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Evaluation of Toxicity Induced by Engineered CuO Nanoparticles in Freshwater Fish, *Labeo rohita*

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Abstract

With the fast development of industries relevant to nanotechnology, the inappropriate disposal of nanoproducts may initiate a new source of pollution in aquatic ecosystems, thus posing a possible danger to aquatic life. This study evaluated the eco-toxicological effects of waterborne copper oxide nanoparticles (CuO-NPs) having a 32.84nm size and rod shape on a freshwater fish, *Labeo rohita*. 96-h LC_{50} of CuO-NPs was 353.98mg/L. Two sub-lethal concentrations equivalent to 1/3rd and 1/5th LC_{50}/96h (70.79 and 117.99 mg/L) of CuO-NPs were selected for 15, 30, and 45-day exposure tests. Bioaccumulation for the 1/3rd 96h LC_{50} was significantly higher compared to 1/5th of 96-h LC_{50} of CuO-NPs. There was a sharp decrease in the CAT activity and this decline ultimately increased the TBARS contents. The highest percentage of damaged nuclei and genetic damage index in fish erythrocytes was recorded at the highest concentration and after 45 days of treatment. The adverse effects of CuO-NPs were examined to be dose and duration dependent with increasing extent during all studied time intervals. Summarizing, exposure to sublethal concentrations of CuO-NPs is sufficient to cause alterations in ecotoxicological endpoints such as metal overload, oxidative stress and genotoxicity after chronic exposure

Introduction

With the rapid development of nanotechnology, metal oxide nanomaterials have received great attention because of their typical physicochemical properties, primarily attributed to their higher surface area compared to their bulk materials. Moreover, factors regarding size, shape, chemical properties, dissolution, and aggregation are specifically advised to be the most crucial parameters that influence the attributes of nanoparticles (Nel *et al*., 2006). Copper oxide nanoparticles (CuO-NPs) commonly synthesized nano-materials due to their good catalytic, antibacterial, and thermo-physical attributes (Prasad *et al*., 2016), are commonly utilized in chemical processes, cells, sensors, electronics, nutrient protection, textile industries, coatings, drug delivery, water management, and agriculture (Keller *et al*., 2017). Aquatic ecosystems are the eventual destinations of pollutants (Yalsuyi & Vajargah, 2017). Inevitably, increased production and use of CuO-NPs results in bio-accumulation in aquatic biota and ultimately entered in higher trophic levels (Zhao *et al*., 2011; Shaw & Handy, 2011), posing a possible danger to non-target living things. For Cu NPs, the PEC (predicted environmental concentration) in some waters were about 60 µg/L (Chio *et al*., 2012). The toxicity of copper ions to aquatic life is well noted and as a redox metal, Cu takes part in Fenton and Haber–Weiss reactions, causing the reactive oxygen species (ROS) formation and oxidative stress (OXS) (Bebianno *et al*., 2004). All aquatic animals have improved systems to defend against damage induced by reactive oxygen

species, including antioxidant enzymes such as CAT, SOD and GPx (Eyckmans *et al*., 2011). Cu nanoparticles caused mortality after severe gill pathology in zebrafish (Griffitt *et al.,* 2007) and their toxicity was fully dependent on the specific environmental conditions (Villarreal *et al*., 2014). Fish are ordinarily used as a model organism in toxicological examinations (Burgos-Aceves *et al*., 2018). Furthermore, among the various fresh water fishes, shares significant contribution in the freshwater production of Indian subcontinent and its demand is dominated in the market due to its good taste. Several reports indicated nanoparticles were toxic for aquatic animals such as fish and crustaceans (Chang *et al*., 2012; Subashkumar & Selvanayagam, 2014). However, there is little data about the mean lethal concentration, accumulation, oxidative stress, and genotoxicity of nanoparticles in fish as a biomarker for freshwater bodies (Karthikeyeni *et al*., 2013). It is likely that a study based on chronic exposure may more distinctly show Nps toxicity and be of more importance ecologically in flourishing our understanding about mechanism of nanoparticles toxicity. In aquatic ecosystems, organisms are usually exposed to different sub-lethal concentrations of metals for a long duration (Javed, 2013). Hence, the aim of the present study was to determine the accumulation of copper in gills and its effect on catalase (CAT), thiobarbituric acid reactive substances (TBARS) levels and genotoxicity after exposing the fish to sub-lethal concentrations of CuO-NPs over 45 days because of the increasing application of copper oxide nanoparticles and their possible hazards to aquatic organisms.

Materials and Methods

Synthesis and Characterization of CuO-NPs

Copper chloride and sodium hydroxide were obtained from Merck via local distributors for the synthesis of CuO-NPs. And both were dissolved in deionized water, separately. A solution of NaOH was added drop by drop into a $CuCl_2$ solution with continuous mixing by a stirrer at room temperature that resulted in the formation of $Cu(OH)_2$ (bluish gel). After that, the collected precipitates were filtered and rinsed with distilled water which after drying and annealing produced CuONPs (Manimaran *et al*., 2014). To study the structure of the prepared copper oxide nanoparticles, the scanning electron microscope (SEM) (JEOL-JSM 5910) was used. The size of copper oxide nanoparticles was measured by the Debye Scherrer equation,

$$D=0.9\lambda/\beta Cos\theta$$

Where λ=wave length of X-ray, β=line broadening at half the maximum intensity in radians, θ=the Bragg angle.

Experimental Fish

This experiment was done in the wet laboratory of Fisheries Research Farms (FRF), University of Agriculture, Faisalabad, Pakistan. *Labeo rohita* were purchased from the Fish Seed Hatchery in Faisalabad, Pakistan. *Labeo rohita* (27±8 cm length, 50±7g weight) was placed in tanks with50L water for acclimation over fifteen days. The temperature was maintained at 30±1°C, while dissolved oxygen and pH were 5.1±0.8 mg/l and 7.5, respectively. After this acclimation, the healthy group of fish was selected for research purpose and fish exhibiting unusual behavior was excluded. Commercial pelleted fish feed (30% Digestible Protein and 3Kcal/g Digestible energy) was used to feed fish.

Experimental Setup

96-h Acute Toxicity Test

The 96-h LC_{50} and LC_{99} of copper oxide nanoparticles (CuO-NPs) for *Labeo rohita* were determined under controlled conditions. NPs were dispersed in deionized water through a sonicator (100W, 40 kHz) for 60 minutes immediately prior to use to obtain a test suspension. Ten fish were exposed to 20, 50, 80, 110, 140, 170, 200, 230, 260, 290, 320, 350, 380, 410, 440, 470 and 500mg/L for 96 h. To maintain fixed level, all the test mixtures were exchanged after 24 hours. The control group was free of CuO-NPs. The fish were not fed during the test to reduce the sorption of the nanoparticles in solid feed and feces. During the experiment, water quality parameters like pH, temperature, total hardness, dissolved oxygen and the natural 12:12 day /night photoperiod were checked Lethal concentrations of CuO-NPs have been calculated by the probit analysis method with a 95% confidence interval (Hamilton *et al*., 1977).

Chronic Toxicity Test

L. rohita were randomly distributed in glass tanks and in triplicatess with 10 fish in each tank. Fish were then exposed to two sublethal concentrations based on 96-h LC_{50} of CuO-NPs for 45 days. The sublethal suspensions of 1/3rd and $1/5^{th}$ of the LC_{50}/96-h (70.79 and 117.99 mg/L) of CuO-NPs were prepared by weighing dry CuO-NPs in the deionized water (pH 7.5), then sonicated for 60 minutes to increase their dispersion. A control group of fish was handled in the same way, but without the exposure to nanoscale CuO-NPs. The experimental conditions were same as that of the acclimation period, and physico-chemical parameters of water was checked. Water CuO-NPs suspensions were changed on the daily basis, and fish were fed 50 min before change of water during chronic exposure.

Accumulation of CuO-NPs in Gill Tissue

Labeo rohita (n=3) were taken out form control and treated groups into small container and clove oil (3-4 drops) was used to anaesthetize them. After dissection, gills samples were removed from the fish. One gram freezed dried samples were digested in a mixture of $HCLO_4$ and HNO_3, then on a hot plate, contents were heated at 100°C untill yellowish color of sample disappeared and hydrogen peroxide (2 drops) was also added in it. Each sample was evaporated and then diluted with distilled water and filtration was done by using Whatmann filter paper (S.M.E.W.W. 2012). Each sample were then being analyzed by Atomic Absorption Spectrophotometer (AAS).

Oxidative Stress Biomarkers

After each interval, sampling was done and oxidative stress in terms of levels of thiobarbituric acid reactive substances (TBARS) and Catalase (CAT) was assessed in the gills of the fish. All samples of *Labeo rohita* were homogenized separately, using chilled PBS (phosphate buffer saline) in 1/4 ratio (weight/volume) by homogenizer. After that, the mixture was centrifuged at 10,000 rpm at 4°C for 15 min. For the analysis of TBARS and CAT, supernatant was used and high quality analytical grade (Merck) reagents were obtained via local distributors. Activity of CAT was determined by measuring its capacity to lessen hydrogen peroxide at a particular wavelength (240nm). It was measured by using the method of Chance and Maehly (1955) with minor modifications. The level of TBARS in the gill tissues was calculated as the index of lipid peroxidation (LPO) as described by Gatta *et al*. (2000). The absorbance of the resultant product was measured at 534nm.

Determination of Genotoxicity

Fish erythrocytes were collected on 15, 30, and 45 days for the comet assay to determine dose and time dependent damage in DNA by the procedure of Singh *et al.* (1988) after exposure to $1/3^{rd}$ and $1/5^{th}$ of 96-h LC_{50} of CuO-NPs along with the negative control group (unstressed) positive control (cyclophosphamide). The positive control group was injected intraperitoneally with cyclophosphamide ($20\mu gg^{-1}$) in 4% saline solution. Blood samples were taken from the caudal vein of

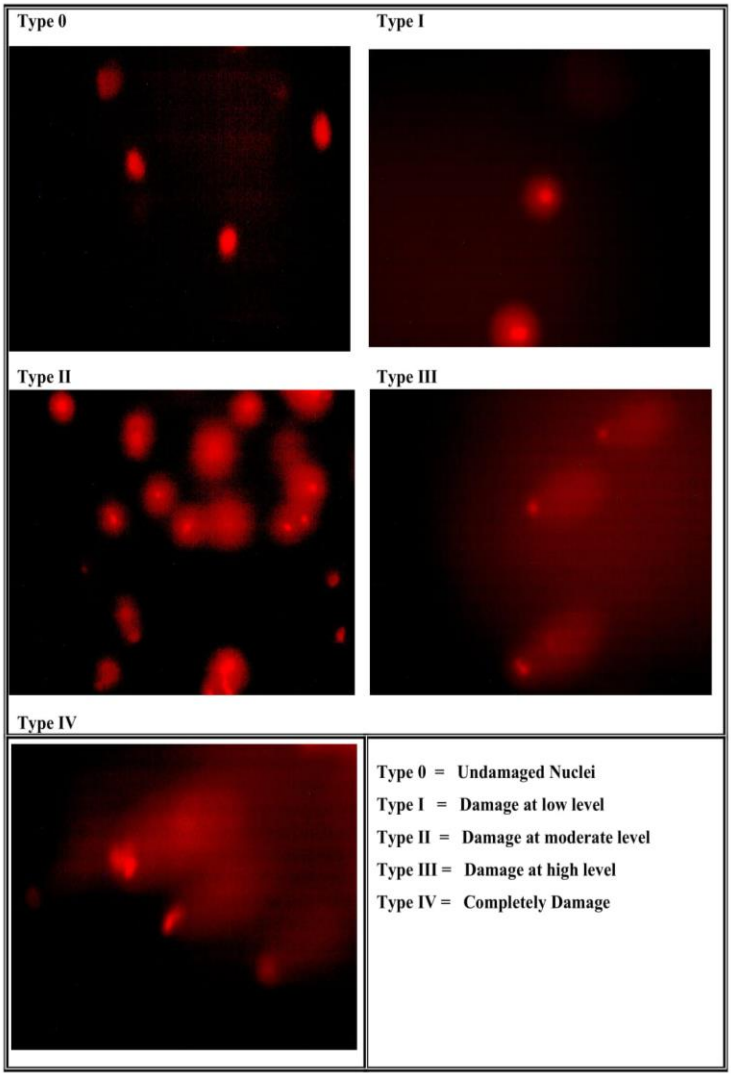

Figure 1. DNA damage classification induced by metal oxide nanoparticles in blood erythrocytes of exposed fish

selected carps (n=3). After the electrophoresis, the prepared slides were placed in a neutralizing solution. Then slides were allowed to stain with ethidium bromide and examined under Epi-fluorescence microscope (N-400M, American scope; USA) at 400X magnification with the mercury light source. DNA damage was assessed by visual categorization of cells into following five categories "comets" related to the tail length (i) Type 0 (Undamaged) (ii) Type I (Low level damage) (iii) Type II (Medium level damage), (iv) Type III (High level damage) (v) Type IV (Complete damage). (Figure 1). The extent of DNA damage was assessed as the mean percentage of cells with medium (Type II), high (Type III) and complete damage (Type IV). Genetic damage index was calculated by following formula:

$$GDI = \frac{(TypeI) + 2(TypeII) + 3(TypeIII) + 4(TypeIV)}{Type0 + TypeI + TypeII + TypeIII + TypeIV}$$

Statistical Analyses

The results were expressed as mean±S.D. All experiments were performed triplicately. SPSS (Statistical Package for Social Sciences) was used to analyze the data. Statistical similarities and differences between all variables were observed by a two-way analysis of variance (ANOVA). The means were compared by Tukey's/Student Newnan-Keul test.

Results

Characterization of Copper Oxide Nanoparticles

SEM images exhibited rod-shaped structure of nanoparticles (Figure 2). The X-ray diffraction (XRD) pattern of CuO NPs is shown in Figure 3. The characteristic peaks corresponding to (110), (002),

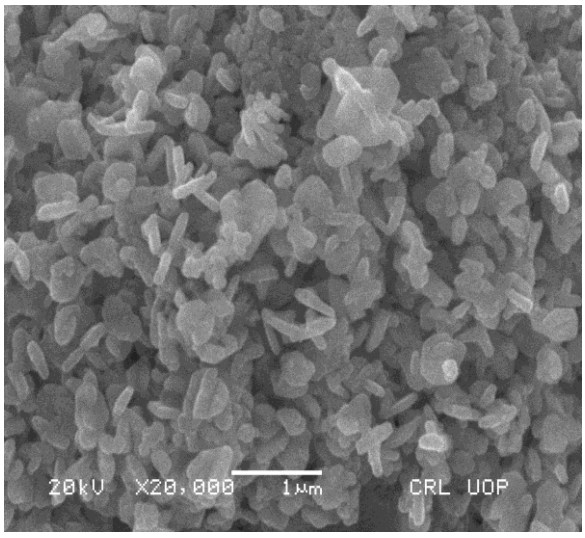

Figure 2. SEM image of CuO-NPs showed uniform distribution of the grains

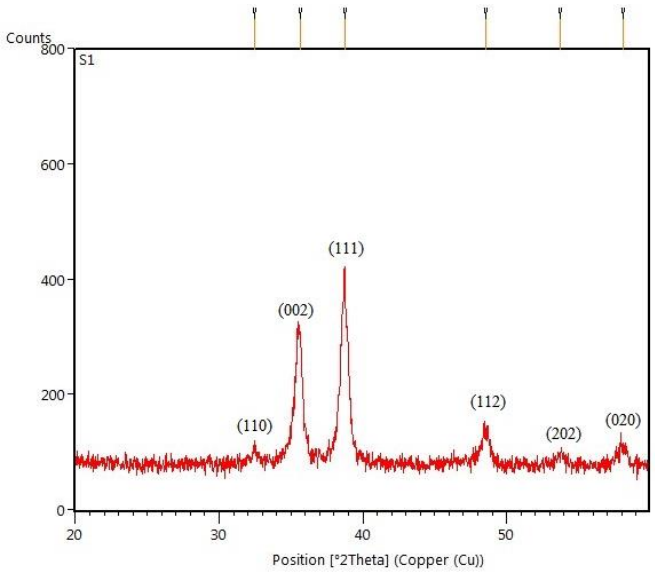

Figure 3. X-ray diffraction Pattern of nanoscale CuO-NPs

(111), (112), (202) and (020) planes are located at 2θ = 32.47°, 35.63°, 38.73°, 48.54°, 53.76°, and 58.12°, respectively (Table 1). The average size was calculated by the Debye-Scherrer formula as 32.84nm.

Acute Toxicity

During aclimatization, no mortality was observed.the rate of mortality was different at various concentrations. The LC_{50} and lethal concentration of CuO-NPs for *Labeo rohita* were 353.98 and 513.18 mg/L, respectively.

Copper Accumulation

The accumulation of Cu in fish gills increased consistently with an increase in copper exposure time and concentration, indicating time and dose dependent accumulation (Figure 4). The fish gills accumulated a significant quantity of copper. In comparison to control group, the increase in Cu concentration in the gills was statistically significant in treatments exposed to sublethal concentrations of CuO NPs ($p<0.05$). The overall accumulation of copper appeared to be significantly higher due to copper oxide nanoparticles at $1/3^{rd}$ of 96-h LC_{50} than at $1/5^{th}$ of 96-h LC_{50} exposure.

Oxidative Stress Bio-Markers

The CAT activity of gill tissues are shown in Figure 5(a). Concerning CAT activity, a significant decrease in both CuO-NPs exposed groups was observed over all periods. In the case of TBARS levels, the CuO-NPs groups showed a significant increase when compared with the control group (Figure 5(b)). High sub-lethal concentration caused bigger oxidative stress than low sub-lethal concentration. Analysis of variance displayed a significant difference in CuO-NPs exposed groups among the all studied durations for both studied oxidative stress biomarkers.

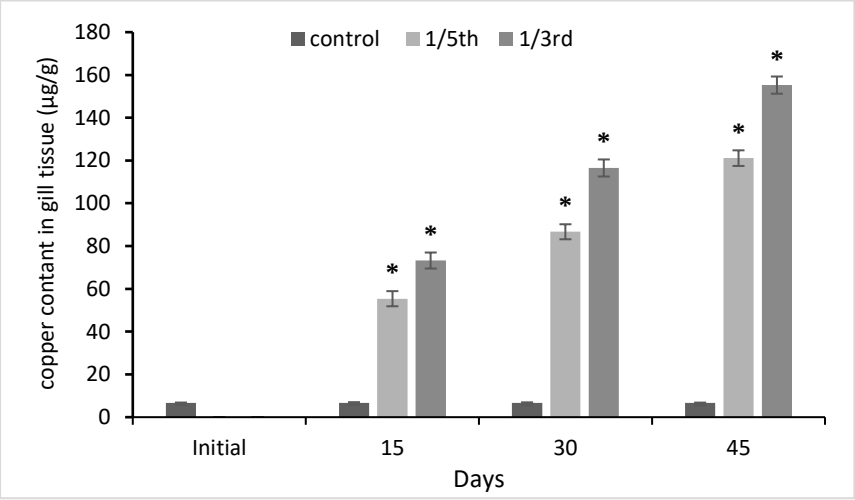

Figure 4. Accumulationof copper in gill tissues of *Labeo rohita.*

Table 1. Physico-chemical parameters used for the experiments

Parameters	Unit	Mean	Analysis Method
Total Hardness	mg/L	100	Titration method
PH	–	7.5	pH meter
Temperature	℃	30	Temperature meter
Dissolved Oxygen	mg/L	5.1	Oxygen meter
Ammonia	mg/L	0.49	Titrimetric method
Electrical Conductivity	µSiemens/cm	545.78	Conductivity meter

Table 2. Peak analysis of CuO-NPs through XRD

Sr.NO	2Theta values(degree)	Theta values (degree)	FWHM (radian)	d.spacing (Å)	D.Particle size(nm)
1	32.5	16.25	0.0016	2.7517	90.2948
2	35.63	17.81	0.0069	2.5257	21.1133
3	38.73	19.3669	0.0034	2.3355	43.2409
4	48.54	24.27	0.0082	1.8931	18.5551
5	53.88	26.88	0.0165	1.7107	9.4245
6	58.12	29.06	0.0110	1.5883	14.4256

Genotoxicity

The exposure of *L. rohita* to different sub lethal concentrations of copper oxide nanoparticles caused variable % of damaged nuclei (Figure 6 (a)) and genetic damage index (Figure 6 (b)). These two indices increased consistently in fish tissues with an increase in exposure time and concentrationThe high sub-lethal concentration (117.99 mg/L) caused significantly (P>0.05) maximum % ofdamaged nuclei and GDI than low sub-lethal concentration (70.79 mg/L).

Discussion

Nanoparticles discharged into aquatic ecosystems have in harmful consequences on non-target animals such as fish and other aquatic organisms (Pagano *et al*., 2017), which are important components of our diet. 96-h LC_{50} test is an appropriate method for showing toxicity of respective pollutant present in environment (Chorehi *et al*., 2013; Vajargah & Hedayati, 2014). However, by applying this test, we can calculate the survival of fish after exposure to various concentrations of pollutants (Vajargah *et al*., 2018). Results of the present research work indicated the LC_{50} of CuO-NPs on *Labeo rohita* was 353.98 mg/l. Various studies have explained the species-specific sensitivity of freshwater fish species after exposure to different toxicants (Eyckmans *et al*., 2011; Sanyal *et al.,* 2017). Kaviani *et al*. (2019) studied the 286.47 mg/l 96-h LC_{50} of CuO-NPs for Caspian Trout. Jevgenij *et al.* (2013) studied LC_{50} values of 31 different types of nanoparticles in *D. rerio* and reported that the CuO-NPs has 400 mg/l 96-h LC_{50}. Zhao *et al*. (2011) determined the toxicity of CuO-NPs and bulk particles (CuO-BPs) toxicity at the different levels (10, 50, 100, 200, 300, 500, and 1000 mg/L), showing that the death rates at all exposure levels were less than 30%, which recommended that CuO-NPs up to 1000 mg/l had no evident acute toxicity for common carp juveniles. Furthermore, a study on the acute toxicity of CuO-NPs on rainbow trout (*O. mykiss*) at the concentrations of 1, 5, 20, and 100 mg/L showed no mortality (Khabbazi *et al*. 2015). According to the above mentioned data copper oxide nanoparticles had low acute toxicity to fish. The toxicity of metal oxide nanoparticles may change significantly in different fish species because of other conditions, such as exposure concentration and duration, the size of fish, the species own specific mechanisms for metabolism of metallic ions, individuals' physiological conditions, and water physicochemical parameters.

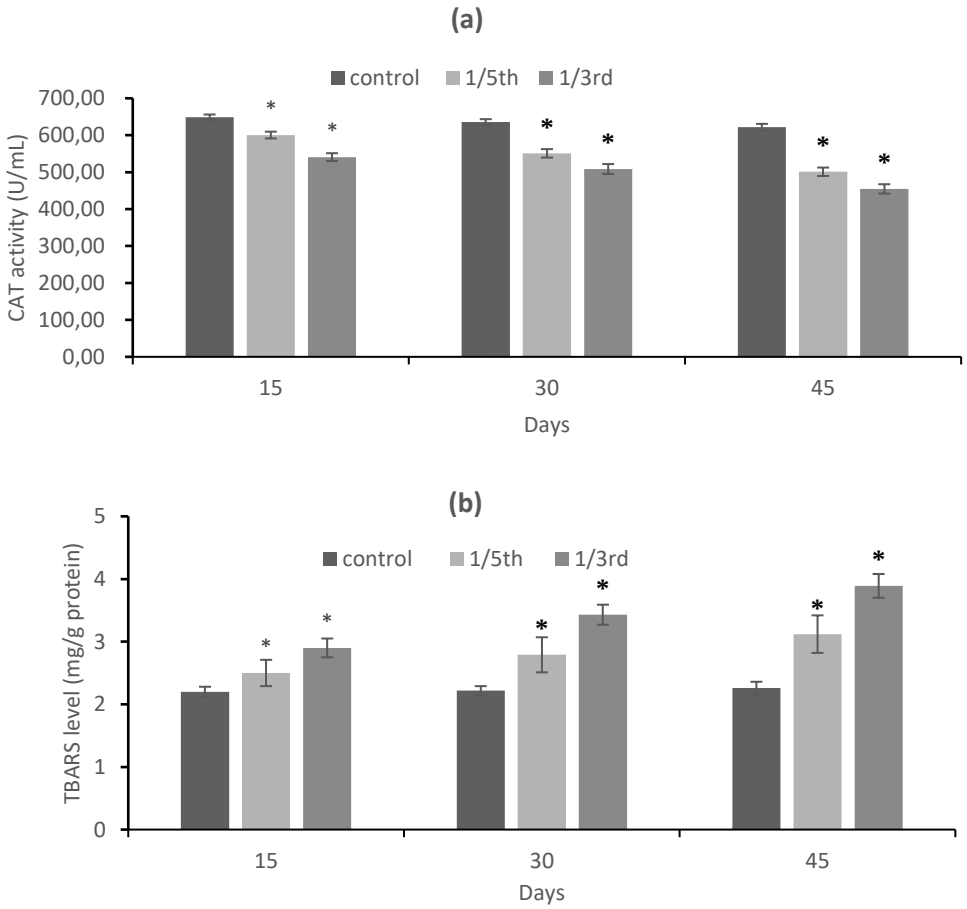

Figure 5. Changes in gills CAT activity (a) and TBARS level (b) of *Labeo rohita* exposed to 1/3rd and 1/5th of LC_{50} of CuO-NPs for the 15th, 30th and 45th day

The different sub-lethal concentrations of heavy metals (i.e., CuO and Ag nanoparticles) can accumulate in fish that are present at the top of the aquatic food chain from water, sediments and food (Torre *et al*., 2013), and also affect humans because fish is an important part of their diet (Al-Yousuf *et al*., 2000). Previously, uptake of various types of nanomaterials by fish species has been reported (Kashiwada, 2006; Moore, 2006). The uptake potency of nanomaterials by fish is one of the crucial factors in evaluating the nanoparticle's toxicity. Therefore, the accumulation level of metals is used as an indicator in different toxicological studies (Birungi *et al*., 2007). Studies from research works exhibited that the bioaccumulation of metallic substances in a tissues/organs is primarily dependent on exposure period and water concentrations of metals and also on several other environmental components such as salinity, pH, oxygen concentration, temperature, hardness, alkalinity and dissolved organic carbon (DOC) of water (Benaduce *et al.,* 2008; Linbo *et al*., 2009; Jitar *et al*., 2014). In *Labeo rohita,* the accumulation of copper oxide nanoparticles increases as dose and time increase.

Fish feeding habits, age and level of lipid contents in tissues could also significantly affect the bioaccumulation of metallic substances in fish (Eneji *et al*., 2011). Metallothioneins are proteins rich in cysteine with a low molecular weight that can selectively attach to heavy metals (Thirumoorthy *et al*., 2007) and their expression increases in fish species present in the polluted environment. These types of proteins attach generally to primary trace elements, such as Cu and Zn and xenobiotic (cadmium, lead, mercury, and nickel) metals, (Ling *et al*., 2016) which can disturb the internal homeostasis of the body. The level of metallothioneins has been projected to be an essential bio-marker of exposure to heavy metals in fish (Schmitt *et al*., 2007). The gills are in constant connection with water due to adherence ability more metal instead of penetrating into the body fluid, stick to the filaments of gills and showed increased metal accumulation in the gills than liver and muscles (Shahzad *et al*., 2017). The gill tissues are the primary target of nanoparticles (Trevisan *et al*., 2014). The assembled particles in tissues induced oxidative stress, which is the most crucial mechanism in nanoparticle toxicity (Nel *et al*., 2006). High reactiveness, and particular surface properties, enhance the capacity of NPs to create more reactive oxygen species by interacting with sub-cellular structures (Gomes *et al*., 2011). Analysis of enzyme activities has

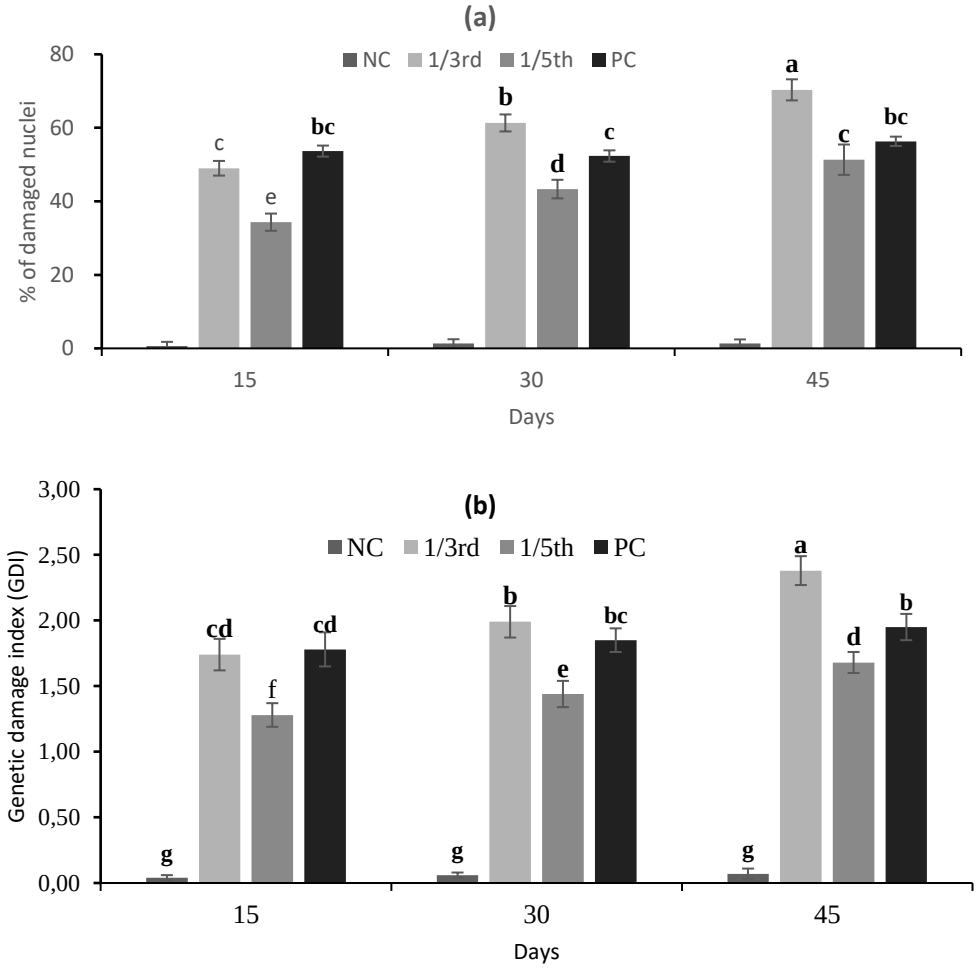

Figure 6. Percentage of erythrocytes with damaged nuclei (a) and genetic damage index (b) in the peripheral blood erythrocytes of *Labeo rohita* exposed to 1/3rd and 1/5th of LC_{50} of CuO-NPs for the 15th, 30th and 45th day

been projected as bio-markers of toxicants in environment because their disturbances indicate a reaction to water toxicants (Borkovic *et al*., 2005). It has been investigated that the existence of some metallic substances such as Fe and Cu in body systems can enhance the oxidative stress levels significantly (Pinto *et al*., 2003). All aquatic organisms have improved systems to defend against damage induced by reactive oxygen species. During the present study, a significant decrease in activity of CAT activity was observed through all periods. Gomes *et al*. (2011) found that Cu nanoparticles repressed the activity of CAT in *M. galloprovincialis* after 15-days exposure to sub-lethal concentrations of CuO-NPs. At the higher exposure concentrations, nano and bulk particles of silver causes concordant decrease in both CAT and SOD activities (Cozzari *et al*. (2015). This suppression may originate due to disequilibrium in reactive oxygen species and the cell antioxidant defensive system (Liu *et al*., 2012). The CAT activity reduction may also be due to the accumulation of free radicals like H_2O_2 (Choi *et al*., 2010). Villarreal *et al*., 2014 studied that engineered CuO-NPs can cause sub-lethal responses related to oxidative stress. Recently, it was shown that the initiation of oxidative stress is a main cause of toxicity related to these nanoparticles. Oxidative stress has also been identified as the origin of toxicity, gills structural damage, and mortality due to CuO-NP exposure in zebrafish (Griffitt *et al*., 2007). TBARS is one of the important symbols used to find the LPO levels in the animals (Ates *et al*., 2015). The outcomes of this research work showed a significant rise in levels of TBARS in the gill tissues of the groups exposed to the dispersion of copper oxide nanoparticles. Wang *et al*. (2012) recommended that exposure to NPs may trigger overproduction of reactive oxygen species within the tissue, which ultimately causes damage to the lipids. During the present study, *Labeo rohita* exhibited an increase in DNA damage in their peripheral erythrocytes with an increase in dose concentration and duration. The findings for the % of DNA damage in of *Labeo rohita* in this work are in concordance with those described in previous research work on other organisms, including *Oncorhynchus mykiss* (Isani *et al*., 2013), *Mytilus trossulus* (Mytilidae) (Chelomin *et al.,* 2017), *Escherichia coli* (Entrobacteriaceae) (Bondarenko *et al*., 2012), and *M. galloprovincialis* (Mytilidae) (Gomes *et al*., 2013). Whereas, there is not much data regarding the underlying mechanisms for genotoxicity of CuO-NPs in aquatic organisms, various possible models have established for class mammalia. Fish are the best accessible vertebrate model to calculate possible risks, because of their accumulation and metabolizing power in their bodies (Diekmann *et al*., 2004). Furthermore, the blood erythrocytes of fish are the most appropriate for analyses of DNA damage because they are nucleated and, therefore, most suited for finding nucleoids for SCGE (single cell gel electrophoresis) (Costa *et al*., 2011) since peripheral blood shows the broad health condition of the aquatic organism. Chelomin *et al*. (2017) described that possible genotoxic mechanisms of copper oxide nanoparticles may be due to a direct effect on the structure and repair system of DNA and indirect or intermediate induction mechanisms. Previous research work has observed that due to their increased surface area and small size, metal nanoparticles can reach to the cell nucleus by penetrating through membranes, react with it (Shukla *et al*., 2010; Chen and Mikecz, 2005) Therefore, further researches on other species are needed to compare the biochemical effects and physiological of NPs in the short and long-term exposure and to point out direct toxicity mechanism.

Conclusion

In this study, the bioaccumulation pattern, oxidative stress, and DNA damage were checked by waterborne exposure to *Labeo rohita*. The high concentration of CuO-NPs in aquatic environments can cause mortality in fish. Sublethal concentrations of CuO-NPs can induce significant bioaccumulation, oxidative stress and genotoxicity in fish. So, appropriate management of the aquatic ecosystem needs toxicity information of toxicants and these type of data can be helpful for proper management of all aquatic ecosystems and also future works.

Ethical Statement

All experiments were carried out by experts in accordance with the code of ethics. The protocols and procedures of this study were approved by the animal use and animal care committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan (DR/175, 05-04-2022).

Author Contribution

Sana Aziz conducted the experiment and wrote the manuscript. Sajid Abdullah helped in compiling the data and in writing the article. All authors have given their approval to the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest

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