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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-Khaled 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 -20°C 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$_2$SO$_4$ until pink colour disappeared which was the end point for p-alkanility.

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

**Total alkalinity:**

Titrated sample p-alkalinity further titrated with 0.02N H$_2$SO$_4$ with added 2 drops of mixed indicator. Titrated it until brick red colour appeared.
Total alkalinity mg/l = \( \frac{ml \ H_2SO_4 \ used \times 1000}{ml \ water \ sample} \)

**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.

Calcium Hardness mg/l CaCO\(_3\) = \( \frac{ml \ EDTA \ used \times 1000}{ml \ water \ sample} \)

**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.

Total Hardness as mg/l CaCO\(_3\) = \( \frac{ml \ EDTA \ used \times 1000}{ml \ water \ sample} \)

**Chlorides**

Took 25 ml water sample in a conical flask. Added 1 ml K\(_2\)CrO\(_4\)as indicator solution. Titrated it with standard Silver Nitrate solution to brick red end point.

Chloride mg/l = \( \frac{ml \ AgNO_3 \times \ Normality \ of \ AgNO_3 \times 35460}{ml \ water \ sample} \)

**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 (H\(_2\)NO\(_3\)) and 2ml perchloric acid (HClO\(_4\)) were added. The contents were then heated on a hot plate in a fume hood at 100°C 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 Whatttman 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 thiobarbituric 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 (H₂O₂). 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 containing 100 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.

![ESEM images of copper oxide nanoparticles (CuO-NPs)](image)

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 (CO₂), 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 (CO₂)       | 0.00 ± 0.00 mg/L  |
Table: 1. Mean (± S.D) values of various physicochemical parameters. \(n=3\)

| Parameter            | Value                      |
|----------------------|----------------------------|
| 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        |

**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.
### 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

| 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\(^b\) | 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\(^b\) |

**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 $1.3167 \pm 0.0153$ U/mg and $2.3330 \pm 0.2080$ U/mg. Lipid peroxidation (LPO) amount in gills showed more as compared to the liver where mean LPO in gills was $6.7000 \pm 0.2000$ nmol/mg and $3.2333 \pm 0.1528$ nmol/mg in liver had been observed.

| Enzymes | Tissues | Treatments |
|---------|---------|------------|
| CAT U/mg | Gills | T0 (0 mg/L) | T1 (0.5 mg/L) | T2 (1.0 mg/L) | T3 (1.5mg/L) |
|         | Liver  | 5.2667 ± 0.1528 | 4.7667 ± 0.1528 | 4.1667 ± 0.1528 | 3.7667 ± 0.1155 |
| SOD U/mg | Gills | 6.8000 ± 0.1000 | 6.5000 ± 0.0577 | 7.5667 ± 0.1528 | 9.3333 ± 0.1155 |
|         | Liver  | 5.7667 ± 0.1528 | 3.7333 ± 0.1528 | 4.0000 ± 0.2000 | 5.2333 ± 0.1155 |
| GSH U/mg | Gills | 3.1667 ± 0.1528 | 2.5633 ± 0.0252 | 1.7267 ± 0.0321 | 1.3167 ± 0.0153 |
|         | Liver  | 3.7667 ± 0.1155 | 3.1330 ± 0.1528 | 2.7333 ± 0.1528 | 2.3330 ± 0.2080 |
| LPO nmol/mg | Gills | 0.4333 ± 0.1528 | 2.6333 ± 0.1528 | 4.8667 ± 0.0577 | 6.7000 ± 0.2000 |
|         | Liver  | 0.8333 ± 0.0577 | 1.5667 ± 0.1528 | 2.1667 ± 0.1528 | 3.2333 ± 0.1528 |

**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 $17.184 \pm 1.271$ at high concentration of CuO-NPs as compared to the control $2.630 \pm 0.938$ 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 ± 0.860 at high concentration T3 (1.5 mg/L) as compared to the control 0.5413 ± 0.2588.
**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 $0.96 \pm 0.015$ 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 $6.7 \pm 0.2$ 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 (O2−) at cell level by converting it into H2O2, whereas, the catalase (CAT) breaks H2O2 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 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 Mytilus 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 *Mytilus 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.
References:

Abdel-Khalek, A. A. (2015). Risk assessment, bioaccumulation of metals and histopathological alterations in Nile tilapia (Oreochromis niloticus) facing degraded aquatic conditions. *Bulletin of Environmental Contamination and toxicology*, 94(1), 77-83.

Abdel-Khalek, A. A., Badran, S. R., & Marie, M. A. S. (2016). Toxicity evaluation of copper oxide bulk and nanoparticles in Nile tilapia, Oreochromis niloticus, using hematological, bioaccumulation and histological biomarkers. *Fish physiology and biochemistry*, 1-12.

Ahamed, M., Siddiqui, M. A., Akhtar, M. J., Ahmad, I., Pant, A. B., & Alhadlaq, H. A. (2010). Genotoxic potential of copper oxide nanoparticles in human lung epithelial cells. *Biochemical and biophysical research communications*, 396(2), 578-583.

Ahmad, I., Oliveira, M., Pacheco, M., & Santos, M. A. (2005). Anguilla anguilla L. oxidative stress biomarkers responses to copper exposure with or without β-naphthoflavone pre-exposure. *Chemosphere*, 61(2), 267-275.

Aitken, R., Chaudhry, M. Q., Boxall, A. B. A., & Hull, M. (2006). Manufacture and use of nanomaterials: current status in the UK and global trends. *Occupational medicine*, 56(5), 300-306.

Alarifi, S., Ali, D., Verma, A., Alakhtani, S., & Ali, B. A. (2013). Cytotoxicity and genotoxicity of copper oxide nanoparticles in human skin keratinocytes cells. *International journal of toxicology*, 32(4), 296-307.

Al-Bairuty, G. A., Shaw, B. J., Handy, R. D., & Henry, T. B. (2013). Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (Oncorhynchus mykiss). *Aquatic Toxicology*, 126, 104-115.

APHA (2005) Standard methods for the examination of water and waste water, 21st edn. American Public Health Association, Washington, DC
Bai, W., Tian, W., Zhang, Z., He, X., Ma, Y., Liu, N., & Chai, Z. (2010). Effects of copper nanoparticles on the development of zebrafish embryos. *Journal of nanoscience and nanotechnology, 10*(12), 8670-8676.

Baker, R. T. M., Handy, R. D., Davies, S. J., & Snook, J. C. (1998). Chronic dietary exposure to copper affects growth, tissue lipid peroxidation, and metal composition of the grey mullet, Chelon labrosus. *Marine Environmental Research, 45*(4), 357-365.

Berntssen, M. H., Lundebye, A. K., & Hamre, K. (2000). Tissue lipid peroxidative responses in Atlantic salmon (Salmo salar L.) parr fed high levels of dietary copper and cadmium. *Fish Physiology and Biochemistry, 23*(1), 35-48.

Bhatt, I., & Tripathi, B. N. (2011). Interaction of engineered nanoparticles with various components of the environment and possible strategies for their risk assessment. *Chemosphere, 82*(3), 308-317.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in enzymology, 52*, 302-310.

Buffet, P. E., Tankoua, O. F., Pan, J. F., Berhanu, D., Herrenknecht, C., Poirier, L., ... & Guibbolini, M. (2011). Behavioural and biochemical responses of two marine invertebrates Scrobicularia plana and Hediste diversicolor to copper oxide nanoparticles. *Chemosphere, 84*(1), 166-174.

Carmona, E. R., Inostroza-Blancheteau, C., Obando, V., Rubio, L., & Marcos, R. (2015). Genotoxicity of copper oxide nanoparticles in Drosophila melanogaster. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 791*, 1-11.

Chang, H., Jwo, C. S., Lo, C. H., Tsung, T. T., Kao, M. J., & Lin, H. M. (2005). Rheology of CuO nanoparticle suspension prepared by ASNSS. *Rev. Adv. Mater. Sci, 10*(2), 128-132.
Chaubey, R. C. (2005). Computerized image analysis software for the comet assay. *Molecular Toxicology Protocols*, 97-106.

Chen, Z., Meng, H., Xing, G., Chen, C., Zhao, Y., Jia, G., ... & Chai, Z. (2006). Acute toxicological effects of copper nanoparticles in vivo. *Toxicology letters*, 163(2), 109-120.

Chupani, L., Niksirat, H., Panáček, A., Lünsmann, V., Haange, S., Bergen, M., Jehmlich, N. & Zusková, E. (2018a). Insight into the modulation of intestinal proteome of juvenile common carp (*Cyprinus carpio* L.) after dietary exposure to ZnO nanoparticles. *Science of the Total Environment*, 613, 62-71.

Chupani, L., Niksirat, H., Velíšek, J., Stará, A., Hradilová, V., Kolařík, J., Panáček, A. & Zusková, E. (2018). Chromic dietary toxicity of zinc oxide nanoparticles in common carp (*Cyprinus carpio* L.): Tissue accumulation and physiological responses. *Ecotoxicology and Environmental Safety*, 147, 110-116

Chupani, L., Zusková, E., Niksirat, H., Panáček, A., Lünsmann, V., Haange, S., Bergen, M. & Jehmlich, N. (2017). Effects of chronic dietary exposure of zinc oxide nanoparticles on the serum protein profile of juvenile common carp (*Cyprinus carpio* L.). *Science of the Total Environment*, 579, 1504-1511.

Claiborne, A. (1985). Catalase Activity. In: *CRC handbook of methods in oxygen radical research* (ed. R. A. Greenland). Boca Raton, pp. 283-284.

Crane, M., & Handy, R. D. (2007). An assessment of regulatory testing strategies and methods for characterizing the ecotoxicological hazards of nanomaterials. *Report of Defra London UK*, 19, 286-91.
Dai, L., Syberg, K., Banta, G. T., Selck, H., & Forbes, V. E. (2013). Effects, uptake, and depuration kinetics of silver oxide and copper oxide nanoparticles in a marine deposit feeder, Macoma balthica. *ACS Sustainable Chemistry & Engineering, 1*(7), 760-767.

Fahmy, B., & Cormier, S. A. (2009). Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells. *Toxicology In Vitro, 23*(7), 1365-1371.

Ferreira, A. D. C., Ciriolo, M. R., Marcocci, L., & Rotilio, G. (1993). Copper (I) transfer into metallothionein mediated by glutathione. *Biochemical Journal, 292*(3), 673-676.

Ferruzza, S., Sambuy, Y., Ciriolo, M. R., De Martino, A., Santaroni, P., Rotilio, G., & Scarino, M. L. (2000). Copper uptake and intracellular distribution in the human intestinal Caco-2 cell line. *Biometals, 13*(2), 179-185.

Figueiredo-Fernandes, A., Ferreira-Cardoso, J. V., Garcia-Santos, S., Monteiro, S. M., Carrola, J., Matos, P., & Fontainhas-Fernandes, A. (2007). Histopathological changes in liver and gill epithelium of Nile tilapia, Oreochromis niloticus, exposed to waterborne copper. *Pesquisa Veterinária Brasileira, 27*(3), 103-109.

Gerber, C., & Lang, H. P. (2006). How the doors to the nanoworld were opened. *Nature nanotechnology, 1*(1), 3-5.

Gomes, T., Araújo, O., Pereira, R., Almeida, A. C., Cravo, A., & Bebianno, M. J. (2013). Genotoxicity of copper oxide and silver nanoparticles in the mussel Mytilus galloprovincialis. *Marine environmental research, 84*, 51-59.

Gomes, T., Pereira, C. G., Cardoso, C., Pinheiro, J. P., Cancio, I., & Bebianno, M. J. (2012). Accumulation and toxicity of copper oxide nanoparticles in the digestive gland of Mytilus galloprovincialis. *Aquatic toxicology, 118*, 72-79.
Gomes, T., Pinheiro, J. P., Cancio, I., Pereira, C. G., Cardoso, C., & Bebianno, M. J. (2011). Effects of copper nanoparticles exposure in the mussel Mytilus galloprovincialis. *Environmental science & technology, 45*(21), 9356-9362.

Griffitt, R. J., Hyndman, K., Denslow, N. D., & Barber, D. S. (2009). Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. *Toxicological Sciences, 107*(2), 404-415.

Gutteridge, J. M. (1995). Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical chemistry, 41*(12), 1819-1828.

Handy, R. D., Sims, D. W., Giles, A., Campbell, H. A., & Musonda, M. M. (1999). Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (Oncorhynchus mykiss) during chronic dietary exposure to copper. *Aquatic Toxicology, 47*(1), 23-41.

Hoyle, I., Shaw, B. J., & Handy, R. D. (2007). Dietary copper exposure in the African walking catfish, Clarias gariepinus: Transient osmoregulatory disturbances and oxidative stress. *Aquatic toxicology, 83*(1), 62-72.

Hu, W., Culloty, S., Darmody, G., Lynch, S., Davenport, J., Ramirez-Garcia, S., ... & Sheehan, D. (2014). Toxicity of copper oxide nanoparticles in the blue mussel, Mytilus edulis: a redox proteomic investigation. *Chemosphere, 108*, 289-299.

Humason, G.L. (1979). *Animal tissue technique*. 4th Edition. W.H.Freeman and Company, San Francisco, pp. 61.

Jollow, D. J., Mitchell, J. R., Zampaglione, N. A., & Gillette, J. R. (1974). Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology, 11*(3), 151-169.
Karlsson, H. L. (2010). The comet assay in nanotoxicology research. *Analytical and bioanalytical chemistry*, 398(2), 651-666.

Karlsson, H. L., Cronholm, P., Gustafsson, J., & Moller, L. (2008). Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. *Chemical research in toxicology*, 21(9), 1726-1732.

Li, N., Hao, M., Phalen, R. F., Hinds, W. C., & Nel, A. E. (2003). Particulate air pollutants and asthma: a paradigm for the role of oxidative stress in PM-induced adverse health effects. *Clinical Immunology*, 109(3), 250-265.

Manahan, S.E. (1991). Water pollution, environment chemistry, 1st edition, Lewis Publishers, London.

Mansouri, B., Maleki, A., Johari, S. A., Shahmoradi, B., Mohammadi, E., Shahsavari, S., & Davari, B. (2016). Copper Bioaccumulation and Depuration in Common Carp (Cyprinus carpio) Following Co-exposure to TiO2 and CuO Nanoparticles. *Archives of environmental contamination and toxicology*, 71(4), 541-552.

Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European journal of biochemistry*, 47(3), 469-474.

Moore, M. N. (2006). Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? *Environment International*, 32(8), 967-976.

Owen, R. & Handy, R.D. (2007). Formulating the problems for environmental risk assessment of nanomaterials. Environ. Sci. Technol., 41(16): 5582–5588.

Ramirez-Prieto, M. T., García-Río, F., & Villamor, J. (2006). [Role of oxidative stress in respiratory diseases and its monitoring]. *Medicina clinica*, 127(10), 386-396.
Scown, T. M., Van Aerle, R., & Tyler, C. R. (2010). Review: do engineered nanoparticles pose a significant threat to the aquatic environment? *Critical reviews in toxicology, 40*(7), 653-670.

Shahzad, K., Khan, M.N., Jabeen, F., Kosour, N., Sohail, M., Khan, M.K.A. & Ahmad, M. (2017). Bioaccumulation of manufactured titanium dioxide (TiO$_2$), copper oxide (CuO) and zinc oxide (ZnO) nanoparticles in soft tissues of tilapia (*Oreochromis mossambicus*). *Punjab University Journal of Zoology, 32*(2), 237-243.

Shaw, B. J., & Handy, R. D. (2006). Dietary copper exposure and recovery in Nile tilapia, *Oreochromis niloticus*. *Aquatic Toxicology, 76*(2), 111-121.

Shaw, B. J., & Handy, R. D. (2011). Physiological effects of nanoparticles on fish: a comparison of nanometals versus metal ions. *Environment International, 37*(6), 1083-1097.

Shaw, B. J., Al-Bairuty, G., & Handy, R. D. (2012). Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): physiology and accumulation. *Aquatic Toxicology, 116*, 90-101.

Singh, D., Nath, K., Sharma, Y. K., & Trivedi, S. P. (2008). Hepatotoxic effect of Cu (II) in freshwater fish, Channa punctatus: a histopathological study. *Res Environ Life Sci, 1*(1), 13-16.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental cell research, 175*(1), 184-191.

Sohail, M., Khan, M. N., Chaudhry, A. S., & Qureshi, N. A. (2016). Bioaccumulation of heavy metals and analysis of mineral element alongside proximate composition in foot, gills and mantle of freshwater mussels (*Anodonta anatina*). *Rendiconti Lincei, 27*(4), 687-696.
Sohail, M., Khan, M. N., Chaudhry, A. S., & Shahzad, K. (2016). Proximate composition and elemental analysis in soft tissues of freshwater mussels (Anodonta anatina) from the Chashma Lake, River Indus Pakistan. *Frontiers in Biology, 11*(4), 331-337.

Sohail, M., Khan, M. N., Qureshi, N. A., & Chaudhry, A. S. (2016). Monitoring DNA damaging in gills of freshwater mussels (*Anodonta anatina*) exposed to heavy metals. Pakistan journal of Zoology 49 (1).

Stone, V., Johnston, H., & Clift, M. J. (2007). Air pollution, ultrafine and nanoparticle toxicology: cellular and molecular interactions. *IEEE transactions on nanobioscience, 6*(4), 331-340.

Tabrez, S., & Ahmad, M. (2011). Components of antioxidative system in Allium cepa as the toxicity monitor of trichloroethylene (TCE). *Toxicological and Environ Chemistry, 93*(1), 73-84.

Velma, V., & Tchounwou, P. B. (2010). Chromium-induced biochemical, genotoxic and histopathologic effects in liver and kidney of goldfish, Carassius auratus. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 698*(1), 43-51.

Vutukuru, S. S., Chintada, S., Madhavi, K. R., Rao, J. V., & Anjaneyulu, Y. (2006). Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, Esomus danricus. *Fish Physiology and Biochemistry, 32*(3), 221-229.

Wang, T., Long, X., Cheng, Y., Liu, Z., & Yan, S. (2014). The potential toxicity of copper nanoparticles and copper sulphate on juvenile Epinephelus coioides. *Aquatic Toxicology, 152*, 96-104.

Wang, T., Long, X., Cheng, Y., Liu, Z., & Yan, S. (2015). A comparison effect of copper nanoparticles versus copper sulphate on juvenile Epinephelus coioides: growth parameters, digestive enzymes, body composition, and histology as biomarkers. *International journal of genomics, 2015*.
Zhao J, Wang Z, Liu X, Xie X, Zhang K, Xing B (2011) Distribution of CuO nanoparticles in juvenile carp (Cyprinus carpio) and their potential toxicity. J Hazard Mater 197:304–310

Zhou, K., Wang, R., Xu, B., & Li, Y. (2006). Synthesis, characterization and catalytic properties of CuO nanocrystals with various shapes. Nanotechnology, 17(15), 3939.