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Evaluating toxicity of copper (II) oxide nanoparticles (CuO-NPs) through water borne exposure to tilapia (*Oreochromis mossambicus*) by tissue accumulation, oxidative stress, histopathology and genotoxicity

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Abstract:

Metal oxide nanoparticles are widely used in industries and peak level can be confirmed in their surroundings. In the present study the sub-lethal effects of CuO-NPs from low to high concentration as 0.5 mg/L to 1.5 mg/L were observed in tilapia (*Oreochromis mossambicus*). Accumulation of copper from CuO-NPs was increased with the increase in doses and maximum accumulation was found in gill than liver and muscles. The increased lipid peroxidation level was observed in gill as compared to liver and the similar results were obtained in catalase and glutathione while superoxide dismutase level was higher in liver than gills. In histological alterations, gills oedema, curved tips, fusion of gill lamellae and thickening of primary and secondary gill lamellae were observed. Necrosis and apoptosis with condensed nuclear bodies and pyknotic nuclei were observed in liver at highest dose concentration. In genotoxic study, the highest value of % tail DNA and olive tail movement was observed with increasing concentrations. Copper oxide nanoparticles has greater potential to accumulate in the soft tissues, which may causes respiratory distress such as oxidative stress, induction of anti-oxidant defense by raising glutathione, organ pathology and genotoxicity.

Keywords: eco-toxicity, fish, DNA breakage, histology

Introduction:

Nanotechnology is attracting worldwide attention and becoming leading edge in the area of research. It is exploring new phenomenon and theories in science, but also leading to industrial revolution, a driving force of economic growth and expected to become a trillion dollar industry in the next few years (Gerber and Lang, 2006). Nanoparticles are the product of nanotechnology help in solving problems like medicine, energy production and environmental sustainability. Due to their unique physical and chemical properties frequently pragmatic in food, cosmetics, agriculture chemicals and inputs, water purification, decontamination, textiles and electronics (Aitken *et al.,* 2006).

The production and application of nanoparticles on large scale in several industries led their release into the environment affecting various components of environmental biota (Bhatt and Tripathi, 2011; Moore, 2006). Their use in domestic appliances and household products create wastewater or effluents in the natural ecosystem produce environmental risks (Crane and Handy, 2007; Owen and Handy, 2007). Recent studies showed that exposure to nanoparticles can affect aquatic animals such as fish at cellular and molecular level (Chupani *et al.,* 2017; Chupani *et al.,* 2018a).

Copper oxide nanoparticles (CuO-NPs) various uses such as catalysts, gas sensor, heat transfer fluids, microelectronics and cosmetics (Chang *et al.,* 2005; Zhou *et al.,* 2006). Due to extensive use, the toxicity of copper oxide nanoparticles is increasing as compared to other metal oxides which results potential danger in natural environment (Buffet *et al.,* 2011).Nanoparticles are more toxic to their bulk ionic counterparts due to high surface area and reactivity which tend to lead bioavailability and toxicity (Bhatt and Tripathi, 2011; Scown *et al.,* 2010).

Resultant by products of nanoparticles cause damage to aquatic organisms such as fish, bacteria, protozoans, crustaceans and algae where they accumulate and cause toxicity to them (Shaw and Handy, 2011). Nanoparticles are associated with the accumulation in the organs of aquatic animals and alter their physiological responses due to release into the water (Chupani *et al.,* 2018) Copper oxide nanoparticles (CuO-NPs) also show the toxic effects because it releases copper ion and nano-forms in in the aquatic environment which the fish exposed and get harm to these (Gomes *et al.,* 2011).

Studies have been conducted to consider the accumulation of CuO-NPs in vertebrates and invertebrates. Shaw *et al.* (2012) studied the accumulation of copper in rainbow trout (*Oncorhyncus mykiss*) treated with waterborne copper nanoparticles and copper sulphate. Gomes *et al.* (2012) worked on the accumulation and toxicity of CuO-NPs in the digestive glands of *Mytilus galloprovincialis* explaining the accumulation and susceptibility of digestive glands to copper nanoparticles. Wang *et al.,* (2014) discovered the potential toxicity and accumulation of copper nanoparticles and copper sulphate on grouper (*Epinephelus coioides*) juvenile. Similarly Zhao *et al.,* (2011) also studied the distribution of CuO-NPs in juvenile carp (*Cyprinus carpio*) and their potential toxicity. Copper had more efficiency to internalize fish tissues elaborate haematological and histological alterations (Abdel-Khalek *et al.,* 2016)

Ahamed *et al.* (2010) assessed genotoxic, cytotoxic and oxidative stress in human lung epithelial cells exposed copper nanoparticles. Shaw *et al.* (2012) studied oxidative stress induced by copper nanoparticles and copper sulphate. Hu *et al.* (2014) elaborated the oxidative damage in blue mussel (*Mytilus edulis*). Another study conducted by Gomes *et al.* (2012) also revealed the oxidative damage in the digestive glans of *Mytilus galloprovincialis*. CuO-NPs also induce oxidative stress and cytotoxicity in airway epithelial cells in human (Fahmy and Cormier, 2009).

CuO-NPs create pathological changes in different organs of fish. Al-Bairuty *et al.* (2013) found pathological alterations in the gills, gut, liver, kidney, brain and muscles of juvenile rainbow trout (*Oncorhyncus mykiss*) exposing them to waterborne copper nanoparticles and copper sulphate. Dietary copper exposure also showed the same pathological alterations in Nile tilapia (*Oreochromis niloticus*) (Shaw and Handy, 2006). CuO-NPs releases more copper in aquatic media, when Nile tilapia (*Oreochromis niloticus*) was exposed to waterborne copper histopathological alteration in liver and gill epithelium were observed (Figueiredo-Fernandes *et al.,* 2007). Wang *et al.* (2015) also studied pathological alterations in the liver and gill of juvenile *Epinephelus coioides*. CuO-NPs have potential toxic effects on the development of zebrafish embryos (Bai *et al.,* 2010). Comet assay is one of the first developed method in assessing DNA strand breakage in neutral and alkaline conditions (Karlsson, 2010). CuO-NPs are the most potent to induce cytotoxicity and DNA damage, induce genotoxicity by damaging the DNA strands (Karlsson *et al.,* 2008).

The goal of present study was to determine the toxicological effects of water borne copper oxide nanoparticles (CuO-NPs) exposure to tilapia (*Oreochromis mossambicus*) and resulted changes with the uptake of these materials in the tissues including bioaccumulation, oxidative stress, histopathological alterations and genotoxicity.

Material and Methods

Copper oxide nanoparticles

CuO-NPs 50<nm were purchased from Sigma-Aldrich Co. LLC GmbH. Germany in the form of nano powder. The shape and surface area were determined by using ESEM (Model: EFI ESEM XL30 Philips). Fig.1 showing elliptical shape and very fit to the nano-scale with average size of 47 nm. Microphotographs were taken at 20000 and 120000 folds with 20 kv power supply.

Animal collection and placement:

Tilapia (*Oreochromis* mossambicus) were collected from aquaculture ponds at Pattoki District Kasur, Pakistan by the ethical permission of ORIC (Office of Innovation and Commercialization), University of the Punjab. Animals were sorted out with average weight of 22.9 ± 0.37 g and size 9.4 ± 0.2 cm. About 150 fish were placed into plastic bags having freshwater and oxygen was diffused into water using oxygen cylinder pipe with no mortality during transportation. Animals were placed in rectangular water glass tanks fitted with aerators and aquarium heaters to maintain oxygen and temperature level. Fish were acclimatized for seven days in the water glass tanks before the start of experiment as described in one of previous study by Shahzad *et al.* (2017).

Experiment Design

Animals were graded into ten experimental water glass tanks (12 fish/tank) with triplicate having dimensions 45.72 x 60.96 x 45.72 cm for 14 days after acclimatization in a semi-static system. Commercial food containing 35% crude protein, 4% crude fats, 5% crude fibre and 12% moisture was given to fish twice a day. Stock solution of CuO-NPs was prepared in Milli Q water by means of sonication. CuO-NPs were sonicated for 30 minutes at 40 KHz frequency in a sonicator (WUC-A06H). Three treatments identified as T1 (0.5 mg/L), T2 (1.0 mg/L) and T3 (1.5 mg /L) of were applied to separate tanks and one control having no CuO-NPs with 3 tanks as replicates per treatment. While exposing to CuO-NPs, the fish were not fed to reduce the adherent of nanoparticles to food. Water was changed each day before the treatment. About 80% of the water along with animals waste were taken out of each tank with the help of a suction pump. Fresh water was then added to the water glass tanks. CuO-NPs were again sonicated and administrated into the glass tank water. The volume of water in each glass tank was 40 litres.

At the end of 14th day animals were taken out one by one into smaller water container. To anesthetize 3 to 4 drops of clove oil were added. Blood was collected into EDTA vials by means of BD syringes from dorsal aorta to assess genotoxicity via comet assay. Animals were slaughtered peacefully and humanely to expose visceral organs. Gills, liver and muscles were excised with the help of scissors. Excised organs were placed in plastic bottles at -20ºC for bioaccumulation and oxidative stress enzymatic and non-enzymatic assessment. For histology tissues were fixed in Bouin's fixative in small glass vials. This experiment design follows as previously described by Shahzad *et al.* (2017).

Water Quality/Physicochemical Parameters

Physicochemical parameters such as temperature and dissolved oxygen (DO) were measured with the help of pro 20 DO meter purchased from Xylem Analytics (YSI), pH was measured by a pH meter (Hoelzle and Chelius 1687) and conductivity and TDS were measured by JENCO conductivity meter. Titration based standard APHA (2005) protocols were followed for p-Alkalinity, total alkalinity, Ca-hardness, total hardness and chlorides. Brief descriptions of these methods are given as follows as previously described by Shahzad *et al.,* (2017)

p-Alkalinity

25 ml water sample was taken in conical flask added 2 drops of phenolphthalein indicator. Stirred it and titrated it with 0.02N H_2SO_4until pink colour disappeared which was the end point for p-alkanility.

$$\text{p-Alkalinity mg/l} = \frac{\text{ml } H_2SO_4 \text{ used x } 1000}{\text{ml water sample}}$$

Total alkalinity:

Titrated sample p-alkalinity further titrated with 0.02N $H_2SO_4$with added 2 drops of mixed indicator. Titrated it until brick red colour appeared.

$$\text{Total alkalinity mg/l} = \frac{\text{ml } H_2SO_4 \text{ used x } 1000}{\text{ml water sample}}$$

Ca-hardness

25 ml water sample was taken in conical flask added 1 ml NaOH for producing pH 12-13. Stirred and added 0.1 gm indicator powder. Titrated it with EDTA with proper stirring to get the proper end point.

$$\text{Calcium Hardness mg/l } CaCO_3 = \frac{\text{ml EDTA used x } 1000}{\text{ml water sample}}$$

Total hardness

25 ml water sample was diluted to about 100 ml with distilled water added 1-2 ml buffer solution to adjust the pH 10-10.1 then dry powder indicator was added. Titrated it with 0.01 M EDTA solution until blue colour appeared.

$$\text{Total Hardness as mg/l } CaCO_3 = \frac{\text{ml EDTA used x } 1000}{\text{ml water sample}}$$

Chlorides

Took 25 ml water sample in a conical flask. Added 1 ml K_2CrO_4as indicator solution. Titrated it with standard Silver Nitrate solution to brick red end point.

$$\text{Chloride mg/l} = \frac{\text{ml } AgNO_3 \text{ x Normality of } AgNO_3 \text{ x } 35460}{\text{ml water sample}}$$

Sample Preparation for Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

One gram of freeze dried samples of gills, liver and muscles were separately taken in digestion flasks to each of which about 10 ml concentrated nitric acid (HNO_3) and 2ml perchloric acid ($HClO_4$) were added. The contents were than heated on a hot plate in a fume hood at 100^oC until the yellow acid digested colour was disappeared. Two drops of hydrogen peroxide were added. Each digested sample was evaporated to 2ml, cooled and diluted with distilled water to 50 ml and filtered with Whattman filter paper. These samples were analysed by using inductively

coupled plasma mass spectrometry (ICP-MS) (APHA, 2005) as previously described by Shahzad *et al.*, (2017).

Biochemical Assay:

Homogenate Preparation:

The samples of gills and liver were excised from each fish, washed with buffer and then soaked in 10% homogenate in 0.1 M phosphate buffer (pH 7.4) in a Teflon tissue homogenizer. The homogenates were centrifuged at 10,000 rpm for 10 min at 4˚C. After centrifugation, the supernatant from each sample was collected and stored in a freezer immediately.

Estimation of Lipid Peroxidation (LPO):

Lipid peroxidation (LPO) was estimated in the freshly prepared homogenate by measuring the formation of thiobarbiturinc acid reactive substances (TBARS) and quantified as MDA equivalents as described by Buege and Aust (1978).

Estimation of Catalase (CAT):

Catalase (CAT) was analysed by following the protocol of Claiborne (1985). The reaction mixture containing 100 µL of sample with 1.90 mL of potassium phosphate buffer (50 mM, pH 7.0) with a final volume of 3.0 mL. The reaction was initiated by the addition of 1 mL of hydrogen peroxide (H_2O_2). The solution was read at 240 nm for 3 min at an interval of 30 seconds.

Estimation of Superoxide Dismutase (SOD):

Superoxide dismutase (SOD) was analysed as described by Marklund and Marklund (1974). The method was based on the ability of superoxide dismutase to inhibit the auto-oxidation of pyrogallol. The reaction mixture in a final volume of 3.0 ml containing100 µL of sample with 2.80 mL of tris-succinate buffer (0.05M, pH 8.2) was incubated at 25°C for 20 min. The reaction was initiated by the addition of 100 µL of 8 mM pyrogallol, and the change in absorbance was

measured at 412 nm for 3 min with an interval of 30 seconds. The activity was measured in units per milligram of protein.

Estimation of Glutathione (GSH):

Glutathione (GSH) was estimated by following the protocols of Jollow *et al.* (1974). Each homogenate and sulfosalicylic acid were taken in equal volumes, mixed and incubated at 4˚C for 1 hour followed by centrifugation at 12,000 rpm for 15 minutes at 4˚C. Each supernatant (0.4 mL) was taken and mixed with 2.2 mL of potassium phosphate buffer (0.1 M, pH 7.4). The reaction was initiated by the addition of 0.4 ml DTNB (5,5'-dithiobis-2-nitrobenzoic acid) and the contents were read at 412 nm within 30 seconds.

Histology:

For histology gills and liver tissues were processed as described by Humason (1979).

The Comet Assay:

The alkaline comet assay procedure was used as described by Singh *et al*. (1988). Microscopic slides were stained with ethidium bromide. The slides were examined with fluorescence microscope at 400 magnifications. Microscopic images of the comets were scored using Comet IV Computer Software (Chaubey, 2005).

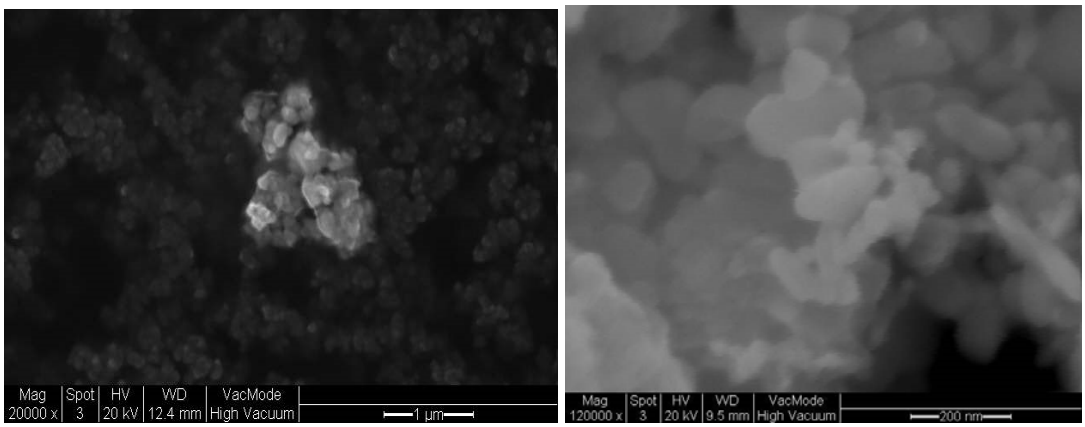
Statistical Analysis:

The data from bioaccumulation, biochemical and comet assays were analysed using Minitab Version 17. The effects of glass tanks were not observed as those were used as replicates during the treatments which were compared in statistical analysis. Analysis of variance (ANOVA) was applied using Tukey's test at 95% level of significance to compare means at $P<0.05$. The histological parameters were not statistical analysed. Instead those were visually examined to observe any potential variations between treatments.

Results:

Copper oxide nanoparticles

Fig.1 (a) and (b) showed the ESEM images of the copper oxide nanoparticles (CuO-NPs). The shapes of the nanoparticles were spherical to elliptical with the average size of 47 nm. The data supports the specification given by the Sigma-Aldrich.



(a) **(b)**

Fig.1 (a) and (b) showing the ESEM images of copper oxide nanoparticles (CuO-NPs) at 20000 and 120000 magnification.

Water quality/physicochemical parameters

Table 1 shows mean values of temperature, pH, dissolved oxygen (DO), conductivity, total dissolved solids (TDS), carbon dioxide (CO_2), p-alkalinity, total alkalinity, Ca-hardness, total hardness of water that was used in this study as previously presented by Shahzad *et al.,* (2017).

Physicochemical parameters	**Present study**
Temperature	27.997 ± 0.0606 ˚C
pH	7.7500 ± 0.0306
Dissolved Oxygen (DO)	7.00 ± 0.153 mg/L
Conductivity	395.67 ± 1.86 µS/m
Total Dissolved solids (TDS)	333.47 ± 1.68 mg/ L
Carbon Dioxide (CO_2)	0.00 ± 0.00 mg/ L

p-Alkalinity	8.677 ± 0.145 mg/ L
Total Alkalinity	202.67 ± 1.20 mg/ L
Ca-Hardness	35.0 ±0.577 mg/ L
Total Hardness	51.667 ± 0.882 mg/L
Chloride	25.00 ± 0.577 mg/ L

Table: 1. Mean (± S.D) values of various physicochemical parameters. *n=3*

Bioaccumulation of CuO-NPs:

The high accumulation of CuO-NPs in the gills, liver and muscles of studied fish (*Orochromis mossambicus*) was observed with the increase in dose concentration. From the studied tissues, the maximum Cu from CuO-NPs was observed in the gills of fish as compared to liver and muscles (Table 2) and the values at highest dose (1.5 mg/L) were 0.9567 ± 0.01528 ppb. The accumulation of Cu show no significant difference between the gills and muscles at high dose. Significant difference was observed in Cu accumulation in liver at higher dose of CuO-NPs and the mean value was Cu was 0.6833 ± 0.0115 ppb. The observed values of Cu accumulation in muscles at various doses of CuO-NPs were as T1 (0.633 ± 0.0208 ppb), T2 (0.6733 ± 0.0208 ppb) and T3 (0.9533 ± 0.0379 ppb) as compared to the control T0 (0.6233 ± 0.0058 ppb). While the lowest concentration was observed in liver such as at T1 (0.9267 ± 0.0153 ppb), T2 (0.7400 ± 0.0100 ppb) and T3 (0.6833 ± 0.0115 ppb) then the control T0 (2.6233 ± 0.0153 ppb). A decreasing trend of Cu accumulation had been observed in liver with the increasing concentration of CuO-NPs (Table 2). The order of Cu from CuO-NPs accumulation in soft tissues of fish was gills > muscles > liver.

Tissues	Treatments			
	T0 (0 mg/L)	T1 (0.5 mg/L)	T2 (1.0 mg/L)	T3 (1.5 mg/L)
Gills	0.5133 ± 0.0153^{h}	0.7133 ± 0.0058^{de}	0.8233 ± 0.0252^{c}	0.9567 ± 0.01528^{b}
Liver	2.6233 ± 0.0153^{a}	0.9267 ± 0.0153^{b}	0.7400 ± 0.0100^{d}	0.6833 ± 0.0115^{ef}
Muscles	0.6233 ± 0.0058^{g}	0.633 ± 0.0208^{fg}	0.6733 ± 0.0208^{efg}	0.9533 ± 0.0379^{b}

Table 2: Mean (± S.D) Cu (ppb) accumulation from CuO-NPs in gills, liver and muscles of fish. Values showing different ^{abc} superscripts in each row were significantly different ($p<0.05$). $n=7$

Oxidative Stress:

Table 3 showed data of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) and lipid peroxidase (LPO) in the gill and liver of tilapia (*Oreochromis mossambicus*). There was an increase in the amount of CAT, SOD and LPO in the gills as compared to the liver where GSH was more observed in liver with the increasing concentration of CuO-NPs treatments. In present study CuO-NPs generated reactive oxygen species (ROS) and free radicals from CuO-NPs held responsible for lipid, protein and DNA damage. This rise and fall in these enzymatic and non-enzymatic biomarkers was due to carbonylation and peroxidation by free radicals and generation of ROS production in result of metal toxicity (Tabrez and Ahmad, 2011).

The amount of catalase (CAT) increased with the increasing concentration of CuO-NPs in gills as compared to the liver where a decrease in its amount had been observed. The mean CAT level was 12.6670 ± 0.1530 U/mg in gills and 3.7667 ± 0.1155 U/mg in liver as compared to the mean control 2.8667 ± 0.0577 U/mg in gills and 5.2667 ± 0.1528 U/mg in liver. Superoxide dismutase (SOD) also showed the same increasing trend in gills as compared to the liver where mean SOD was 9.3333 ± 0.1155 U/mg in gills and 5.2333 ± 0.1155 U/mg in liver as compared to the mean control 6.8000 ± 0.1000 U/mg in gills and 5.7667 ± 0.1528 U/mg in liver, therefore,

there was no significant difference had been found in liver at high concentration when compared to mean control. Glutathione (GSH) had been more observed in liver at higher concentration where mean GSH level in gills was 1.3167 ± 0.0153 U/mg and 2.3330 ± 0.2080 U/mg. Lipid peroxidation (LPO) amount in gills showed more as compared to the liver where mean LPO in gills was 6.7000 ± 0.2000 nmol/mg and 3.2333 ± 0.1528 nmol/mg in liver had been observed.

Enzymes	Tissues	Treatments			
		T0 (0 mg/L)	T1 (0.5 mg/L)	T2 (1.0 mg/L)	T3 (1.5mg/L)
CAT U/mg	Gills	2.8667 ± 0.0577^{h}	8.2667 ± 0.1528^{c}	$10.633 \pm 0.0.153^{b}$	12.6670 ± 0.1530^{a}
	Liver	5.2667 ± 0.1528^{d}	4.7667 ± 0.1528^{e}	4.1667 ± 0.1528^{f}	3.7667 ± 0.1155^{g}
SOD U/mg	Gills	6.8000 ± 0.1000^{c}	6.5000 ± 0.0577^{c}	7.5667 ± 0.1528^{b}	9.3333 ± 0.1155^{a}
	Liver	5.7667 ± 0.1528^{d}	3.7333 ± 0.1528^{g}	4.3000 ± 0.2000^{f}	5.2333 ± 0.1155^{e}
GSH U/mg	Gills	3.1667 ± 0.1528^{b}	2.5633 ± 0.0252^{cd}	1.7267 ± 0.0321^{e}	1.3167 ± 0.0153^{f}
	Liver	3.7667 ± 0.1155^{a}	3.1330 ± 0.1528^{b}	2.7333 ± 0.1528^{c}	2.3330 ± 0.2080^{d}
LPO nmol/mg	Gills	0.4333 ± 0.1528^{g}	2.6333 ± 0.1528^{d}	4.8667 ± 0.0577^{b}	6.7000 ± 0.2000^{a}
	Liver	0.8333 ± 0.0577^{g}	1.5667 ± 0.1528^{f}	2.1667 ± 0.1528^{e}	3.2333 ± 0.1528^{c}

Table 3: Mean (± S.D) values of different enzymes in various fish tissues. Values with different ^{abc} superscripts were significantly different ($p<0.05$). $n=7$

Histology:

Histological alterations were observed in the gills and liver of tilapia (*Oreochromis mossambicus*) shown in Fig. 2 and Fig. 3.Fig.2 (a) reference control showing normal arrangement of primary and secondary gill lamellae. Fig.2 (b) to (d) reference treated with CuO-NPs which varied from the reference control showing alterations in the arrangement and distribution of primary and secondary gill lamellae, oedema and curved tips.

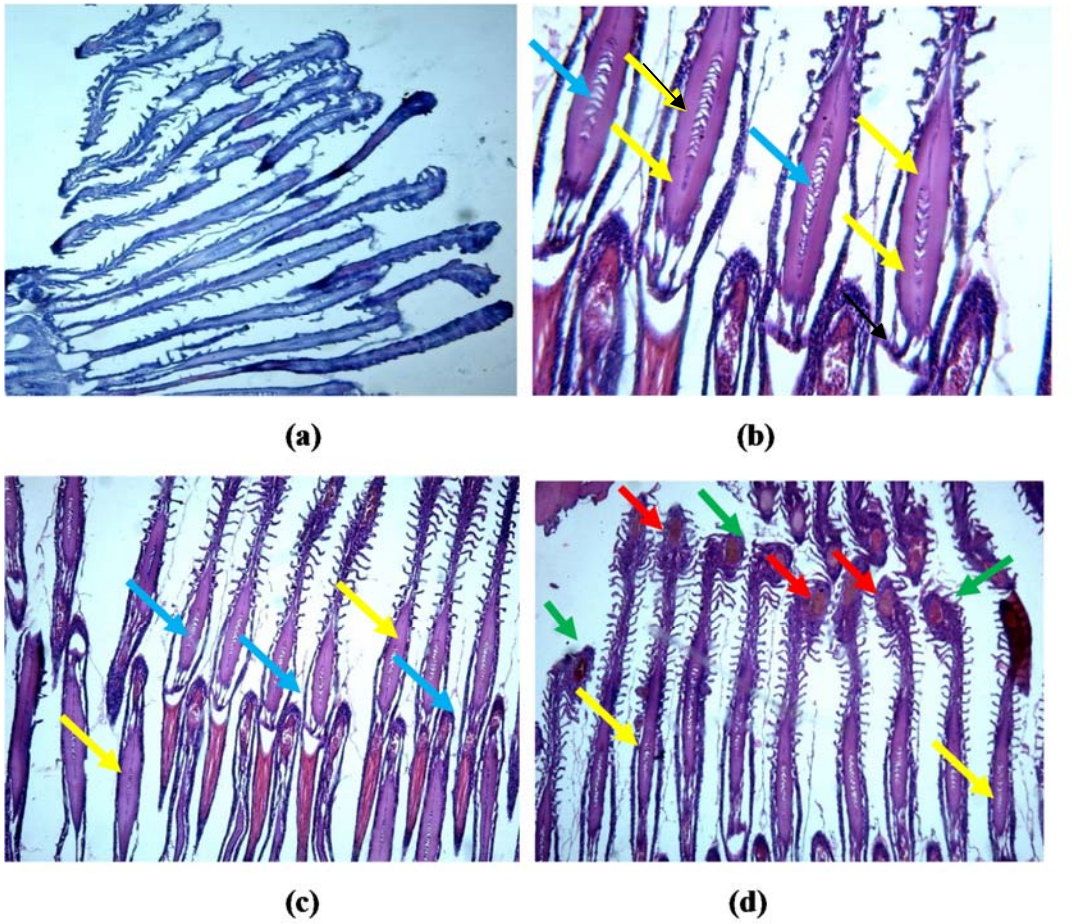


Fig.2 a-d Sections about 5 µm of reference and treated fish gills. (a) The gill of control fish showing normal arrangement of primary and secondary gill lamellae. (c) to (d) The reference treated gill were showing oedema (red arrows), curved tips (green arrows), fusion of gill lamellae (blue arrows) and thickening of primary and secondary gill lamellae (yellow arrows).

The liver histology shown alterations in the hepatic cells as compared to the reference control Fig.3. (a) reference control liver histology whereas, (c) to (d) elaborated the necrosis, apoptosis with condensed nuclear bodies. More apoptosis was observed with large amount of nuclei aggregation in cluster form. Pyknotic nuclei and cells having oedema were also observed.

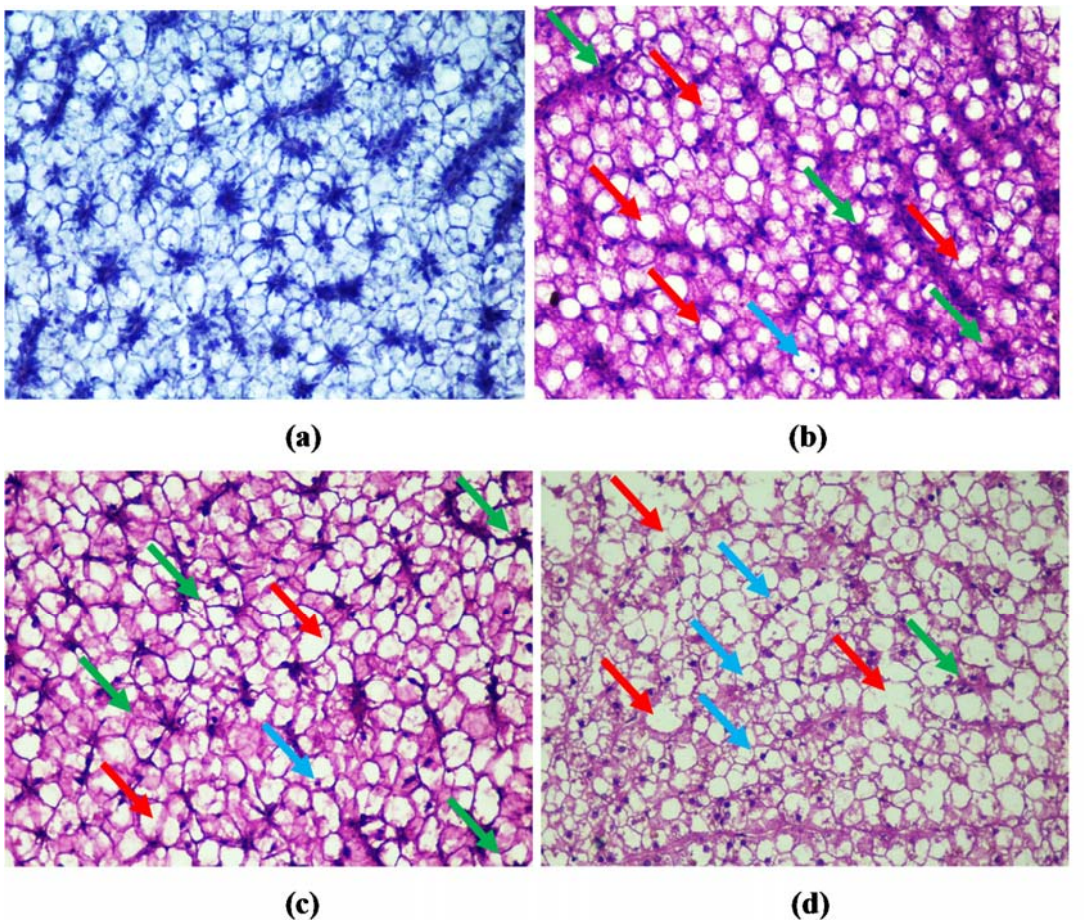
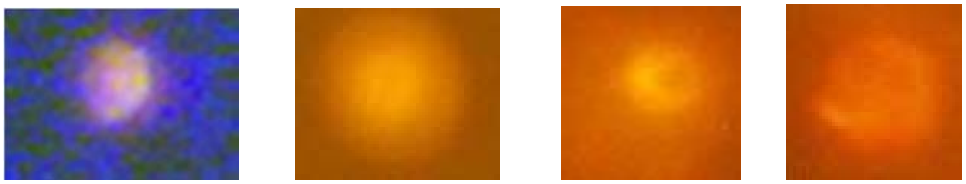


Fig.3 a-d Sections about 5 µm of reference and treated fish liver. (a) The liver of control fish showing normal arrangement and distribution of hepatocytes. (c) to (d) Reference treated liver showing necrosis and apoptosis with condensed nuclear bodies (green arrows), pyknotic nuclei (blue arrows) and oedema (red arrows).

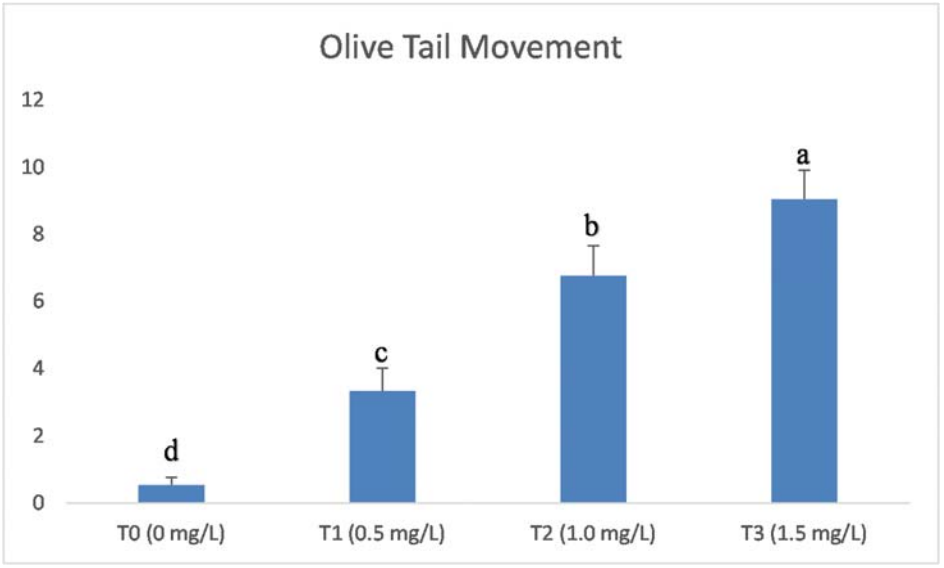
Comet Assay:

Alkaline comet assay was performed to measure the potential of CuO-NPs to induce DNA damage to erythrocytes of fish (*Oreochromis mossambicus*) (Fig.4). DNA damaged increased with the increasing concentration of CuO-NPs exposure as compared to the control. % Tail DNA maximum observed as 17.184 ± 1.271 at high concentration of CuO-NPs as compared to the control 2.630 ±0.938 showing significant difference. Significant difference has been observed

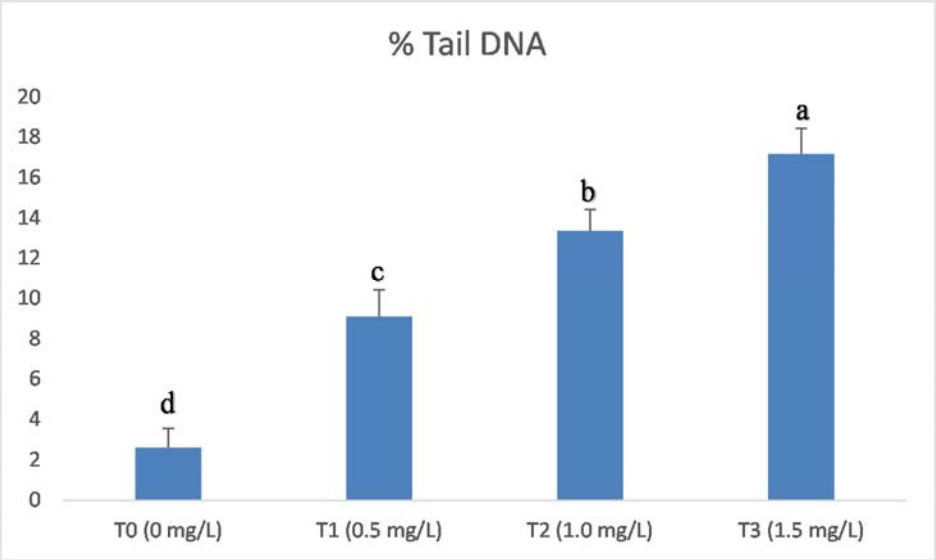
throughout increase in CuO-NPs dose concentration from T0 (0 mg/L), T1 (0.5 mg/L) T2 (1.0 mg/L) and T3 (1.5 mg/L). Similarly olive tail movement was observed maximum with the increasing concentration of CuO-NPs. Significant difference was found among all the treatments where the olive tail movement was observed maximum 9.052 ± 0.860at high concentration T3 (1.5 mg/L) as compared to the control 0.5413 ± 0.2588.



(a) **(b)** **(c)** **(d)**



(e)



(f)

Fig.4 Genotoxicity of CuO-NPs in erythrocytes. Comet assay: (a) Reference Control olive (b) to (d) Reference treated comet. Olive tail movement (e) and percentage of tail DNA (f). Data are expressed as mean ± S.D ($p<0.05$)

Discussion:

In the present study copper was found to be accumulated maximum in the gills of tilapia as 0.96 ± 0.015 ppb. Shaw *et al.* (2012) resulted more Cu accumulation in gill and intestine as compared to spleen, brain and muscles of rainbow trout (*Oncorhyncus mykiss*) by elaborating the accumulation and physiological effects of waterborne copper nanoparticles and copper sulphate. Another study conducted by Griffitt *et al.* (2009) while exposing zebrafish gill with copper and silver nanoparticles concluded with the result that the gill was more susceptible to copper and silver nanoparticles. The same results have been found during the present study where more copper is accumulated in the gill of tilapia which might because gills are in direct contact with aquatic media. Shaw and Handy (2007) exposed the Nile tilapia (*Oreochromis niloticus*) to diet borne copper resulted maximum copper accumulation in liver as compared to the gill and intestine. Mansouri *et al.*, (2016) came up with the results that more copper was found in liver as compared

to the gills, muscles and intestine of common carp (*Cyprinus carpio*) while co-exposing it with titanium and copper nanoparticles. The distribution of Cu^{+2}during the study of potential toxicity and distribution of CuO nanoparticles in juvenile carp (*Cyprinus carpio*) had been more observed in intestine than the gill, muscles, skin and scales, liver and brain (Zhao *et al.,* 2011), whereas Abdel-Khalek *et al.,* (2016) made the same observation where more Cu was accumulated in the liver as compared to the kidney, gills, skin and muscles which are different from the present study where more Cu was accumulated in the gills as compared to the liver and muscles. In another previous study, the freshwater mussels were exposed to various doses of metals and more accumulation in soft tissues was observed as the dose was increased (Sohail *et al.,* 2016).

Lipid peroxidation (LPO) activity during this study was found to be high in the gills of present fish as 6.7 ± 0.2 nmol/mg than liver (Table 3). It is proposed that the oxidative stress a common process of cell damage induced by different types of nanoparticles (Stone *et al.,* 2007). We may hypothesized that the toxicity induced by CuO-NPs exposure to fish in our study could be mediated by the generation of oxidative stress in them. Most of the metal oxides nanoparticles had potential ability to induce oxidative stress by inducing ROS viability. Cells respond to oxidative stress by enhancing their antioxidant defence mechanism in order to protect themselves from any oxidative damage. Therefore, it transpires if cells fail to neutralize the oxidative damage, protein oxidation, lipid peroxidation, DNA damage, mitochondrial perturbation and apoptosis occurs (Ramirez-Prieto *et al.,* 2006; Gutteridge, 1995 and Li *et al.,* 2003). Copper is known to exaggerate oxidative stress responses in fish (Ahmad *et al.,* 2005). Two reports showed that exposure of dietary copper induced lipid peroxidation in fish (grey mullet and Atlantic salmon) and hepatic fatty change in rainbow trout. (Barker *et al.,* 1998; Berntssen *et al.,* 2000 and Handy *et al.,* 1999). The TBARS assay measures the presence of lipid peroxidase and an increase in

TBARS has been observed in our case where more LPO level was observed in the gills as compared to the liver by MDA quantification. Similar results had been observed in the rainbow trout where more TBARS were found in gills (Shaw *et al.,* 2012). Hoyle *et al.* (2007) found the same results in African walking catfish while exposing with dietary copper.

In present study CuO-NPs mediated antioxidant activity showed elevated level of CAT 12.667 ± 0.153 U/mg as and SOD 9.33 ± 0.115 U/mg in gills as compared to liver. Antioxidant enzymatic parameters (Table 3) were showing the CuO-NPs induced toxicity. The activity of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were dependent on type and amount of stressor. According to Vutukuru *et al.* (2006) superoxide dismutase (SOD) lowers the superoxide radical (O_2^-) at cell level by converting it into H_2O_2, whereas, the catalase (CAT) breaks H_2O_2 that have the ability to penetrate into all the biomembranes and halt the activities of other enzymes. Wang *et al.* (2014) while exposing juvenile *Epinephelus coioides*to copper nanoparticles and copper sulphate where antioxidant enzymes activity more found in gills. Previously more CAT and SOD activity had been observed in digestive glands of *Mytillus galloprovincialis* by Gomes *et al.* (2012) while exposing the mussel with copper oxide nanoparticles. Glutathione (GSH) activity found to be maximum in liver as 2.33 ± 0.208 U/mg being central metabolic hub than gills. As glutathione is an important copper carrier and chelator of copper in cells (Ferreira *et al.,* 1993; Ferruzza *et al.,* 2000). The rise in glutathione level is due to copper regulation, whereas Berntssen *et al.,* (2000) observed a decrease in total glutathione level in gills and liver as compared to the intestine in rainbow trout. Hoyle *et al.* (2007) observed more GSH activity in intestine as compared to the gill.

Histological alterations can be observed in the gills of tilapia oedema, curved tips, fusion of gill lamellae and thickening of primary and secondary gill lamellae during exposure to CuO-

NPs in this study. Conversely liver histopathology revealed necrosis and apoptosis with condensed nuclear bodies, pyknotic nuclei, and oedema in figure 2 and 3. Al-Bairuty *et al* (2013) made the same observation in the gills and liver of rainbow trout while exposing them with copper nanoparticles and copper sulphate. Chen *et al.* (2006) studied in vivo toxicological effects of copper nanoparticles in the liver of mice made the same results. Figueiredo-Fernandes *et al.* (2007) studied the histopathological changes in the gills and liver of Nile tilapia (*Oreochromis niloticus*) and come up with the same histopathological alterations in the tissues. Wang *et al.,* (2015) by making histology as a biomarker to compare the toxic effects of copper nanoparticles verses copper sulphate on juvenile *Epinephelus coioides*, resulted same histopathological alterations in the soft tissues. Abdel-Khalek *et al.* (2016) also proposed the same alterations in the gills and liver of Nile tilapia. All these study were similar to the present study where we found histopathological changes in gill and liver. Chen *et al.* (2006) studied in vivo toxicological effects of copper nanoparticles in the liver of mice made the same results.

Gills are the primary site for gaseous exchange and liver is the main body metabolic organ, CuO-NPs induces a number of changes in their structure to which the functions alter. Therefore, oedema, curved tips, fusion of gill lamellae and thickening of primary and secondary gill lamellae showed permanent rupture in gill leading it to become non-functional and impaired gaseous exchange and reduced the uptake of oxygen for gaseous exchange (Abdel-Khalek, 2015). Liver being main detoxifying organ when come in contact with absorbed xenobiotics and lacerations often liked with aquatic pollutants (Velma and Tchounwou, 2010). The liver during the present study showed a number of alterations in connection with Singh *et al.,* (2008) where due to extensive necrosis and hypertrophy a rupture in the outer membrane of *Channa punctatus* liver resulting high metabolic activity in liver to which hepatocytes disappeared. Manahan (1991)

argued the results, the deteriorating necrosis is the result of damage in cellular membrane integrity and loss of proteins synthesis and carbohydrate metabolism.

CuO-NPs induced genotoxic potential resulted in DNA strand breakage like % tail DNA and olive tail movement (OTM) to erythrocytes of existing tilapia. Present study could be compare with similar results observed by Gomes *et al.* (2013) where genotoxicity by copper oxide and silver oxide nanoparticles in the mussel *Mytillus galloprovincialis* prompted rise in % tail DNA and OTM. Carmona *et al.,* (2015) observed the genotoxic effects of copper oxide nanoparticles in *Drosophila melanogaster* and found the same results, rise in % tail DNA and olive tail movement with increasing concentration of copper nanoparticles. In another previous study, freshwater mussels exposed for the various heavy metals in laboratory conditions and more values of DNA damage was observed in Cu-exposed mussels in comparison with other metals (Sohail *et al.,* 2016). Ahamed *et al.* (2010) found copper with potential genotoxic effects in human lung epithelial cells. Dai *et al.* (2013) studied the effects, uptake and depuration kinetics of silver and copper oxides nanoparticles in marine deposit feeder *Macoma balthica* where they did not find out the genotoxic effects of both silver and copper nanoparticles. Another study on the cytotoxicity and genotoxicity of copper oxide nanoparticles by Alarifi *et al.,* (2013) resulted in DNA damage to human skin keratinocytes cells. Genotoxic effects of CuO-NPs were studied in fruit fly *Drosophila melanogaster* (Carmona *et al.,* 2015).

Conclusion:

The present study determines the toxic effects of manufactured copper (II) oxide (CuO) nanoparticles to tilapia (*Oreochromis mossambicus*), but they are not lethal ranges from 0.5 mg/L to 1.5 mg/L. Therefore, a number of changes has been observed during this study. The highest Cu accumulation has been observed at highest dose 1.5 mg/L in gills. The oxidative stress which is

induced found more LPO, CAT, GSH and SOD with the increasing dose concentration. CuO-NPs generate a lot of damage to the tissues of fish. Genotoxicity is also observed in DNA damage to erythrocytes where % tail DNA and olive tail movement more observed at high dose. This study suggests that CuO-NPs are sub-lethal to aquatic organism ranges mentioned above which make them a threat to alter their structural and physiological characteristics.

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