Histopathological changes and antioxidant responses in Common carp (*Cyprinus carpio*) exposed to copper nanoparticles

Aasma Noureen[1,5], Farhat Jabeen*[1], Tanveer A. Tabish[2], Muhammad Ali[1], Rehana Iqbal[3], Sajid Yaqub[1], Abdul Shakoor Chaudhry[4]

1. Department of Zoology, Government College University Faisalabad, 38000, Pakistan
2. College of Engineering, Mathematics and Physical Sciences, University of Exeter, Stocker road, Exeter, EX4 4QF, United Kingdom
3. Department of Zoology, Bahauddin Zakariya University, Multan, 60800, Pakistan
4. School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom
5. Virtual University Lahore, Pakistan

* Address correspondence to farhatjabeen@gcuf.edu.pk

Abstract:

Despite the rapid increase of nanotechnology in a wide array of industrial sectors, the biosafety profile of nanomaterials remains undefined. The accelerated use of nanomaterials has increased the potential discharge of nanomaterials into the environment in different ways. The aquatic environment is mainly susceptible as it is likely to act as an ultimate sink for all contaminants. Therefore, this study assessed the toxicological impacts of waterborne engineered copper nanoparticles (Cu-NPs) on histology, lipid peroxidation (LPO), catalase (CAT) and glutathione (GSH) levels in the gills of common carp (*Cyprinus carpio*). Nanoparticles were characterized by XRD and SEM techniques. Before starting the sub-acute toxicity testing, 96hrs LC50 of Cu-NPs for *C. carpio* was calculated as 4.44mg/l. Then based on LC50, *C. carpio* of 40-45g in weight were exposed to three sub-lethal doses of waterborne engineered Cu-NPs (0 or 0.5 or 1 or 1.5 mg/l) for a period of 14 days. The waterborne Cu-NPs have appeared to induce alterations in gill histology and oxidative stress parameters in a dose-dependent manner. The gill tissues showed

degenerative secondary lamellae, necrotic lamella, fused lamella, necrosis of the primary and secondary lamella, edema, complete degeneration, epithelial lifting, degenerative epithelium and hyperplasia in a dose-dependent manner. In the gill tissues, waterborne Cu-NPs caused a decreased level of CAT and elevated levels of LPO and GSH in the fish exposed to the highest dose of 1.5mg Cu-NPs /l of water. Our results indicate that the exposure to waterborne Cu-NPs was toxic to the aquatic organisms as shown by the oxidative stresses and histological alterations in *C. carpio,* a freshwater fish of good economic value.

Keywords: Cu nanoparticles, toxicity, histopathology, oxidative stress, antioxidant enzymes, *Cyprinus carpio*

1. Introduction

Many nanomaterials (NMs) have widely been exploited in electronics, supercapacitors, environmental and medicinal applications owing to their unique features such as specific surface area, stability, inertness, optical properties and size related tuneable surface chemistry as compared to their bulk counterparts[1,2]. The research and development of functional NMs have become an emerging technological arena, thanks to the improved physicochemical properties which make them ideal candidates for use in real-world applications. Physicochemical properties and physiological nature of NMs are closely related to their environmental toxicology as a result of their release into the environment. Nevertheless, understanding the contribution of these physicochemical characteristics to the availability of NMs to the environment remains unexplained. The fast-growing commercial applications of NMs have raised serious concerns about their possible eco-toxic effects as a result of their release into the environment[3,4]. Therefore, the legislation and standardization are more important for the environmental exposure of NMs[5-7].

Among numerous types of NMs, copper nanoparticles (Cu-NPs) have been used as doping materials in semiconductors, chemical sensors and antimicrobial agents due to their distinct features of high reactivity, good electrical properties, cost-effective preparation, and size-dependent optical features. Few studies are also available on the preparation of composites by combining Cu-NPs with polymers, which have the ability to release metal ions to control the growth of pathogenic microorganisms [8]. Moreover, Cu-NPs have

extensively been used in cosmetics and sunscreens to prevent skin infections, and develop antifouling coatings in oceanic industries [9, 10]. The rapid mobility of Cu-NPs in aquatic ecosystems has resulted in extraordinary hazards on human and ecosystem health, which need to be addressed. Aquatic systems are considered to represent the ultimate 'sink' for the accumulation and uptake of NMs from the surrounding environment[11]. However, current knowledge on the toxicological impacts of Cu-NPs is unlikely to provide answers for the fundamental questions regarding their toxicity mechanism, transformation, and clearance from eco- and living systems. The toxicity of Cu-NPs is still under consideration by many research groups to trace their bioavailability and fate. Short term exposure of CuO-NP and TiO_2-NP in *Cyprinus carpio* results in severe histological anomalies in its gills and other organs[12]. The toxicological impacts in terms of histological alterations in gills of *C. carpio* exposed to CuO-NPs are enhanced in combination with TiO_2 NPs[13]. Moreover, the histological alterations (hyperplasia, fused lamella, and blood clotting) are also reported in gills of *C. carpio* exposed to different doses of CuO-NPs[14]. Cu induce alternation in the mitochondrial function by increasing proton leakage in mitochondria which results in the decrease of respiratory control ratios in response to CuO-NP and Cu exposure which in turn increase ROS levels [15]. Rod-shaped CuO NPs induce significant decrease in reproduction, feeding inhibition and increase in ROS generation in Neotropical fish species (*Ceriodaphnia silvestrii* and *Hyphessobrycon eques*) [16].

Fish is a useful model system for revealing the possible environmental toxicity of Cu-NPs which can be accumulated in fish. Many Cu-NPs have toxic effects on fish. The main cause of Cu toxicity to fish is the rapid binding of Cu to the gill membranes, which damage gills filaments and impair the osmoregulatory function of gills [17]. Therefore, identification of physical and chemical factors that may influence the transformation, accumulation, and transport of Cu-NPs is essential to model their biosafety profile to solve real-world clinical problems. With this scenario, the current study assessed the waterborne Cu-NPs induced toxicity by testing the oxidative stress and histological profiles in *Cyprinus carpio* by assuming the following pathways of their possible effects on fish (Scheme 1). *C. carpio* was selected as an experimental model for its hardy and tolerant nature and adaptability to various conditions and habitats.

Material and Methods

1.1. Fish specimens, housing, and feeding

Specimens of *Cyprinus carpio* (*C. carpio*) (n= 330; 40-45g) were purchased from a fish farm, Faisalabad, Pakistan. The fish specimens were transferred to oxygenated water containers which were then immediately transported to the GC University Labs. These fish were then group housed in three tanks (100 l; 76x30x46 cm) and fed with a commercial fish feed (65% protein and 10% fat) at the rate of 5% body weight. De-chlorinated tape water was used in this experiment with forced aeration by an aquarium air pump (Electric RS-348A, Sea Work Fish Aquarium Center Lahore, Pakistan). Water quality parameters were tested and maintained at 6.6–7.6 mg/l dissolved Oxygen (DO), 6.9-7.5 pH; 47-52 ppm hardness, 25°C temperature and provision of 12 h light and 12 h dark photoperiod. The fish stock was acclimatized for 1 week to these conditions before the start of this study. The current study is the continuation of our on-going project.[18,19]

1.2. Chemicals

The manufactured Cu-NPs (CAS Number 7440-50-8; 60–80 nm and 99.5 % purity) were purchased from Sigma Aldrich Ltd. The working solution of Cu-NPs was made in ultra-pure water and sonicated to obtain a homogeneous dispersion of particles. The stock solution was exposed to the ultrasound sonication bath (Sonorex Super 10P. Bandelin Electronic GmbtH & Co.KG) for 1 h prior to each use and was immediately transferred into the exposure glass aquaria to ensure uniform particle suspension.

2.3. Characterization of Nanoparticles

The X-Ray diffraction (XRD) pattern of Cu-NPs was observed by Advance X-ray diffractometer (Bruker D8). The scanning electron microscopic image of the sample was taken by an FEI Helios Dual-Beam SEM-

based measuring system, which was equipped with a high-performance electron beam column and sample stage. The spatial resolution used for this measurement was 1 nm at optimum settings (15 kV accelerating voltages).[18]

2.4. Dynamic light scattering (DLS), Zeta Potential (ZP) measurements and Stability Tests

The stock solution (10mg/l) of Cu-NPs was made in ultra-pure water and sonicated for one hour by sonication bath (Sonorex Super 10P. Bandelin Electronic GmbtH & Co.KG) to obtain a homogeneous dispersion of particles. The hydrodynamic size distribution was analysed using particle size analyser (90 Plus Particle Size Analyser, Brookkhaven Instruments, USA). The zeta potential of nanoparticle dispersions was measured using SZ100 nanoparticle analyser (Horiba Scientific, Japan). Samples (i.e., NP dispersed in water in fish aquarium @ concentration of 0.5 or 1 or 1.5 mg/l, pH~8.2) were taken from the tanks at every 12 h for analysis. Samples were transferred into a 4 ml cuvette without any filtration to determine the size and assess the extent of aggregation. The stability and aggregation kinetics of the CuO NPs in aquarium water was studied by using dynamic light scattering method. The mean hydrodynamic size of each nanoparticle concentration was recorded at different kinetic intervals of 0, 06, and 12 hrs using the particle size analyser (90 Plus Particle Size Analyser, Brookkhaven Instruments, USA).

2.5. Determination of LC50 of Cu-NPs for C. carpio

The acute toxicity tests were conducted to determine the 96 h LC50 values in *C. carpio* (n= 210 taken from stocking tanks; 40-45g in weight and 13.5-14.5cm in length) after their exposure to Cu-NPs[18]. The experiment was carried out in glass aquaria with maximum water capacity of 40 liters. All exposures were performed in triplicate in glass aquaria containing 10 fish per aquarium with continuous aeration by an aquarium air pump (Electric RS-348A, Sea Work Fish Aquarium Center Lahore, Pakistan). For dosing, stock solution was made in ultrapure water followed by sonication for 1 hour (DSA100-SK1-2.8l Sonicator). The solution of Cu-NPs was added in the experimental aquaria to obtain 0 or 0.5 or 1 or 1.5 or

3 or 6 or 12 mg/l concentration at the similar levels of physicochemical parameters as maintained during acclimatization period. Feeding of fish and water change of aquaria were stopped during test period of 96h. The dead fish were removed and the levels of LC50 calculated at intervals of 24, 48, 72 and 96h from the relevant tanks and then pooled to present deaths over 96h for each tested dose of Cu-NPs. The 96h LC50 was calculated as 4.44mg/l by Probit Analysis (Minitab 17 software)[18,19].

2.6. Experimental Design and Routines for sub-acute toxicity testing

A completely randomized design was used to evenly distribute 120 fish (taken from stock aquaria) into twelve aerated glass tanks (10 fish/ tank) where 3 tanks represented each of the 3 doses of Cu-NPs and one control group. Based on the LC50, three sub-lethal doses (0.5, 1 and 1.5 mg/l represented by Cu-NP1, Cu-NP2, and Cu-NP3, respectively) were selected for sub-acute toxicity assessment. All procedures performed in this study involving fish handling were in accordance with the research ethical standards approved by the Ethics Committee of the Government College University Faisalabad, Pakistan on Animal Experimentation. The fish in triplicated tanks were exposed to different concentrations (0, 0.5, 1 and 1.5 mg/l) of Cu-NPs for 14 days. About 85% of water change was performed after every 12 h with re-dosing of Cu-NPs to help to retain water quality. Water samples were daily assessed during the experiment to maintain pH, temperature, dissolved oxygen, total ammonia, and water hardness. Five fish per aquarium were randomly sampled at day 0 and 15 for the analysis of oxidative stress enzymes (LPO, GSH, and CAT) in gill tissues and gill histology. 2-3 mm of each fish gill was fixed in fixative for histology and the remaining part was used for oxidative stress enzymes analysis. Fish mortality and behaviour were also recorded.

2.7. Fish Dissection and Tissue Sampling

For tissue sampling, fish were anesthetized by simply adding 2-6 drops of clove oil to a bucket of 1-2 litre water. The fish were then immersed in this water and the effect of anaesthesia was confirmed when the fish lost equilibrium. Dissections were carried out by using acid washed instruments, to minimize any cross-

contamination between samples. From each of the sampled fish, 1g of gill tissues was used promptly for the preparation of homogenate. Also, small pieces of gill tissues were fixed in a mixture containing 60 ml alcohol, 30 ml formaldehyde and 10 ml of glacial acetic acid for histological studies.

2.8. Preparation of gill homogenates

The gills were removed, washed and then homogenized in Tris EDTA buffer (pH 7.4), using a homogenizer (Potter-Elvejham Homogenizer). After centrifugation at 10,000 rpm for 20 minutes at 4 °C, supernatants were collected, stored at −80 °C and used as homogenate for the assessment of oxidative stress enzymes [19] as described below.

2.9. Estimation of lipid peroxidation (LPO)

LPO was assessed by using the method reported by Ohkawa et al. [20]. For the estimation, 0.2 ml of gills homogenate were mixed with 0.25 ml SDS (8.1%), 1.52 ml acetic acid (20 %) and 1.52 ml thiobarbituric acid (0.8%). Then distilled water was added to the mixture to make its volume 4 ml. The reaction mixture was then boiled in a water bath (95°C) for 1 hour followed by the addition of 1:15 mixture of pyridine and n-butanol. The mixture was then shaken and the absorbance was taken at 532 nm by spectrophotometer (U-2800 Hitachi, 200-1100) against tetramethoxypropane as a standard and expressed in nM/mg of gill tissues.

2.10. Estimation of Glutathione (GSH)

The GSH contents were estimated by following the protocol reported by Sedalk and Lindasy [21] with slight modifications. The gills homogenate was mixed with trichloroacetic acid (50%) and centrifuged at 1000 rpm for 7 minutes to collect the supernatants. Each supernatant (0.5 ml) was mixed with Tris-EDTA buffer (2 ml, 0.2 M; pH 8.9) and 0.01 M 5'5'- dithio-bis-2-nitrobenzoic acid (0.1 ml) and the mixture was kept for 5 minutes before the absorbance was measured at 412 nm on the spectrophotometer (U-2800 Hitachi (200-1100). The GSH contents were expressed as µM /g of gill tissues.

2.11. Estimation of catalase (CAT) enzyme

The CAT in gills was assessed by following the method reported by Aebi [22]. For this purpose, 50 µl (10 % w/v) gills homogenate was taken into the cuvette (3.0 ml) that already contained phosphate buffer (1.95 ml, pH 7.1). The absorbance was taken after 30 sec at 240 nm (30-sec intervals) after adding 30 mM hydrogen peroxide (1 ml). The CAT was expressed as U/ml of gill tissue homogenate.

2.12. Histology

Sera (60 ml alcohol, 30 ml formaldehyde and 10 ml of glacial acetic acid) was used to fixed the samples which were further processed for histological analysis. After 5-7 hours of fixation, the process of dehydration was done by using 70, 80, 90, 95, and 100 % of ethanol. Paraffin wax was used to make the block of dehydrated samples. The tissue sections of 3.5-4 µm were cut by a microtome (Microtome CUT5062 by Nikon Instruments) and stained by using the hematoxylin-eosin stain[23]. The photographs were taken using a microscope (Nikon E200POL) with a digital camera.

2.13. Statistical Analysis

The data were analyzed by one-way Analysis of Variance in Minitab17 software. The treatment means were compared with the post hoc Tukey's test. The differences between means were considered significant if $p<0.05$. All data values were expressed as mean $\pm$ SEM in different tables of this paper .

2. Results and Discussion

The XRD pattern of the observed Cu-NPs is presented in Fig.1.The main three characteristic diffraction peaks for Cu were observed around $2\theta = 42.292°, 49.859°, 73.644°$ which corresponded to 999, 450, 220 crystallographic planes of face-centered cubic (FCC) Cu phase. The average particle size of Cu-NPs was assessed by using the Debye-Scherrer formula [24]:

$D = 0.89\lambda / (\beta * \cos\theta)$ ..... (2)

Where:

D = Particle diameter (Average crystallite size)

β =Full Width at Half Maximum (FWHM)

θ = Bragg angle

λ =X-Ray Wavelength, (Cu) Kα emission (λ=1.54056A°)

Average crystallite size of Cu-NPs was in the range of 78.33 nm

Fig. 2 presents the SEM of Cu-NPs. The samples were photographed at 20,000x and 30,000x magnification at 1.2 µm fields of view, which provided a good balance between high spatial details and particle density. The result showed uniformly dispersed Cu-NPs with cubical morphology. The dispersion of particles was homogeneous and the average particle size was in the range of 65-90 nm.

The mean hydrodynamic diameter of Cu-NPs (0.5, 1 and 1.5 mg/l) was found to be 93, 95 and 99 nm, respectively which was greater than the particle size measured in XRD and SEM techniques. The average hydrodynamic diameter was less than 100nm showing the nanoparticles stability for toxicity assessment. The surface potential of dispersed Cu-NPs was measured to be -21.6±1.25 mV in aquarium water. The magnitude of the ZP can be taken as one of the parameters to understand the colloidal stability of the NP[25]. NP with ZP values greater than +30 mV or less then −30 mV typically have a high degree of stability in suspension[25]. The ZP remained constant over time, which indicated good colloidal stability of the NPs as reported in other studies[26].

Fig. 3 shows the 96 hours LC50 value of Cu-NPs for *C. carpio* as 4.44±0.67 mg/l. Table 2 presents the physicochemical parameters of aquarium water used in the experiment. All the parameters were according to EPA criteria suitable for freshwater life. In this study, a dose-dependent alteration in the oxidative stress enzymes was found in the gills of Cu-NPs exposed fish. Table 3 presents the concentrations of oxidative stress enzymes (LPO, GSH, and CAT) in the gills of *C. carpio* among different treatment groups. The levels

of LPO in gills were found in the order of Cu-NP3>Cu-NP2>Cu-NP1>control. The concentration of GSH in the gills was found in the order of Cu-NP3>Cu-NP2>Cu-NP1>control. The lowest CAT was detected in the gills of *C. carpio* in the Cu-NP3 group. Overall, oxidative stress enzymes showed significant differences when the treatment groups of fish were compared amongst themselves and with the control fish.

The histology of gills was investigated to evaluate the toxicological impact of Cu-NPs in fish. As the histological investigation is an important sensitive tool to specify alteration in tissues or organs under stress if any. Fig. 4A shows the normal histology of the control fish gills with well-defined gill filaments and lamellae. Photomicrographs of different treatment groups showed dose-dependent histological changes in fish gills such as degeneration, necrosis, and fusion of gill lamellae along with edema, epithelial lifting and hyperplasia (H) (Fig. 4B- D). The intensity of gill histological alterations is presented in Table 4.

Both natural and anthropogenic activities are the most abundant sources of Cu in aquatic systems [17] where Cu is an important nutrient for aquatic organisms. The high levels of Cu in an aquatic environment can cause mortality whereas chronic exposure may induce adverse effects on the survival, growth, and reproduction of aquatic organisms. The high concentration of Cu may also alter brain function, enzyme activity, blood chemistry and metabolism of aquatic organisms [17]. The Cu-NPs may cause specific and altered toxicological impacts compared to regular Cu microparticles. When the bulk Cu is converted into NPs, this chemical conversion generally can change their physicochemical properties as a result of the change in size and surface area[27]. Nevertheless, these altered and improved physicochemical properties might be facilitating the absorption and translocation of NPs [28].

The previous studies have confirmed the oxidative stress induced toxicity of Cu and its counterpart nanoparticles in aquatic organisms [29-32]. The cellular metabolism of molecular oxygen produces reactive oxygen species (ROS). These ROS could produce a number of severe abnormalities in living organisms. The living organisms are equipped with different antioxidant enzymes that have the ability to compensate for the toxic effect of oxidants (enzymatic and nonenzymatic). The major enzymatic antioxidants are SOD, CAT, and GSH [1, 33-34]. CAT in animals has the ability to lyse hydrogen peroxide (H_2O_2) into O_2 and water

[35]. Significant changes in antioxidant profiles were confirmed in the current study in fish exposed to different concentrations of Cu-NPs showing that CAT was reduced while LPO and GSH concentration was elevated in gills. The dose-dependent significant increase in the gill LPO might be due to ROS production. In the current study, the decrease in CAT levels in the gills indicated that the Cu-NPs treatment-induced toxicity. The results of our study are also in line with Manke et al. [36] and Abdel-Khalek et al. [37] who demonstrated that MDA contents in gill tissues of Nile Tilapia were significantly increased when treated with various concentrations of Cu-NPs for 30 days. LPO induced by Cu-NPs was also reported in other biological models, indicating that the toxicological effects of Cu-NPs were induced by oxidative stresses [38-43]. The reduction in CAT with increasing Cu-NPs concentrations may be a consequent of limited production of CAT or/and accumulation/uptake of Cu ions into CAT enzymes which eventually deactivated its functionality [44]. Sevcikova et al. [45] also reported the lower levels of CAT at higher concentrations of Cu in *C. carpio*.

After 14 days of exposure to waterborne Cu-NPs, the treated groups showed major histological alterations and abnormalities in the gills. The histological alterations included degeneration, necrosis and fusion of lamellae alongwith edema, epithelial lifting and hyperplasia. In the present study, the histological response of the gills to Cu-NPs induced toxicity was dose-dependent where alterations in gill histology increased with the increasing dose of Cu-NPs. Similar findings are also reported in previous study in which *C. carpio* exposed to different doses of CuO-NPs, showed severe histological alterations including hyperplasia, fused lamella and blood clotting in gills [14]. Cu-NPs exposures produce similar gill abnormalities as identified by soluble Cu. Combined effects of Cu-NPs dissolution and particle size cause severe toxicological effects on gills morphology [46]. Overall, the current study confirmed the highly toxic effects (dose-dependent manner) of waterborne Cu-NPs in *C. carpio*.

Conclusion:

It appeared that the waterborne engineered Cu-NPs were harmful to the defense system of the fishes as indicated by the histological alterations and oxidative stress in *C. carpio*. The waterborne Cu-NPs induced alterations in gill histology and oxidative stress parameters in a dose-dependent manner. The gill tissues showed degenerative secondary lamellae, necrotic lamella, fused lamella, necrosis of the primary and secondary lamella, edema, complete degeneration, epithelial lifting, degenerative epithelium and hyperplasia in a dose-dependent manner. In the gill tissues, waterborne Cu-NPs caused a decreased level of CAT and elevated levels of LPO and GSH in the fish exposed to the highest dose of 1.5mg Cu-NPs /l of water. Therefore, ecological contamination of such toxic NPs must be avoided through both awareness campaigns and regulatory procedures. Further research is recommended to investigate the impact of chronic exposure of other fish species or animal models to Cu-NPs or other NP. This may help us to determine the optimum tolerance levels of different freshwater fish to Cu-NPs and various other NPs.

Conflicts of Interest: The authors declare no conflict of interest.

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