile tilapia, *Oreochromis niloticus*, using hematological, bioaccumulation and histological biomarkers

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Abstract The increased industrial applications of nanoparticles (NPs) augment the possibility of their deposition into aquatic ecosystems and threatening the aquatic life. So, this study aimed to provide a comparable toxicological effects of nano-CuO and bulk CuO on a common freshwater fish, *Oreochromis niloticus*. Fish were exposed to two selected doses (1/10 and 1/20 of the LC_{50}/96 h) of both nano-/bulk CuO for 30 days. Based on the studied hematological parameters (RBCs count, hemoglobin content and hematocrit%), the two selected concentrations of CuO in their nano- and bulk sizes were found to induce significant decrease in all studied parameters. But, nano-CuO-treated fish showed the maximum decrease in all recorded parameters among the all studied groups especially at the low concentration of 1/20 LC_{50}/96 h. Hematological status was also confirmed using the calculated blood indices (MCV, MHC and MCHC). In case of bulk CuO-treated groups, the significant decrease in the studied hematological parameters was not followed by any change in MCV and MCH (normocytic anemia), while fish that exposed to NPs showed a significant increase in all calculated blood parameters reflecting erythrocytes swelling which is related to the intracellular osmotic disorders (macrocytic anemia). Regarding metal bioaccumulation factor, the results showed that CuO NPs had more efficiency to internalize fish tissues (liver, kidneys, gills, skin and muscle). The accumulation pattern of Cu metal was ensured by histopathological investigation of liver, kidneys and gills. The histopathological analysis revealed various alterations that varied between adaptation responses and permanent tissue damage.

Keywords *Oreochromis niloticus* · CuO (BPs and NPs) · Hematological parameters · Histopathology

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Introduction

As a result of their unique properties, NPs gained growing attention because of their excessive surface area and tiny size that completely differ from their bulk particles (BPs) counterpart of the same materials (Jahanbakhshi et al. 2015). So, comparative studies of nanoscale and microscale materials are important because the intrinsic characteristics of NPs may be directly related to their toxicity (Hao et al. 2013; Ribeiro et al. 2013). Over the last decade, several studies have reported that metal oxide NPs are potentially toxic, but few attempts have been done to assess the ecotoxicity of nano-metal oxides in aquatic systems (Miller et al. 2010; Adam et al. 2015). Copper is one of the essential elements in the biological system that has an important role in homeostasis

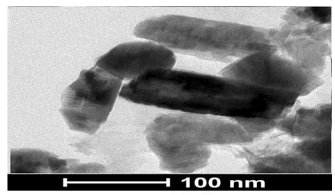
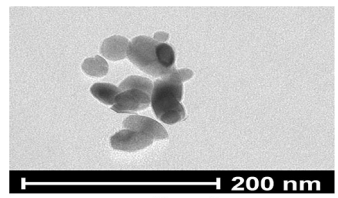
maintenance. So, any change in Cu concentration may lead to sever adverse effects. Many toxicological studies have been focused on atmospheric or inhalation exposures to CuO NPs, while few studies have analyzed the toxicological impacts of CuO NPs on fish (Khabbazi et al. 2015). In the aquatic environment, CuO NPs are considered as a significant source of contamination due to its widespread applications in antifouling paints that used in boats and immersed structures (Perreault et al. 2012). Therefore, the potential toxicity CuO NPs should not be ignored. The use of biological markers assists in the identification of causal relationship between the exposure of toxic materials and the expected toxicological effects on individual and population levels (Gomes et al. 2011). So, there is an urgent need to study the impacts of metal oxides like CuO NPs on different fish species because fish is considered as one of the main nontarget aquatic organisms that affected by pollution (Jahanbakhshi et al. 2015). Studying different hematological parameters and calculated blood indices in fish has become an important health indicator and has an important role in understanding either normal or pathological processes (Saravanan et al. 2011). Toxic responses affect the normal physiological functions, endanger the health of fish and lead to fish death in sever conditions. Therefore, any variations in hematological parameters indicate disturbances in physiological processes. Moreover, the histopathological endpoints have been recommended as a highly relevant methodology during the assessment of aquatic contaminations since it reflects the true health state of the affected organism (Velma and Tchounwou 2010). The pathological alternations that occur in many vital organs are reasonably well known in fish that exposed to waterborne copper (Singh et al. 2008). Metal accumulation in internal tissues is the major route through which a certain pollutant can transfer across the food chain and finally assimilated by human consumers resulting in health risks (Abdel-Khalek 2015). Therefore, determination of accumulated metals in fish is really significant from toxicological point of view. The present study aimed to provide a comparative study between CuO (BPs and NPs) at 1/10 and 1/20 LC_{50}/96 h concentrations to declare their accumulation levels and toxic effects using Nile tilapia, *Oreochromis niloticus*, as an animal model. In addition, this study depended on several hematological and histopathological end points to give a complete picture about the health state of the exposed fish.

Martials and methods

Materials preparation and experimental design

CuO BPs were purchased from El-Nasr pharmaceutical chemicals Co., Egypt, while CuO NPs (non-coated) were purchased from Sigma-Aldrich, St. Louis, MO, USA, with an average size <50 nm and 99 % purity level. Nile tilapia, *O. niloticus*, were obtained from fish farm fed on unpolluted water located in El-Ismailia governorate, Egypt. The initial body length and weight of fish were 15.4 ± 0.74 cm and 57.3 ± 7.27 g, respectively. Fish were transported in plastic container with continuous aeration to the laboratory where they maintained for 14 days in glass aquaria with 50 l aerated, dechlorinated tap water (eight fish/aquarium). The water temperature was kept at 25 ± 1 °C, while dissolved oxygen and pH were 6.5–7.8 mg/l, and 7.1–7.3, respectively. The feeding was done once a day with commercial pellets (20 % crude protein, 4 % crude fat, 5 % crude fiber, 12 % crude ash and 10 % crude moisture) throughout the acclimatization period. The water was partially changed daily, and any fish wastes, dead fish or abnormal fish were excluded. As detailed in our previous study Abdel-Khalek et al. (2015), the LC_{50}/96 h of bulk CuO (2205 mg/l) and nano-CuO (150 mg/l) was determined by probit analysis, using statistical program (SPSS software, version 16.0, IBM, Chicago, IL, USA). Also, a complete characterization of CuO particles was done using field emission transmission electron microscopy (FETEM; JEM-2100F, JEOL Inc., Japan) at an accelerating voltage of 200 kV for size determination; Nano-Zetasizer-HT, Malvern Instrument, UK, for dynamic light scattering (DLS) determination; and a Malvern Zeta sizer Nano ZS instrument for zeta potential determination as summarized in Table 1. Two suspension concentrations of both bulk and nano-CuO forms (1/10 and 1/20 LC_{50}/96 h) were prepared by weighing dry CuO powder into the dechlorinated water (pH 7.4) and then ultrasonicated (100 W, 40 kHz) for 1 h to increase their dispersion. Then fish were randomly distributed in glass aquaria (40 × 70 × 26 cm) in triplicates of five experimental groups (eight fish/

Table 1 Characterization of CuO BPs and NPs as described in Abdel-Khalek et al. (2015)

Parameters	CuO BPs	CuO NPs
Size from TEM (nm)	140–200	30–40
Average hydrodynamic size (nm)	954	588
Zeta potential (mV)	11.5	−5.32
Purity (%)	>99	>99
Shape	 Rod	 Granular

aquarium): 1/20 of LC_{50}/96 h value of CuO BPs, 1/10 of LC_{50}/96 h value of CuO BPs, 1/20 of LC_{50}/96 h value of CuO NPs, 1/10 of LC_{50}/96 h value of CuO NPs and control (handled identically but without exposure to CuO). The water conditions were kept as in acclimatization period, and water was constantly checked for pH, temperature and dissolved oxygen using Corning Checkmate II multi-parameter meter. Water was changed every 2 days, and partially retreating process was done (80 % water change every 2 days with retreating with CuO particles to keep the concentration after each change constant). Fish were fed 40 min. before each water change to minimize the adsorption of metal particles on food pellets.

Hematological analysis

After 30 days, blood samples were withdrawn from the caudal vein of the studied fish using heparin as anticoagulant. The needle was run as deep as possible through the midline just behind the anal fin in a dorso-cranial direction. By drawing the needle gently backwards, blood is usually sucked into the syringe in order to determine the hematological parameters.

Blood samples were mixed and diluted with physiological saline solution (0.9 % NaCl). The red blood cells (RBCs) were counted using improved Neubauer hemocytometer according to Dacie and Lewis (1991).

Hemoglobin (Hb) concentration was determined by Drabkin (1964) method, and cyanomethemoglobin was measured spectrophotomerically at 540-nm wavelength. Hematocrit (Hct) was carried out in small heparinized Hct tubes using Hct centrifuge at 3000 rpm. for 15 min, and then the percentage volume of the RBCs to total blood volume was calculated.

Blood indices were calculated according to Gupta (1977) as below:

The mean corpuscular volume (MCV) was calculated from the following formula: MCV = Hct × 10/RBCs (million/mm^3). The mean corpuscular hemoglobin (MCH) was calculated from the following formula: MCH = Hb content (gm/dl) × 10/RBCs (million/mm^3). The mean corpuscular hemoglobin concentration (MCHC) of hemoglobin in 100 ml packed cell volume was calculated from the following formula: MCHC = Hb content (gm/dl) × 100/Hct.

Determination of the accumulated Cu and bioaccumulation factor in the studied tissues

Accumulated Cu in tilapia fish of all studied groups was determined using a flame atomic absorption spectrophotometer (Perkin-Elmer-2280, USA) according to APHA (2005). Fish were dissected, and then vital tissues named liver, kidneys, gills, skin and muscles were obtained. The isolated tissues were dried, acid-digested by concentrated HCl using the dry-ashing procedure proposed by Issac and Kerber (1971) and Hseu (2004) and then diluted with deionized water to known volume. In order to correct the background absorption, the procedural blanks were aspirated throughout the measurement process. Samples with known concentration of standard solution were

measured during the analysis procedure to check the measurement accuracy. Also, the analysis accuracy was checked by standard reference material (Lake Superior fish 1946 NIST, National Institute of Standards and Technology, USA), and the metal recovery ranges lied between 95 and 110 %. The accumulated Cu was expressed as mg/kg dry weight. Bioaccumulation factors of Cu were calculated according to the following equation: BAF = Cu concentration in tissue (mg/kg)/Cu concentration in water (mg/l).

Histopathological examination

Liver, kidneys and gills of the studied fish were preserved in Bouin's fixative and then transferred to 70 % alcohol, and clearing in xylene and paraffin wax embedding were followed. The selected tissues were sectioned at 4 μm and then stained using hematoxylin and eosin (Carleton et al. 1967).

Statistical analyses

The results were expressed as mean ± SE. Data were statistically analyzed using Student *t* test and Duncan's multiple range test to determine difference in means ($p < 0.05$) using Statistical Processor Systems Support, SPSS software, version 16.0, IBM, Chicago, IL, USA.

Results and discussion

In comparison with its bulk-sized materials of the same chemical composition, nano-sized materials often display unique chemical and physical characteristics. As shown in Table 1, the characterization results revealed that NPs (30–40 nm) had granular shape, well distribution pattern with less aggregation (confirmed by DLS and zeta potential) in comparison with rodlike BPs that showed much aggregation potency. These unfamiliar characteristics have special tendency to exhibit more toxic effects. Gaiser et al. (2011) reported that silver and cerium dioxide NPs have more toxic effects on *Daphnia magna* than their micro-sized counterparts because of the smaller size of the particles. Khabbazi et al. (2015) stated that the data that deal with the effects of CuO NPs on hematological parameters are scarce. The toxic substances cause fluctuations in many hematological parameters, either via enhancing their number or concentration by promoting their biosynthetic activities or via falling their number or concentration in by suppressing their biosynthetic sites (Dar and Borana 2014). In comparison with control group, RBCs that counted from 1/10 LC_{50}/96 h of CuO BPs-treated groups showed a significant decrease than those exposed to 1/20 LC_{50}/96 h of CuO BPs, while RBCs count showed significant decrease in fish that exposed to 1/20 LC_{50}/96 h than those exposed to 1/10 LC_{50}/96 h in case of NPs-treated groups (Fig. 1a). The Hb content of 1/10 LC_{50}/96 h of CuO BPs exposed groups showed a significant decrease, while a nonsignificant difference was observed in fish that exposed to 1/20 LC_{50}/96 h. However, Hb content revealed a significant decrease at the concentration of 1/20 LC_{50}/96 h than those exposed to 1/10 LC_{50}/96 h of CuO NPs when compared with the control group (Fig. 1b). Concerning Hct, fish that exposed to 1/10 LC_{50}/96 h of CuO BPs showed a significant decrease than those exposed to 1/20 LC_{50}/96 h of CuO BPs, while Hct values showed a significant decrease when fish exposed to both concentrations of CuO NPs compared with the control groups (Fig. 1c).

These results were in accordance with Dhanapakiam and Ramasamy (2001) who found a reduction in RBCs, Hb and Hct values in the common carp that exposed to waterborne metals including Cu for 30 days. The continuous Cu exposure leads to reduction in Hb content and Hct% via impairing the hemopoietic processes and accelerating the disintegration of erythrocyte membranes. The erythrocytes destruction leads to a reduction in the RBCs count and consequently decreases Hb content and Hct values. The decrease in the studied blood parameters may be attributed to RBCs hemolysis which confirmed by the current intravascular hemolysis in the blood vessels of the liver and kidneys. This reduction in RBCs count may be related to the impaired intestinal absorption of iron or possible disruption of hematopoietic tissues that induced by Cu exposure (Dar and Borana 2014) or due to osmoregulatory dysfunction that increase the rate of erythrocyte destruction in hematopoietic organs (Cogun et al. 2012).

Khabbazi et al. (2015) suggest that CuO NPs cause respiratory restrictions which led to changes in the number of RBCs. The change in RBCs may be also related to gills damage which disturb the respiratory process and this confirmed by the present histological

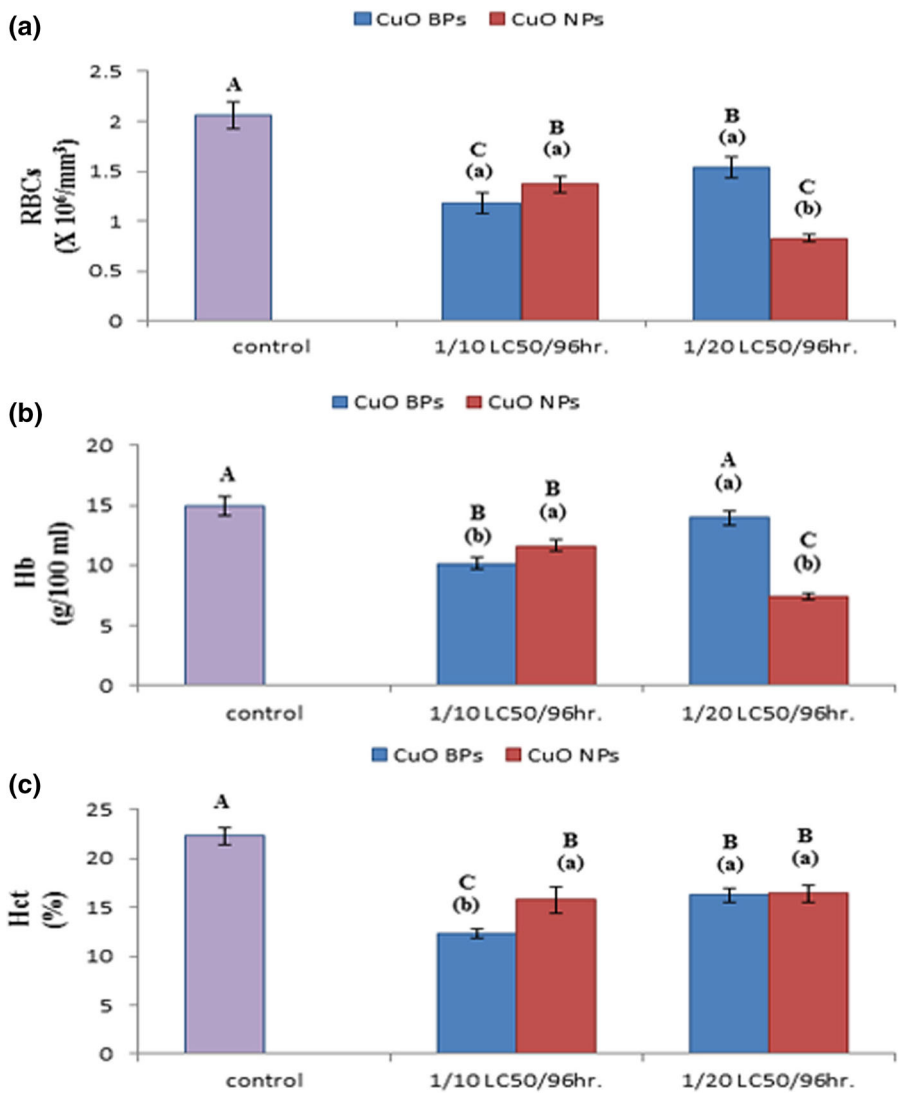


Fig. 1 RBCs count, Hb content and Hct values in *Oreochromis niloticus* exposed to 1/10 and 1/20 LC_{50}/96 h of CuO (BPs and NPs) for 30 days. **a** Data are represented as means of eight fish in each group ±SE. **b** The small letters represent the Duncan's test ($p < 0.05$) between BPs and NPs groups of the same concentration, while capital letters represent Duncan's test ($p < 0.05$) between both concentrations of BPs or both concentrations of NPs compared with control groups. **c** Columns with same letters are not significantly different; otherwise, they do

examination. Regarding MCV and MCH, the results revealed nonsignificant difference fish that exposed to CuO BPs, while it showed a significant increase in fish that exposed to CuO NPs compared to control groups (Fig. 2a, b). In case of MCHC, a significant difference in case of fish that exposed to CuO BPs was observed at the both studied concentrations, while a significant difference in case of fish that exposed to CuO NPs was observed at 1/10 LC_{50}/96 h concentration (Fig. 2c).

The significant decrease in RBCs, Hb and Hct values that not followed by any change in MCV and MCH as in BPs-CuO-treated groups reflects that the mean corpuscular size and Hb content are still normal (normocytic anemia), while NPs-treated fish showed significant increase in all blood indices. This elevated indices reflecting erythrocytes swelling which is related to intracellular osmotic disorders (macrocytic anemia). The enlarged RBCs have been associated with hypoxia that may be caused by gills damage (confirmed by the present histopathological examination). Moreover, the change in MCH and MCHC could be attributed to hemolysis of RBCs and the reduction in RBCs production in the hemopoietic tissues under the action of the accumulated NPs. These results were similar to Dharam et al. (2008) who reported that blood indices showed a significant increase when *Channa punctatus* exposed to Cu during 15 and 30 days.

Fish may absorb trace metals from the surrounding media, and then they accumulate it in various tissues in

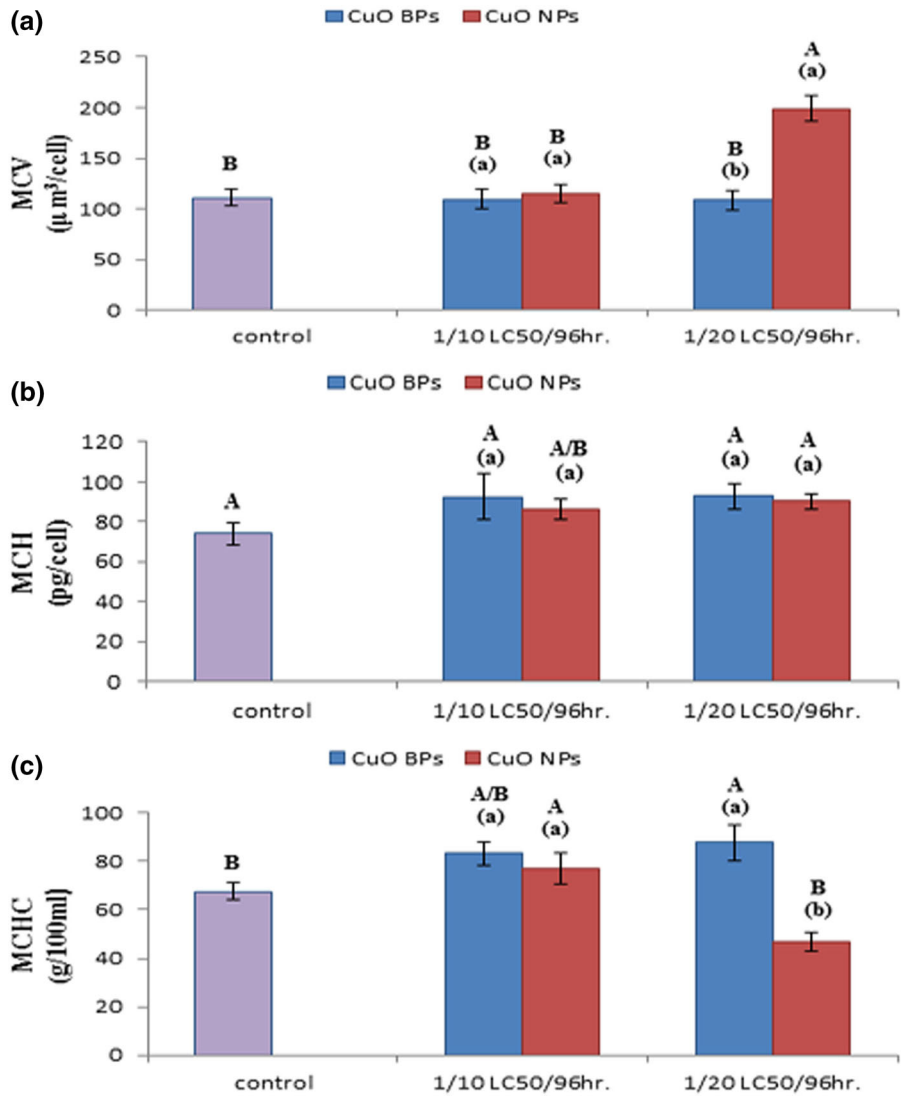


Fig. 2 Calculated blood indices in *Oreochromis niloticus* exposed to 1/10 and 1/20 LC_{50}/96 h of CuO (BPs and NPs) for 30 days. **a** Data are represented as means of eight fish in each group ±SE. **b** The small letters represent the Duncan's test ($p < 0.05$) between BPs and NPs groups of the same concentration, while capital letters represent Duncan's test ($p < 0.05$) between both concentrations of BPs or both concentrations of NPs compared with control groups. **c** Columns with same letters are not significantly different; otherwise, they do

significant amounts above those found in their environment (Abdel-Khalek 2015). The accumulated Cu metal and BAF in some vital tissues (liver, kidney, gills, skin and muscles) of Nile tilapia were recorded in Table 2.

Cu accumulation showed a tissue-specific pattern with the following descending order: liver > kidney > gills > skin > muscles. The active metabolic organs such as liver, kidney and gill are suffering from many toxicological effects as they accumulate more amounts of metals than other inactive metabolic tissues like muscle (Uysal et al. 2008). The pollutants may be distributed uniformly but accumulate differently within the various tissues. The high Cu accumulation in liver can be explained by its relation to low molecular weight proteins (metallothionein-like) which are concentrated in hepatic tissues (Abdel-Khalek 2015). In addition, the concentrated CuO NPs in liver may be due to the formation of metal sulfur proteins in liver tissue induce by NPs (Isani et al. 2013). The accumulation of NP metals in gills as stated by Hao et al. (2013) might be due to the adsorption of NPs directly on the gill surface with static negative charges and subsequently penetrate gill membrane. The high metal accumulation in the gills could be due to metals combination with the mucus covering gill lamellae. Also, lower pH values at the gill surface (due to the respired CO_2) may dissolve

Table 2 Copper accumulation and bioaccumulation factor (BAF) in different tissues of *O. niloticus* exposed to 1/10 and 1/20 LC_{50}/96 h of CuO BPs and NPs for 30 days

	CuO BPs		CuO NPs	
	Accumulated Cu (mg/kg dry weight)	BAF	Accumulated Cu (mg/kg dry weight)	BAF
Liver				
Control	114.34 ± 11.84C	–	114.34 ± 11.84B	–
1/10 LC_{50}/96 h	452.04 ± 98.86B	2.05	238.14 ± 18.56A	15.88
1/20 LC_{50}/96 h	700.78 ± 87.41A	6.29	146.16 ± 22.71B	19.48
Kidney				
Control	98.82 ± 36.93B	–	98.82 ± 36.93A	–
1/10 LC_{50}/96 h	403.78 ± 64.30A	1.83	131.59 ± 8.79A	8.77
1/20 LC_{50}/96 h	540.53 ± 35.96A	4.90	178.34 ± 27.98A	23.78
Gills				
Control	32.59 ± 11.19B	–	32.59 ± 11.19B	–
1/10 LC_{50}/96 h	269.51 ± 20.99A	1.22	63.76 ± 6.51A	4.25
1/20 LC_{50}/96 h	325.57 ± 37.76A	2.95	28.45 ± 6.10B	3.79
Skin				
Control	10.07 ± 0.62B	–	10.07 ± 0.62B	–
1/10 LC_{50}/96 h	331.11 ± 82.79A	1.5	22.77 ± 4.67A	1.52
1/20 LC_{50}/96 h	271.23 ± 102.93A	2.46	11.15 ± 1.16B	1.49
Muscle				
Control	4.76 ± 0.60C	–	4.76 ± 0.60B	–
1/10 LC_{50}/96 h	76.50 ± 4.14B	0.34	9.81 ± 0.57A	0.65
1/20 LC_{50}/96 h	37.61 ± 2.02A	0.34	10.13 ± 0.98A	1.35

Data are represented as means of eight fish in each group ±SE

Means with the same capital letter in the same column for each tissue are not significantly different; otherwise, they do (Duncan's test)

copper particles to become freely diffuse into the gill tissues (Masoud et al. 2007). Accumulation patterns of metals in kidneys of teleosts are dependent on both uptake and elimination rates. Cu also showed a significant accumulation in renal tissues which probably due to hepatic Cu-metallothionein reabsorption and subsequent renal thionein synthesis (Isani et al. 2013). The lower muscle Cu concentration in the present study was in accordance with Cogun et al. (2003) who observed that muscle of *O. niloticus* accumulates the lowest Cu concentrations after 30 days of exposure. Comparison between BAF values in the studied tissue samples showed higher values of CuO bioaccumulation at NPs-treated fish than that of BPs at all studied tissues except skin especially at the low concentration (1/20 LC_{50}/96 h). This indicated that NPs had more efficiency to internalize the tissues as confirmed by Chang et al. (2012) who reported that ion channels and transporter proteins permit NPs to cross the plasma membrane and some NPs enter cells via endocytosis at which the membrane wraps around them and vesicles transport NPs into cells. The higher skin BAF of BPs may be due to the tendency of CuO BPs to adhere to skin than NPs.

Histopathological investigations have been recognized to be reliable biomarkers during toxicity evaluation of various pollutants (Mela et al. 2007). Figures 3, 4 and 5 show the histopathology of liver, kidneys and gills of studied fish groups. The control groups (Fig. 3a, b) showed normal liver structure with compactly arranged hepatocytes, and sinusoids were scattered randomly all over the hepatocytes, while liver sections of fish that exposed to 1/10 and 1/20 LC_{50}/96 h of CuO BPs (Fig. 3c, e) showed mild

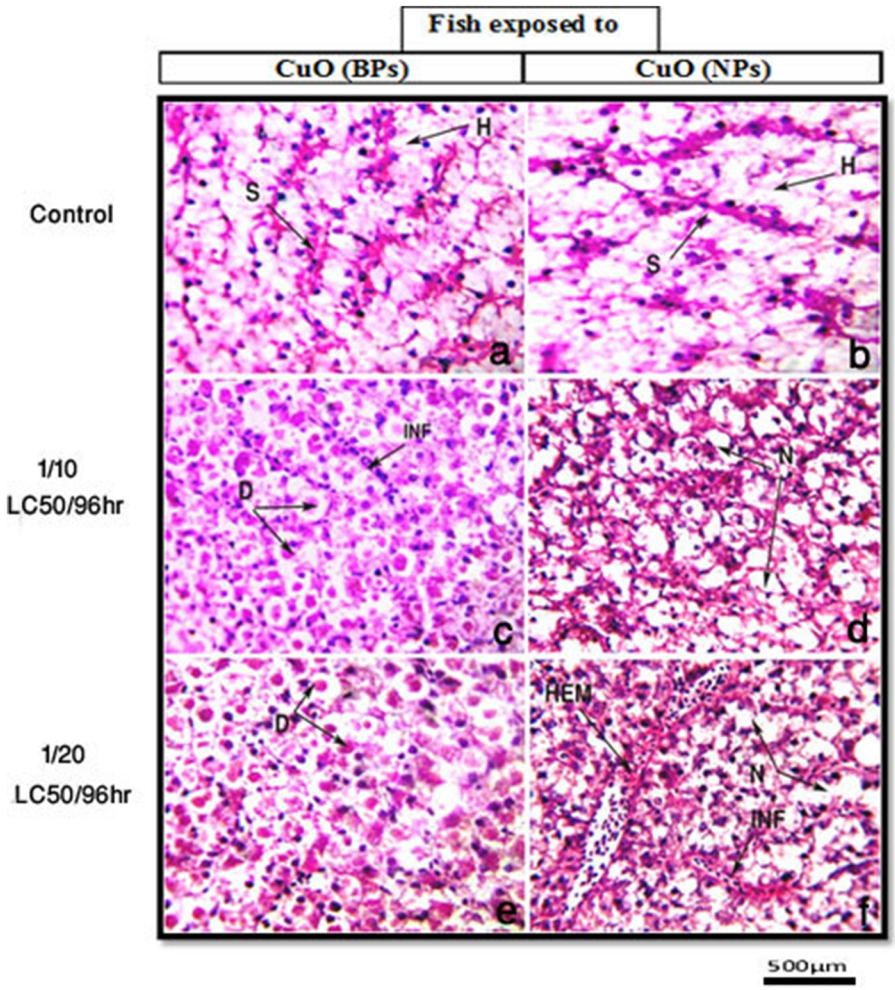


Fig. 3 Histopathological sections in livers of *Oreochromis niloticus* exposed to 1/10 and 1/20 LC_{50}/96 h of CuO (BPs and NPs) for 30 days. *S* sinusoid, *H* hepatocytes, *D* degeneration, *INF* infiltration, *HEM* hemolysis, *N* necrosis

histopathological changes as infiltration of blood cells, damage of parenchymal cells (degeneration). Also, the liver samples collected from fish that exposed to 1/10 and 1/20 LC_{50}/96 h of CuO NPs (Fig. 3d, f) showed deterioration in liver histoarchitecture marked by tissue disorientation with rupture of parenchyma cells, necrosis and RBCs infiltration.

Liver is considered as the main organ of detoxification that comes into contact with absorbed xenobiotics, and its lesions are often associated with the exposure to aquatic pollutants (Velma and Tchounwou 2010). In the present study, liver samples showed many alterations which in agreement with Singh et al. (2008) who found that the outer membrane of *C. punctatus* liver was significantly ruptured in addition to the extensive necrosis and hypertrophy that resulting in disappearance of hepatocytes after 30 and 45 days of exposure to Cu(II). These histopathological changes suggest a high metabolic activity in hepatocytes in response to the uptake of metals. According to Manahan (1991), degenerative-necrotic state (showed in NPs-treated groups) is a consequence of damages in the cellular membrane integrity and disturbances in the synthesis of proteins and carbohydrate metabolism. Kidney sections of the control groups (Fig. 4a, b) showed a normal structure with uniformly functional tubules, and the interstices of the tubules contain hematopoietic tissue. Renal tissues of fish that exposed to 1/10 and 1/20 LC_{50}/96 h of CuO BPs declared a glomerulus hyperplasia, edematous fluid and glomerular shrinkage with increase in Bowman's space (Fig. 4c, e) where fish that exposed

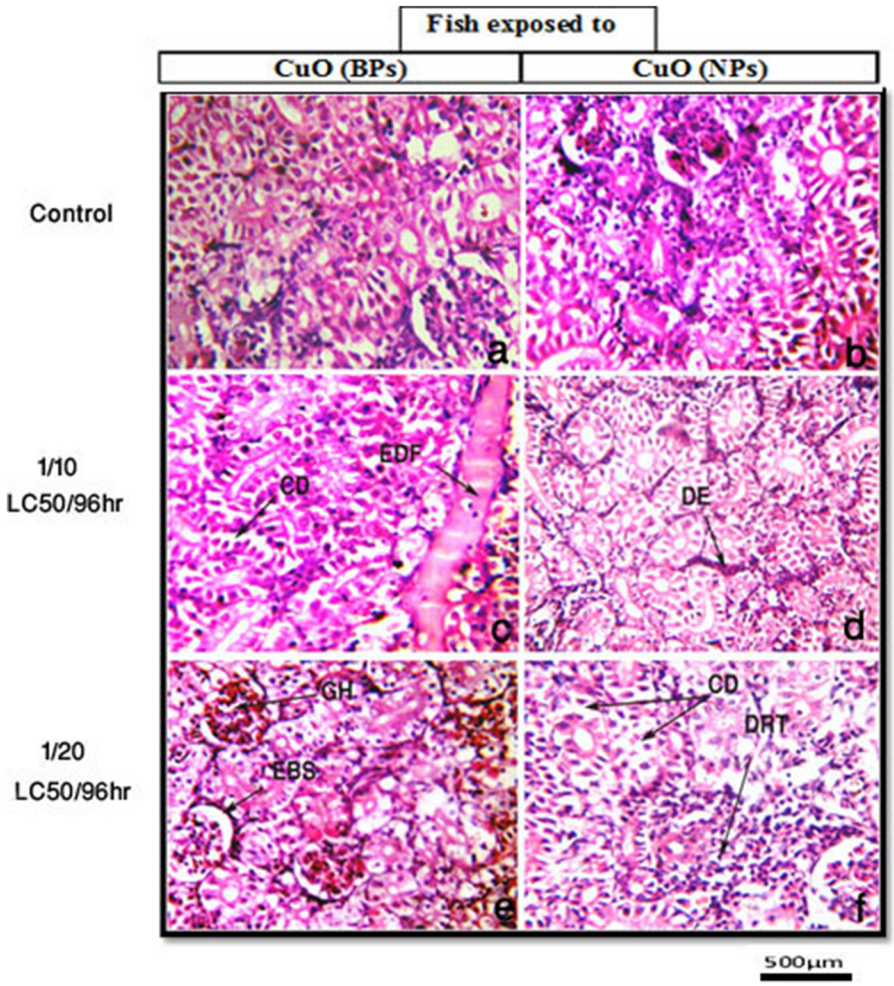


Fig. 4 Histopathological sections in kidneys of *Oreochromis niloticus* exposed to 1/10 and 1/20 LC_{50}/96 h of CuO (BPs and NPs) for 30 days. *CD* cellular degeneration, *EDF* edematous fluid, *EBS* expansion of Bowman's space, *DE* diffusion of erythrocytes in the interstitial fluid, *DRT* degeneration and deformation in renal tubules architecture, *GH* glomerulus hyperplasia

to 1/10 and 1/20 LC_{50}/96 h of CuO NPs showed progressive damage of kidney tubules associated with cellular degeneration, diffusion of the erythrocytes in the interstitial fluid, degeneration and deformation in renal tubules architecture (Fig. 4d, f).

The kidneys of fish receive largest proportion of post-brachial blood; therefore, renal lesions might be expected to be a good indicator of environmental pollution. Degeneration of tubular epithelial cells and tubular necrosis may be due to the accumulation of inflammatory cells associated with metals toxicity (Velma and Tchounwou 2010). The observed alternations were in consistency with Abdel-Khalek (2015) who described the disintegrations of kidney tubules in *O. niloticus* that inhabit metal contaminated aquatic habitats. Moreover, the tubular deformations may probably be as a result of the impaired glomerular filtration due to ion exchange and reabsorption disorder in renal tubules leading to protein leakage in the filtrate and decreased osmotic pressure (Ferguson 1989). Gills of the control groups (Fig. 5a, b) showed well-structured primary filaments and secondary lamellae with flat epithelial and chloride cells located at the bases of the secondary lamellae. Gills sections of fish that exposed to both concentrations of CuO BPs and NPs showed hyperplasia and fusion of the respiratory epithelium at the primary and secondary lamellae as well as edema with epithelial lifting at the base of secondary lamellae (Fig. 5c–f); in addition, congestion in the lamellar blood vessels, necrosis and shortening in the secondary lamellae were obvious in the NPs-treated groups.

Gills are directly affected by contaminants, owing to their anatomical location that lead to continuous

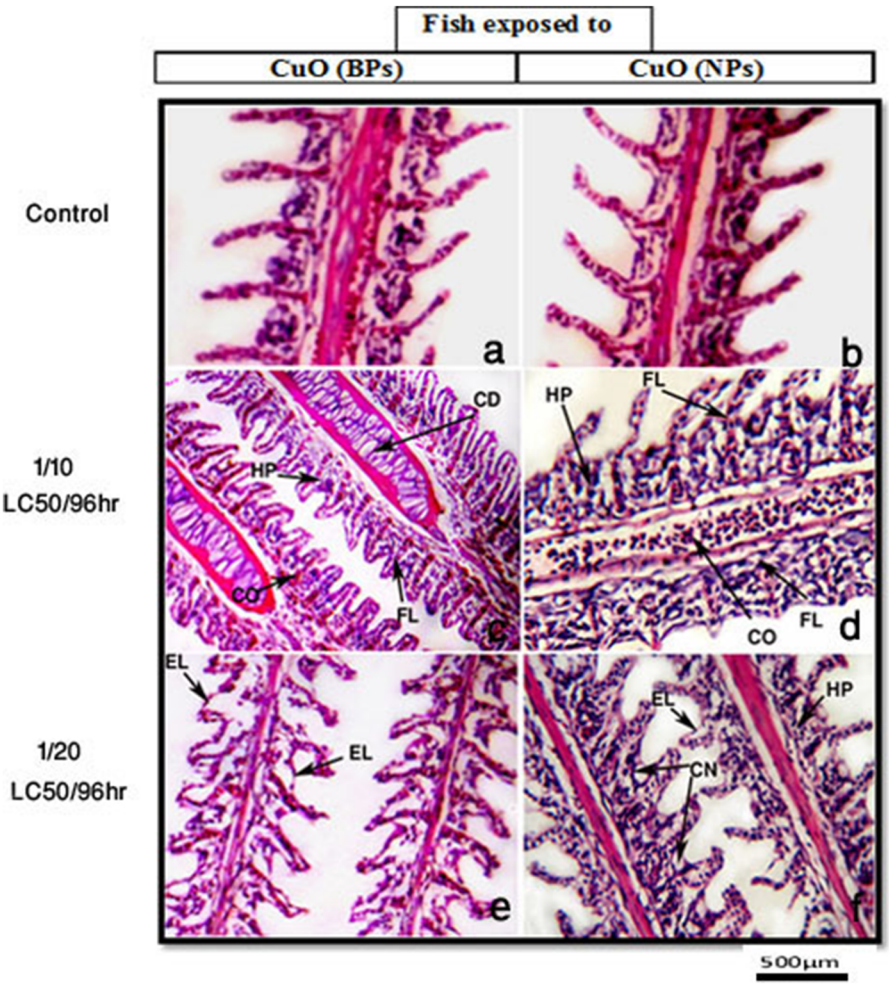


Fig. 5 Histopathological sections in gills of *Oreochromis niloticus* exposed to 1/10 and 1/20 LC_{50}/96 h of CuO (BPs and NPs) for 30 days. *HP* hyperplasia, *CO* congestion, *CD* cartilage deformation, *FL* fusion of the secondary lamellae, *EL* epithelial lifting, *N* necrosis, *CN* cellular necrosis in the secondary lamellae

contact with the external pollutants. Alterations like epithelial lifting, hyperplasia and hypertrophy of the epithelial cells, besides partial fusion of some secondary lamellae, are considered as adaptation mechanisms. These mechanisms result in increased distance between external environment and blood serving as a barrier against the entrance of contaminants (Hadi and Alwan 2012), while necrosis, lamellar rupture and shedding of gill epithelium are considered as permanent injuries which lead to non-functional gills and impair the respiratory functions as well as reduce the O_2 uptake (Abdel-Khalek 2015).

The results of this work indicated that CuO particles in their nanoscale had much toxicological effects on the studied hematological and histological endpoints than that of their bulk counterpart. These toxicological effects were obvious in the NPs-treated groups especially at 1/20 LC_{50}/96 h. This observation could be due to the low tendency of NPs to interact with each other because the tendency of interaction decreases with the concentration decrease. This lower interaction could increase the free NPs that can penetrate fish as smaller agglomerates may be taken easily in cells than larger ones as confirmed by CuO characterization in our pervious study Abdel-Khalek et al. (2015). The high surface/volume ratio of NPs increases their catalytic potency, reactivity and consequently biological toxicity (Adam et al. 2015). Therefore, RBCs and many organs might indirectly be damaged via reactive oxygen species (ROS) induction (Kumar et al. 2011) formed through nanoparticles–cell environment interaction. The toxic mechanism of NPs was mainly

dependent on the interaction between NPs and biomolecules resulting in protein unfolding, thiol cross-linking and enzymatic activity loss (Chang et al. 2012). On the basis of our biomarkers, the damage effects showed size and dose dependent. These results were supported by findings of Wang et al. (2008), which showed that the pathological changes induced by ZnO NPs were depending on both size and concentration.

Conclusions

This study provided us with comparable toxicity data between two concentrations of different forms of CuO (BPs and NPs). In the present investigations, CuO NPs had more toxic effect than that of CuO BPs with respect to the studied hematological and histopathological biomarkers. On the basis of metal bioaccumulation, CuO NPs had more efficiency to internalize the vital organs and this may be due to the different entrance mechanisms of these nano-sized particles. Further studies are needed to monitor the behavior of CuO NPs in aquatic ecosystems and to show the exact toxicity mechanisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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