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Assessment of copper nanoparticles (Cu-NPs) and copper (II) oxide (CuO) induced hemato- and hepatotoxicity in *Cyprinus carpio*

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Abstract

Recently, Cu based nanoparticles have drawn considerable attention for their various fascinating roles in multiple biological systems. It is recognized that their frequent use can create compatibility challenges for the recipient systems. Nevertheless, it is unclear how various biological interactions affect the compatibility of Cu oxide II (CuO) and Cu oxide nanoparticles (Cu-NPs) for different organisms. Consequently, it has been difficult to perform structured risk assessments for their use in biological systems. Therefore, this study compared the effects of different doses of waterborne Cu-NPs and CuO on blood and liver of selected groups of *Cyprinus (C) carpio*. These fish whilehoused in suitable water tanks were exposed to one of the following treatments for 14 days. Control (no added Cu) or 0.5 or 1 or 1.5 mg Cu as Cu-NPs or CuO /L of water. We found significant changes in all assessed blood parameters of fish in response to increasing doses from 0 to 1.5 mg of Cu-NPs or CuO. Similarly, increased levels of lipid peroxide and reduced glutathione were also observed in the livers of *C. carpio* in Cu-NPs or CuO treated groups. Enhanced levels of LPO and GSH were also recorded in Cu-NPs treated groups compared with CuO treated groups in a dose dependent manner. The lowest catalase (CAT) activity was observed in the liver of *C. carpio* treated with the higher dose of Cu-NPs. Cu-NPs or CuO exposure
induced significant histological alterations in the liver of *C. carpio* including focal necrosis, cloudy swelling of hepatocytes, degenerative hepatocytes, vacuolization, pyknotic nuclei, damaged central vein, nuclear hypertrophy, dilated sinusoid, vacuolated degeneration, congestion, and complete degeneration in a dose dependent manner. Substantial alterations in blood and liver specimens were observed in the Cu-NPs treated fish when compared with the CuO treated fish. It appeared that the Cu-NPs were more toxic than the CuO as shown by the hematoo-and hepatotoxicity in *C. carpio* of this study.

Keywords *Cyprinus carpio*. Cu Nanoparticles. CuO. hepatotoxicity. Hematotoxicity

1. Introduction

Due to their small size, the nanoparticles (NPs) can enter the small biological structures and in turn, may interrupt cell composition and function [1]. Nanotechnology has multitude potential to advance biomedical science and healthcare management, but the use of NPs poses unknown threats to the environment and human health [2]. Indeed the NPs have the ability to facilitate the direct generation of harmful reactive oxygen species (ROS) inside the cells [3]. This results in oxidative stress which suggests potential interactions between biological systems and NPs [4]. With the extensive use of NPs, the associated health and environmental risks of NPs exposure are of great concern [3]. Thus, a detailed evaluation of potential NPs toxicity *in vivo* must be evaluated before their utilization in any application.

Copper is needed for the normal physiological functioning of xenobiotic and carbohydrate metabolism and it is also required for the antioxidant defense system [4]. The living organisms can be exposed to copper through inhalation, food and water. Currently, copper nanoparticles (Cu-NPs) are widely used in a variety of established and developing technologies. Also Cu-NPs are used as catalysts, solar energy converters, and antimicrobial agents, due to their distinct morphological and physiological properties [5]. Despite the tremendous applications of Cu-NPs containing products, there is insufficient information about the risks of their exposure [6]. Previously *in vitro* studies have reported the cytotoxic effect of Cu-NPs as their use showed increased levels of ROS in various human cells [7]. It was also reported that Cu-NPs induced more toxicity than their oxide form (CuO-NPs) in human leukemia cell line [8]. To our knowledge,
very few reports have explored the toxicity of Cu-NPs in vivo, using fish as a model animal. Therefore, it is necessary to evaluate the toxicity of Cu-NPs after sub-chronic exposure for the purpose of risk assessment in fish (C. carpio) and other organisms. The present study was designed to evaluate the toxicity of Cu-NPs and their bulk counterpart (copper II oxide, CuO) in the blood and liver of C. carpio. C. carpio was selected as an experimental model because of its resistant and tolerant nature and adaptability to various habitats such as stagnant or slow water flows with potentially high metal contents. This study involved the liver and blood parameters for toxicity assessment because liver is the major organ for detoxification of all toxins which enter the organisms, and blood is a transport medium for a variety of chemicals and toxins. Furthermore, metals and metal-NPs are known to induce ROS which alters the antioxidant defense system to counterbalance the toxic effects of metals in living systems. Therefore, this study assessed the in vivo toxicity of Cu-NPs and CuO in C. carpio through haematological parameters, oxidative stress and histological alterations in the liver.

2. Materials and Methods

2.1 Fish Procurement and Acclimatization and Water Parameters
A fish colony was established prior to the start of this experiment by maintaining the fish health through water quality management in the aquarium of the Department of Zoology, Government College University Faisalabad (GCUF), Pakistan. Samples of C. carpio of similar body weight (40-45g) were procured from the Fish Seed Hatchery Satiana Road, Faisalabad, transported in plastic containers with continuous aeration to this GCUF laboratory and acclimatized in a tank with 100 L capacity for two weeks prior to the experiment. Un-chlorinated tap water was used for the experiment and the water was analysed for its physicochemical parameters. During acclimatization period, the water temperature was maintained at 26°C, while dissolved oxygen and pH were maintained at 6.5–7.4 mg/l, and 6.7–7.2, respectively. Ammonia (NH₃) concentration, total hardness and total dissolved solids were recorded as 0.4-0.6 ppm, 47-52 ppm and 6.5-7.8 ppt, respectively. Photoperiod was provided at 12 hrs light: 12 hrs dark cycle. The fish were fed twice daily with a commercial fish feed (Taijia, fish meal 65% of animal feed, Wudi Taijia Agriculture Development Co., Limited China). The experimental tanks were semistatic and about 80% water was changed daily and any dead fish as well as any fish showing unusual symptoms were excluded from the study.
2.2 Chemicals Used in Study

The high quality analytical and molecular grade chemicals were used in the study. Cu-NPs (60-80 nm, CAS Number 7440-50-8; 99% pure) and cupric oxide (copper II oxide, <10 µm; CAS Number 1317-38-D) were purchased from Sigma-Aldrich.

2.3 Characterization of Nanoparticles

The Cu-NPs used in the study were characterized by using X-ray diffraction (XRD), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). XRD characterized the primary average crystallite size of Cu-NPs in the range of 78.33 nm. SEM revealed the homogeneous dispersion of particle size that was within the range of 65-90 nm. Conversely, TEM revealed the individual particle size of 70-90 nm having irregular morphology with more particles having the cubical crystalline form.

2.4 Preparation of Exposure Solutions of Cu-NPs and CuO

For the preparation of exposure medium, the method of Shaw et al. [9] was followed with some modifications. Briefly the required amounts of Cu-NPs (60-90 nm) and CuO (<10 µm) powder were dispersed in deionized/ ultra pure water (Millipore, 18.2 MΩm resistance and unbuffered) without any solvent in polypropylene tubes. To achieve maximum dispersion, the suspension was homogenized by vortex (3 h at 2000 rpm) followed by ultrasonication (100W, 40 kHz) for 1 h before their immediate transfer into different glass aquaria. The solutions were made fresh before each dosing. Ultrasonication was used for the preparation of mixed aqueous nano-suspensions, which is an accepted technique for dispersing the highly entangled or aggregated nanoparticles [10, 11]. In fact, ultrasonic irradiation is known to enhance the reactivity of metal surfaces. Because of this, the sonochemical activation of various metals has become a routine synthetic technique. The gross morphology (particle size, degree of particle aggregation) is not known to be changed significantly by even lengthy ultrasonic irradiation [12, 13]. Surfactants were not used for the dispersion of nanoparticles as surfactants can also induce added toxicity to organisms during in vivo studies with fish, for example.

2.5 Sub-acute Toxicity
The *C. carpio* were exposed to either Cu-NPs or CuO to investigate their toxic effects on hematological parameters, oxidative stress and histological alterations in the liver. All procedures performed in this study involving fish handling were in accordance with the research ethical standards with the approval of the GCUF Ethics Committee on Animal Experimentation.

The dosing was adopted based on the sub lethal doses for 96-hrs LC50 of Cu-NPs (13%, 23% and 34%) and CuO (11%, 22% and 29%) for *C. carpio* which were established for this study. For this purpose, 210 specimens of *C. carpio* of similar body weight (40-45 g) were selected from the acclimation aquaria, divided into 7 main groups in triplicates. These sub-groups were then randomly transferred into twenty one aerated experimental glass aquaria (10 fish/ aquarium). Each aquarium had 40 liters of water with the same physicochemical parameters that were maintained during the acclimatization period. The fish were acclimated to their respective aquaria for 48 hrs prior to the experiment (Fig. 1). Three aquaria per treatment were randomly allocated and fish were exposed to one of the following treatments involving sub-acute toxicity of Cu-NPs/CuO for 14 days. Control (no addition or 0 Cu-NPs or CuO) or 0.5 or 1 or 1.5 mg/l Cu as Cu-NPs or CuO. The exposure water in the aquaria was replaced daily with the freshly prepared solutions to maintain the concentration of Cu-NPs and CuO at a constant level. The fish were fed at the rate of 2.5% of their body weight with commercial fish meal twice daily. Moreover, the aquaria water samples were regularly analysed from the start to the end of experiment to assess the pH, temperature, dissolved oxygen, total ammonia and water hardness.

### 2.6 Sample collection

At the end of this experiment, five fish from each aquarium were immediately anesthetized with 75 µl/ l of clove oil in a bucket for 4 minutes [14]. Blood samples were collected from the caudal vein in heparinized tubes for the hematological parameters. The fish were dissected to collect liver tissues for the analysis of oxidative stress parameters. For this purpose small pieces of liver tissues from the anterior sides (4–5 mm) of each liver tissue were fixed in a solution (60% ethanol + 30% formalin + 10% acetic acid) for histological observations [15]. The remaining fish were humanly killed and properly disposed off. The detailed protocols of various analyses are given in the following sections.
2.7 Hematological Analysis
The blood samples were collected from the caudal vein and stored in two tubes containing calcium EDTA @1.5mg/ml of blood (Cangzhou Yongkang Medical Devices CO., Ltd, China) for complete blood count (CBC). The samples were used for the estimation of different variables, including red blood cells (RBC), white blood cells (WBC), hemoglobin (HGB), platelets (PLT), lymphocytes (LYM), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and hematocrit (HCT) by using a hematology auto-mated analyzer (Beckman Coulter UniCel DxH) according to the manufacturer guidelines.

2.8 Histological protocol
The samples fixed in sera (containing 60 ml alcohol, 30 ml formaldehdye and 10 ml of glacial acetic acid) were further processed for dehydration by using 70, 80, 90, 95, and 100 % of ethanol. The tissues were then placed in cedar wood oil until clear. The samples were embedded into paraplast. The paraplast was changed after 30 minutes and tissues were again placed in an incubator for 12 hours at 60 ºC. The paraplast was changed third time and placed in an incubator for 12 hours at 60 ºC. The box blocks of each tissue were made and mounted into plastic casters (HAION Caster Industries Co. Ltd., Taiwan). The tissue sections of 3-4 μm were cut by microtome (SLEE Rotary Microtome CUT5062 by Nikon Instruments Europe). Each section was transferred to a clean slide and stretched on Fisher slides (Thermoscientific Shandon™, Colormark™ Slides) which werewarmed and maintained for 24 hours. De-parafinization of specimens was done with xylene (Sigma CAS No: 1330-20-7) followed by rehydration with 50 to 100 % ethanol and staining by the hematoxylin-eosin [16]. The slides were washed with tap water, stained with haematoxyline, dipped again in water for bright coloration and stained with eosin. The slides were then moved to absolute alcohol for complete dehydration. Two drops of DPX (histology Mountant) was put on each slide which was covered with a cover slip for complete spreading. The sections were examined under light microscope (Nikon E200 POL) equipped with a digital camera and photographed. The quantification of histological alterations was performed by using a five graded examination scheme: (−) no histological alterations (normal histological structure); (+/-) mild histological alterations; (+) moderate histological alterations; (++) severe histological alterations; and (+++) very severe histological alterations.
2.9 Oxidative stress Analysis

2.9.1 Preparation of liver homogenate
The liver was removed, washed and then homogenized in Tris-EDTA buffer (pH 7.4), using a Potter-Elvejham homogenizer at 4 °C. The crude homogenate was then centrifuged at 10,000 rpm for 20 min at 4 °C and the collected supernatant was stored at −80 °C for the estimation of oxidative stress parameters [17].

2.9.2 Estimation of lipid peroxidation
Lipid peroxidation (LPO) was estimated by measuring the malondialdehyde (MDA), by the method of Okhawa et al. [18]. The reaction mixture contained 0.2 ml of liver homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% solution of thiobarbutaric acid (aqueous). The pH of 20% acetic acid was pre-adjusted to 3.5 by using NaOH. The final volume of mixture was made up to 4 ml by distilled water and heated (95°C) for 1 hour in a water bath, and then mixed with 1 ml of distilled water along with 5 ml of a mixture containing n-butanol and pyridine (15:1). The mixture was shaken on a vortex mixer (Super mixer. MC-05). The absorbance was taken at 532 nm against tetramethoxypropane. The values of lipid peroxidation were expressed in nM/mg of tissues.

2.9.3 Estimation of glutathione (GSH)
The level of reduced glutathione (GSH) was measured by following the method of Sedlak and Lindsay [19] with minor modification. The liver homogenate was precipitated with trichloroacetic acid (50%) and then centrifuged at 1000 rpm for 7 min. The 0.5 ml of supernatant was mixed with 2.0 ml of Tris-EDTA buffer (0.2 M; pH 8.9) and 0.1 ml of 0.01 M 5′5′-dithio-bis-2-nitrobenzoic acid, after 5 minutes the absorbance was measured at 412 nm on the spectrophotometer (U-2800 Hitachi (200-1100). The levels of GSH were expressed as µM /g of tissues.

2.9.4 Estimation of catalase enzyme activity
The level of catalase (CAT) in liver was assessed by following the method of Aebi [20]. For this purpose 50 µl (10% w/v) of liver homogenate was transferred into 3.0 ml cuvette that already contained 1.95 ml of phosphate buffer (50 mM, pH 7.0). Then 1 ml of hydrogen peroxide (30 mM)
was added and the absorbance was measured after 30 sec at 240 nm at 30 sec intervals. CAT activity was expressed as unit per mg (U/ml) of tissue homogenate.

2.10 Statistical Analysis
The data were analyzed by Minitab17 software using the General Liner Model (ANOVA). Data of all the parameters regarding control, Cu-NPs and CuO treatments were expressed as mean±SD. Tukey’s test was used for the comparison of means. The differences between means were declared significant if P<0.05.

3. Results and discussion
Copper (Cu) as a trace element is essential for the proper functioning of organs and metabolic processes in all living organisms. It is also required for enzyme systems which are integral parts of metabolic processes that are required to sustain life. The excess or deficiency of Cu in the body of organisms adversely affects health by altering different metabolic processes [12]. The main source of Cu in aquatic systems is natural (geological deposits, volcanic activity, weathering and erosion of rocks and soils) and anthropogenic (mining, agriculture, metal, electrical manufacturing, sludge and pesticide) [22]. The antifouling paints which are used for coatings on ship structures are one of the major sources of Cu in the marine environment. Cu is toxic at higher concentrations for aquatic organisms. Its acute effects include mortality after severe pathological abnormalities while chronic exposure to Cu can induce adversarial effects on the growth and reproduction by altering the enzymatic systems, blood composition and metabolic processes [22].

The Cu-NPs might cause specific and diverse toxicity from that of Cu bulk salt. When normal-sized Cu is converted into nano-sized Cu, its physicochemical properties change and its distinct physicochemical characteristics can be modified by its particle size, surface properties, and shape. These distinctive physicochemical properties might enhance the toxicological behavior in vivo by aiding the cellular uptake and translocation of these particles in the body [23]. Currently, with the widespread use of NPs, health risks and environmental effects related to NPs exposure are of great concern [24]. Moreover, the physiological state also influences the interaction between biological systems and NPs, so it is important to determine the fate and biosafety of NPs [25]. Generally, the impacts of Cu-microparticles and Cu-NPs exposure have been investigated in different animals, including some fish species followed by different routes of administration [3,
It has been reported that Cu-NPs induced greater toxicity and also enhanced translocation of particles as compared to the larger particles [27-28].

In the current study, the exposure to Cu-NPs or CuO was maintained by re-dosing after 12 hours with water change as mentioned by the Organization for Economic Cooperation and Development (OECD) for acute fish test criteria [29]. In the current study the fish mortality was not observed on exposure of these fish to any treatment of Cu-NPs or CuO. However, some behavioral changes like frequent opercular movements, increased mucus secretion and decreased fish movements were observed which indicated possible stress in experimental fish in a dose dependent manner.

A significant (p<0.05) change was observed in all assessed blood parameters in response to stress of Cu-NPs and CuO with respect to the control group in a dose dependent manner. Hemoglobin (Hb) was high in the control group while low in GN2 (nanoparticles @ 1mg/l), GN3 (nanoparticles @1.5 mg/l), GS2 (Cu bulk salt @1mg/l) and GS3 (Cu bulk salt @1.5 mg/l) groups. The highest hematocrit value was recorded for the control group while it was lowest in high dose of Cu-NPs treated group (GN3). The RBC were highest in the control group and lowest in high CuO treated group (GS3). The highest WBC and PLT were found in GN3 as compared to the control while other groups showed significant (p<0.05) differences. It was observed that level of WBC and PLT was increased with the increased concentration of Cu-NPs or CuO. The MCV, MCH and MCHC were highest in the control group and lowest in the GN3, a decline in MCV, MCH and MCHC was observed with an increase in concentration of Cu-NPs or CuO. The significant (p<0.05) decrease for MCV, MCH and MCHC was also observed in other treatment groups. Moreover, the Cu-NPs compared to CuO treatment showed more decline in MCV, MCH and MCHC concentration (Table 1). The Cu-NPs and CuO treatments showed non-significant (p>0.05) effect on all blood parameters of *C. carpio* (Hb, RBC, WBC, MCV, MCH, MCHC and Platelet counts) except Hct (p<0.05) (Table 1). Highly significant (p<0.001) changes were observed in all the blood parameters of *C. carpio* treated with different doses of Cu-NPs and CuO (Table 1).

In the present study, MCH, MCV, HCT, MCHC and RBCs levels were significantly (P<0.001) decreased as compared to the control group with an increase in the dose of Cu-NPs or CuO after 14 days of fish exposure. Whereas, the levels of WBCs and Platelets increased in fish that were exposed to the increased concentration of Cu-NPs or CuO treatments. The increased...
levels of WBC indicated the defense response of the immune system against the stress condition due to Cu-NPs or CuO. Moreover, the significant increase in platelet counts indicated the proficient and supportive immune responses. This could be the effect of Cu-NPs or CuO to induce blood thickening that in return induced damage to platelets. A severe microcytic anemic state (decrease in Ht, RBC, Hb and MCV) was also reported by Ajani and Akpoilih [30] in *C. carpio* exposed to different concentrations of Cu. Thangam et al. [31] revealed that the levels of RBC and Hb were decreased in *C. carpio* exposed to sub-lethal dose of Cu for 35 days, but there was no effect on the level of Hct. Abdel-Khalek et al. [32] also reported that hematological parameters including RBC count, hemoglobin content and hematocrit were significantly decreased at all concentrations of Cu-NPs and CuO. It was observed that Cu-NPs treated fish showed the maximum alteration in all the recorded parameters as compared to the CuO treated fish. Abedi et al. [33] observed a non-dose dependent decrease in Ht, Hb and RBC in *Clarias gariepinus* exposed to 25-200 mg/l of lead. The findings of Khabbazi et al. [34] exhibited that the Cu-NPs affected the WBC, Hct, MCH, MCHC and MCV but not the hemoglobin. In the current study, Cu-NPs or CuO treatment showed non-significant differences in all the parameters of hematology other than Hct which was declined in Cu-NPs treatment as compared to the CuO treatment. These findings suggested that Cu-NPs or CuO are clearly hemolytic to the *C. carpio* at their concentrations of 0.5, 1 and 1.5 mg/l. Similarly Amr et al. [35] reported the hemato-toxicity of CuO bulk and NPs treatments in Nile Tilapia.

### 3.2 Assessment of Oxidative Stress

Table 2 presents the concentration of oxidative stress parameters (LPO, GSH and CAT) in the liver of *C. carpio* among different treatment groups. The concentration of LPO in liver was found in the order GN3>GS3>GN2>GS2>GN1>GS1>control. The concentration of GSH in the liver was found to be in the order of GN3>GN2>GN1>GS3>GS2>GS1>control. The lowest CAT activity was observed in the liver of *C. carpio* in GN3 group. Overall oxidative stress enzymes showed significant differences between control and other treatment groups in a dose
Table: 1 Mean (± SD) concentration of Hb, Hct, RBC, WBC, MCV, MCH, MCHC and Platelet counts in the control and different treated groups of *C. carpio*.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Hb (g/dl)</th>
<th>Hct (%)</th>
<th>RBC (x10⁶/M)</th>
<th>WBC (x10³/µL)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>Platelet count (x10³/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNC</td>
<td>7.27±1.21A</td>
<td>22.72±2.08A</td>
<td>1.82±0.03A</td>
<td>1.34±0.02E</td>
<td>146.40±5.20A</td>
<td>41.38±1.05A</td>
<td>33.18±1.03A</td>
<td>172.20±6.12G</td>
</tr>
<tr>
<td>GN1</td>
<td>6.73±1.15AB</td>
<td>18.42±1.36C</td>
<td>1.42±0.07B</td>
<td>64.40±4.26C</td>
<td>140.20±4.15C</td>
<td>40.06±1.06B</td>
<td>32.72±1.14A</td>
<td>198.40±6.25D</td>
</tr>
<tr>
<td>GN2</td>
<td>5.70±0.53C</td>
<td>16.56±2.12D</td>
<td>1.24±0.02C</td>
<td>67.43±3.25B</td>
<td>132.40±3.21E</td>
<td>37.48±1.18D</td>
<td>29.61±1.32C</td>
<td>1012.50±9.21C</td>
</tr>
<tr>
<td>GN3</td>
<td>5.60±1.30C</td>
<td>15.08±2.05E</td>
<td>1.04±0.02DE</td>
<td>68.50±4.10A</td>
<td>128.50±3.35G</td>
<td>33.48±1.22F</td>
<td>25.37±1.19E</td>
<td>1123.30±10.12A</td>
</tr>
<tr>
<td>GSC</td>
<td>7.27±1.21A</td>
<td>22.72±3.08A</td>
<td>1.82±0.03A</td>
<td>1.34±0.02G</td>
<td>146.40±4.20A</td>
<td>41.38±1.05A</td>
<td>33.18±1.03A</td>
<td>172.20±6.12G</td>
</tr>
<tr>
<td>GS1</td>
<td>6.53±1.32AB</td>
<td>23.52±1.43A</td>
<td>1.37±0.03B</td>
<td>41.37±3.29F</td>
<td>142.30±3.20B</td>
<td>39.40±1.45C</td>
<td>31.24±1.22B</td>
<td>186.50±5.35F</td>
</tr>
<tr>
<td>GS2</td>
<td>6.00±1.10BC</td>
<td>19.39±1.52B</td>
<td>1.12±0.01D</td>
<td>56.53±3.15E</td>
<td>137.30±4.06D</td>
<td>37.17±1.04D</td>
<td>29.46±1.48C</td>
<td>197.30±6.15E</td>
</tr>
<tr>
<td>GS3</td>
<td>5.37±1.15C</td>
<td>17.34±2.28D</td>
<td>0.98±0.02E</td>
<td>60.40±4.26D</td>
<td>129.60±3.31F</td>
<td>34.88±1.18E</td>
<td>28.37±1.47D</td>
<td>1062.10±9.06D</td>
</tr>
</tbody>
</table>
G= Group, C= Control, N= Cu-NPs, S= CuO, 1=0.5mg/l, 2=1mg/l, 3=1.5mg/l
Means with different letters in the same column differ significantly (P<0.05)
dependent manner. Whereas the Cu-NPs and CuO treatments showed non-significant (p>0.05) effect on LPO and GSH activities but significant (p<0.05) effect on CAT activities (Table 2).

Table: 2 Mean (±SD) concentration of LPO, GSH and CAT in the liver of *C. carpio* among different treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>LPO (nmol/g)</th>
<th>GSH (µmole/g)</th>
<th>CAT (unit/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNC</td>
<td>326.10±5.66E</td>
<td>1245±8.65F</td>
<td>2.40±0.01A</td>
</tr>
<tr>
<td>GN1</td>
<td>331.70±5.03E</td>
<td>1326±10.03D</td>
<td>2.23±0.02C</td>
</tr>
<tr>
<td>GN2</td>
<td>465.30±4.05C</td>
<td>1462±8.07B</td>
<td>2.16±0.01D</td>
</tr>
<tr>
<td>GN3</td>
<td>545.8±4.33A</td>
<td>2040±11.22A</td>
<td>2.10±0.01E</td>
</tr>
<tr>
<td>GSC</td>
<td>326.10±5.66E</td>
<td>1245.0±8.65F</td>
<td>2.40±0.01A</td>
</tr>
<tr>
<td>GS1</td>
<td>329.40±4.55E</td>
<td>1270.0±8.96E</td>
<td>2.35±0.21B</td>
</tr>
<tr>
<td>GS2</td>
<td>415.1±3.34D</td>
<td>1387.0±9.34C</td>
<td>2.26±0.12C</td>
</tr>
<tr>
<td>GS3</td>
<td>517.70±4.67B</td>
<td>1455±11.21B</td>
<td>2.16±0.12D</td>
</tr>
</tbody>
</table>

G= Group, Cu-NPs= N, CuO=S, C=control, 1=0.5mg/l, 2=1.0 mg/l, 3= 1.5 mg/l
Means with different letters in the same column differ significantly.

Oxidative stress is a well-known mechanism of Cu toxicity to fish [36]. Reactive oxygen species (ROS) are produced from molecular oxygen as a result of normal cellular metabolism. The body of living organisms is equipped with a variety of antioxidants that serve to counterbalance the effect of oxidants (enzymatic and nonenzymatic). The major enzymatic antioxidants of the lungs are SOD, CAT, and GSH [37-38]. CAT is the key enzyme in antioxidant defense systems, animal CAT are enzymes that contain heme group and convert H₂O₂ to water and oxygen. The CAT enzymes are confined in subcellular organelles such as peroxisomes. The mitochondria and the endoplasmic reticulum also contain CAT enzyme in small quantities [39]. The GSH enzyme is abundant in all cell compartments and major indicator of oxidative stress. The GSH enzyme has been showing its antioxidant effects in several ways as it detoxifies H₂O₂ and lipid peroxides by donating its electron to H₂O₂ to reduce it into H₂O and O₂. The ROS can induce LPO and disrupt the membrane of lipid bilayer arrangement that may inactivate membrane-bound receptors and enzymes and increase tissue permeability [40]. Products of LPO, such as MDA and unsaturated aldehydes, are capable of inactivating many cellular proteins by forming protein cross-linkages [41]. Altered antioxidant status was confirmed in the current study in fish exposed to different concentrations of Cu-NPs and CuO which showed a decrease in CAT and an increase in LPO and GSH in the liver as compared to controls in a dose dependent manner. The elevated LPO levels
might be due to the oxidation of oxygen to produce superoxide radicals. The estimation of LPO is generally used as a marker of oxidative stress in the detection of toxicity. This reaction produced H₂O₂ that resulted in the production of MDA by triggering peroxidation of unsaturated fatty acids in the membrane. The increase in LPO and generation of ROS may reduce cell viability. In the current study, the decrease in CAT levels in the liver indicated that the CuO or Cu-NPs treatment induced toxicity. Catalase enzyme has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of H₂O₂ to H₂O and O₂ each second. H₂O₂ is a harmful by-product [42-43]. The results of the present study are in line with Manke et al. [44], who reported that NPs induce oxidative stress due to prooxidant effects of NPs which were attributable to their physicochemical properties including surface reactivity, particle size, surface charge, chemical composition, and the presence of transition metals. The key pathophysiological outcomes of oxidative insults during metal NPs exposures involve cell membrane damage, lipid peroxidation, protein denaturation, and alteration of calcium homeostasis. The results of the current study are also in line with Abdel-Khalek et al. [2], who reported that MDA contents in the liver tissues of Nile Tilapia exposed to both concentrations Cu-BPs and Cu-NPs exhibited increased levels of MDA after 30 days exposure. Similarly, Metwally [45] demonstrated that MDA concentrations in serum and tissues of Nile Tilapia and catfish increased with induction of heavy metal toxicity. Moreover, Roméo et al. [46] also reported that cadmium and copper induced increase in MDA levels. LPO induced by Cu-NPs was also reported in other biological models, indicating that oxidative stress is a common pathway for Cu-NPs toxicological effect [47-49]. The metabolism of heavy metals results in the formation of ROS which are known to extract hydrogen atom from unsaturated bonds, thereby altering lipid structure or function [50]. Farombi et al., [51] investigated the effects of heavy metals found in waters of Nigeria Ogun River on African Catfish and Padmini et al. [52] reported the toxic effects of heavy metals found in Grey Mullet inhabiting Indian Estuarine water on catfish and showed that that the accumulation of metals (Cu) in the liver and gills of fish at high concentration induced lipid peroxidation. GSH plays an important role in non-enzymatic antioxidant system, since it acts as a reductant in conjugation with xenobiotics [53]. In the present study, GSH contents in the liver tissues of *C. carpio* exposed to Cu-NPs and CuO showed a significant increase. These results are in line with Sevcikova et al. [54] who also reported increase in GSH activity in gills of *C. carpio*, and Abdel-Khalek et al. [55], who also reported an increase in GSH at different concentrations of CuO and Cu-NPs in Nile Tilapia. In the present
study, CAT activities recorded a significant inhibition in the liver tissues of the fish exposed to Cu-NPs or CuO and more reduced activities of CAT were reported in the Cu-NPs treatment. A decline in CAT activity at the highest copper concentration might be related to the inhibition of enzyme synthesis, or the direct binding of copper to –SH groups of this enzyme [55], or the exhaustion of its activity due to the flux of superoxide radicals [56]. Sevcikova et al. [54] also reported a decline in CAT activity at highest Cu concentrations in C. carpio. Abdel-Khalek et al. [2], reported that CuO-NPs induced decrease in the CAT activity in tilapia. Cozzari et al. [57] reported that Ag-NPs and bulk Ag-particles exposure caused consistent decrease in CAT activities particularly at the higher exposure concentrations. This inhibition may arise due to imbalance in ROS formation and the antioxidant defense system of the cells [58]. Also, at higher concentrations, chemicals may directly inhibit the activity of enzymes, or indirectly reduce the concentration of the enzymes by damaging cell organelles [59]. The reduction of CAT activity may also result from the accumulation of H₂O₂ and other oxyradicals [60].

The present study investigated more toxic effects of Cu-NPs than CuO-microparticles in the liver tissues of C. carpio for most oxidative stress parameters. Hu et al. [61] suggested that smaller-sized NPs caused more oxidative stress than larger particles of similar compounds. NPs can produce ROS as a consequence of their disproportionately large surface area compared to the bulk materials [62]. Chang et al. [63] revealed that the oxidative stress was induced by Cu NPs in zebrafish (mature female) exposed for 48 h at very high levels as compared to their bulk counterpart. Also, most of the results of biochemical determination in the present study showed that Cu-NPs had more toxic effect than Cu-BS. The distinctive physical properties of NPs are mainly attributed to their large proportion of the atoms that has been exposed on the surface compared to the microparticles. While in bulk materials, the surface atoms constitute only a few percent of the total number of atoms, in NPs most of the atoms lay close to the surface [64]. The toxicity of Cu-NPs is likely explained by the combination of high surface reactivity and large surface area, thus constituting a double impact which has been discussed regarding their inflammatory potential [65].

3.3 Histological Assessment
Histological investigation is an important sensitive tool to specify alteration in any tissue or organ undergoing a potential stress. Thus in the current study the histology of liver was carried out to evaluate the toxicological impact of Cu-NPs and CuO.

Figures 1-7 show the photomicrographs of the liver where control group shows the normal hepatocytes and liver parenchymatus tissues while the treatment groups show the significant histological alterations in a dose dependent manner including focal necrosis (FN), cloudy swelling of hepatocytes (CSH), degenerative hepatocytes (DH), vacuolization (V), pyknotic nuclei (PN), damaged central vein (DCV), nuclear hypertrophy (NH), dilated sinusoid (DS), vacuolated degeneration (VD), congestion (C), and complete degeneration (CD). These alterations were more intense at high dose of Cu-NPs and Cu-BS (Fig. 1-7). The intensity of histological alterations in different treatment groups is presented in Table 3. In the present study, after 14 days of exposure to Cu-NPs and CuO, the exposed groups showed significant histological alterations in the liver of *C. carpio*. The oxidative stress induced by Cu-NPs and CuO in the liver of *C. carpio* in the present study was reflected by the histological alterations in liver. The liver histology of treated *C. carpio* showed significant histological abnormalities, including focal necrosis (FN), cloudy welling of hepatocytes (CSH), degenerative hepatocytes (DH), vacuolization (V), pyknotic nuclei (PN), damaged central vein (DCV), nuclear hypertrophy (NH), dilated sinusoid (DS), vacuolated degeneration (VD), congestion (C), and complete degeneration (CD) in a dose dependent manner after 14 days of exposure. Many hepatocytes in the liver of treated groups showed necrosis as compared to the control groups, which may be due to the naturally occurring physiological apoptosis as described by Roos and Kaina [66] and Soliman et al. [67]. The histological alterations in liver are frequently assessed and used as biomarkers of various stressors [68]. The liver has the great potential to degrade toxic compounds by enzymatic reactions, but it itself might be adversely affected by the high concentrations of these enzymes [69]. Histopathological alterations in fish liver due to exposure of numerous NPs were reported in many previous studies. For example, Al-Bairuty et al., [70] reported similar findings in the liver of juvenile rainbow trout, treated with CuSO₄ /CuNPOs, Ostaszewska et al., [71] found similar histological alterations in the liver of Siberian Sturgeon, which were treated with CuNP @0.01, 0.05 or 0.5 mg/l. The intensity of histopathological alterations in liver of *C. carpio* increased in a concentration dependent manner. Similar findings were also reported by Hao et al. [72] in the liver of *C. carpio* exposed to TiO₂-NPs and by Al-Bairuty et al. [70] in the liver of rainbow trout exposed to Cu-NPs and by
Ostaszewska et al. [71] in the liver of Siberian sturgeon. The possible reason could be the bio-accumulation of triglycerides in the hepatocytes which ultimately could result in the formation of vacuoles which lead to necrosis (70, 72). In the current study, the liver hepatocytes showed shrinkage and necrosis when exposed to the highest concentrations of Cu-NPs or CuO. Progressive degeneration of hepatocytes indicated in the present study was also reported in other studies [60]. Despite their potential for a variety of applications, copper nanoparticles induce very strong inflammatory responses and cellular

![Photomicrograph (H&E; X400) of liver of control C. carpio showing normal hepatocytes (H).](image1)

![Photomicrograph (H&E; X400) of liver of control C. carpio showing normal hepatocytes (H).](image2)
Figure 2 Photomicrograph (H&E; X400) of liver of *C. carpio* treated with 0.5 mg/l Cu-NPs (GN1) showing focal necrosis (FN), cloudy swelling of hepatocytes (CSH), degenerative hepatocytes (DH), vacuolization (V), pyknotic nuclei (PN), nuclear hypertrophy (NH), dilated sinusoid (DS), vacuolated degeneration (VD) and congestion (C).

Figure 3 Photomicrograph (H&E; X400) of liver of *C. carpio* treated with 1.0 mg/l Cu-NPs (GN2) showing focal necrosis (FN), cloudy swelling of hepatocytes (CSH), degenerative hepatocytes (DH), vacuolization (V), pyknotic nuclei (PN), nuclear hypertrophy (NH), dilated sinusoid (DS), vacuolated degeneration (VD), congestion (C), and complete degeneration (CD).

Figure 4 Photomicrograph (H&E; X400) of liver of *C. carpio* treated with 1.5 mg/l Cu-NPs (GN3) showing focal necrosis (FN), cloudy swelling of hepatocytes (CSH), degenerative hepatocytes (DH), vacuolization (V), pyknotic nuclei (PN), nuclear hypertrophy (NH),
dilated sinusoid (DS), vacuolated degeneration (VD), congestion (C), and complete degeneration (CD).

Figure 5 Photomicrograph (H&E; X400) of liver of *C. carpio* treated with 0.5 mg/l CuO (GS1) showing focal necrosis (FN), cloudy swelling of hepatocytes (CSH), degenerative hepatocytes (DH), vacuolization (V), pyknotic nuclei (PN), damaged central vein (DCV), nuclear hypertrophy (NH), dilated sinusoid (DS), vacuolated degeneration (VD), congestion (C), and complete degeneration (CD).

Figure 6 Photomicrograph (H&E; X400) of liver of *C. carpio* treated with 1.0 mg/l CuO (GS2) showing focal necrosis (FN), cloudy swelling of hepatocytes (CSH), degenerative hepatocytes (DH), vacuolization (V), pyknotic nuclei (PN), nuclear hypertrophy (NH), dilated sinusoid (DS), vacuolated degeneration (VD), congestion (C), and complete degeneration (CD).
Photomicrograph (H&E; X400) of liver of *C. carpio* treated with 1.5 mg/l CuO (GS3) showing focal necrosis (FN), cloudy swelling of hepatocytes (CSH), degenerative hepatocytes (DH), vacuolization (V), pyknotic nuclei (PN), nuclear hypertrophy (NH), dilated sinusoid (DS), vacuolated degeneration (VD), congestion (C), and complete degeneration (CD).

<table>
<thead>
<tr>
<th>Histological Alterations</th>
<th>Control</th>
<th>GN1</th>
<th>GN2</th>
<th>GN3</th>
<th>GS1</th>
<th>GS2</th>
<th>GS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal necrosis</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
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<td>+++</td>
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<tr>
<td>Cloudy swelling of Hepatocytes</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
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<td>+++</td>
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<tr>
<td>Degenerative hepatocytes</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Vacuolization</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Pyconotic nuclei</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>Damaged central vein</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
<td>-</td>
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<td>-/+</td>
</tr>
<tr>
<td>Dilated sinusoid</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
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<tr>
<td>Nuclear hypertrophy</td>
<td>-</td>
<td>-/+</td>
<td>-/+</td>
<td>+</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>Vacuolated degeneration</td>
<td>-</td>
<td>-/+</td>
<td>-/+</td>
<td>+</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
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<tr>
<td>Congestion</td>
<td>-</td>
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<tr>
<td>Complete degeneration</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
</tr>
</tbody>
</table>

(-) no histological alterations (normal histological structure); (+/-) mild histological alterations; (+) moderate histological alterations; (++) severe histological alterations; (+++) very severe histological alterations in the liver histology.
toxicity [73] moreover, nanoparticles also induced injury to the liver and heart [74] which is in line with the present study. Coulibaly et al. [75] also reported histological alterations of gills, liver and kidney in Black-Chinned Tilapia (*Sarotherodon melanotheron*) when exposed to water contaminated by heavy metals including Cu. These findings are in line with the current study.

4. Conclusions

It is concluded that Cu-NPs and CuO are toxic to fish health as these induced several adverse effects, including changes in the liver antioxidant enzyme activities, oxidative stress, marked histological and hematological alterations in *C. carpio*. We investigated that Cu-NPs have more toxic potential as compared to CuO. A dose dependent increase in hemato- and hepatotoxicity was observed in *C. carpio* exposed to Cu-NPs or CuO.

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