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RESEARCH ARTICLE

DNA Damage and Oxidative Stress in Economically Important Fish, Bighead Carp (*Hypophthalmichthys nobilis*) Exposed to Engineered Copper Oxide Nanoparticles

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A B S T R A C T

Copper oxide nanoparticles (CuO-NP) are widely studied nanoparticles due to their broad applications in various fields. Nonetheless, little data exists regarding their chronic toxicity to aquatic animals causing aquatic pollution. This study was designed to analyze the toxicological effect of different concentrations of CuO-NPs concentrations on the bighead carp (*Hypophthalmichthys nobilis*). They were synthesized using co-precipitation method and characterized by X-Ray diffraction, Fourier-transform infrared (FT-IR) and UV-visible spectroscopy that confirmed 32.84nm size, elemental composition and reduction level, respectively. The acute toxicity in terms of 96-h LC_{50} was determined using bighead carp as a model organism. After acute toxicity testing, DNA damage in blood peripheral erythrocytes of fish was observed by comet assay and oxidative stress in terms of superoxide dismutase (SOD), glutathione S-transferase (GST), catalase (CAT) and lipid peroxidation (LPO) levels were evaluated after chronic exposure of different concentrations ($1/3^{rd}$ and $1/5^{th}$ of 96-hLC_{50}) of CuO-NPs in gills, liver, heart and muscle tissues of bighead carp. Samplings were conducted after exposure periods of 15, 30, and 45 days. The significantly higher damaged nuclei (%) and genetic damage index were observed in bighead carp after $1/3^{rd}$ of exposure concentration than $1/5^{th}$ of 96h LC_{50}. Significant changes in enzyme activities and LPO level occurred regarding dose and time-dependent manner. The activity of SOD and GST increased for 30 days, followed by the decrease at both experimental doses. CAT activity significantly declined as exposure time was elevated, while the level of LPO also increased in various fish organs as compared to the control group. This study indicates that CuO-NPs have a stronger impact on fish and toxicity increases as exposure time and dose increases.

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INTRODUCTION

In the last few decades, nanotechnology has attracted the focus of biologists due to its use in biotechnology, medicine, environmental challenges, energy and space exploration, wastewater treatment, catalysts, fuel cells, biodetectors and for the environmental rectification (Aitken *et al*., 2006; Ahmad *et al*., 2021). Nanoparticles comprise sizes from 10-100nm (Khan *et al*., 2019), they have been in the biosphere for a considerably long period. In (2004), approximately 2000tonnes of nanoparticles were present, but now, they have increased to more than 58,000 tonnes estimated by 2020 (Nowack and Bucheli, 2007). Metal oxide nanoparticles may leak into natural aquatic habitats in their life cycles (production, consumption, storage and transportation). Various nanoparticles appear naturally by dissolution or attaining large size after particle aggregation in the environment. Contrary, the altered nanoparticles stabilized through fixing and capping factors and therefore, are more persistent than the natural ones.

Nanotoxicity investigation is gaining concern because NPs may contain hazardous elements (in concentrations or structural forms) that do not happen in nature and ultimately produce environmental hazards to aquatic biota (Canli *et al*., 2018). Exposures of NPs to aquatic

organisms have revealed possible associated ecological, food chain risks and generally have some inherent physiological toxicity for aquatic life (Banerjee *et al.*, 2014). Therefore, it remains pertinent that several aquatic organisms seem to have adapted well to survive with nanoparticles because their toxicity level depends on the configuration, overall size, size range distribution and composition, the properties of NPs (Khan *et al*., 2019). 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). Therefore, high rates of manufacturing and their widespread usage frequently result in bioaccumulation in the aquatic biota and ultimately incorporated in higher trophic levels (Canli *et al*., 2018; Tahir *et al*., 2021), with likely threats to non-target aquatic fauna. Cu nanoparticles caused mortality after severe gill pathology in zebrafish and their toxicity was fully dependent on the specific environmental conditions (Villarreal *et al*., 2014). In Pakistan, carps are considered as most common food source due to consumer preferences. But now a day, water pollution becomes a major issue in Pakistan and has a severe impact on aquatic life. In aquatic environments, organisms are usually exposed for a long duration to sublethal concentrations of metals (Javed, 2013; Akram *et al*., 2021). The aims of the present study, therefore, focused on assessing the toxicological impacts of nanoparticles on the bighead carp (*Hypophthalmichthys nobilis*) to induce DNA damage and oxidative stress after exposure to sub-lethal doses ($1/3^{rd}$and $1/5^{th}$ of its respective 96-h LC_{50}) for 45 days (Sharif *et al*., 2021).

MATERIALS AND METHODS

Synthesis and Characterization of CuO-NPs: Copper chloride and sodium hydroxide were dissolved separately in deionized water. Incorporation of sodium hydroxide solution was added dropwise into a solution of copper chloride with continuous stirring at room temperature that resulted in the formation of a bluish gel of copper hydroxide. The resultant precipitates were filtered and rinsed with distilled water. After successive drying, nanoparticles were synthesized. To determine size, elemental composition and reduction of nanoparticles, X-ray diffraction, FT-IR and UV visible spectroscopy were used.

Experimental fish: Fingerlings of Bighead carp (*Hypophthalmichthys nobilis*) were obtained from Fish Seed Hatcheries, Faisalabad. All fish (10 fish/ aquarium) were acclimatized for two weeks in tanks with dechlorinated tap water. The physico-chemcial variables of water were maintained (Table 1). During this period, the carp was fed only once daily with the pelleted commercial feed comprising 30% digestible protein and 3Kcal/g digestible energy.

Experimental design: The CuO-NPs solutions were prepared in the deionized water, then ultrasonicated (100W, 50kHz) for 60 minutes. The bighead carp were randomly transferred in glass aquaria. In the first phase of experiments, 96-h LC_{50} of nanoparticles were measured for bighead carp and then $1/3^{rd}$ and $1/5^{th}$ LC_{50} /96 h value of CuO-NPs were selected for 45 days to check DNA damage and oxidative stress in fish in triplicates. A control set of fish was free of test suspension. To maintain a fixed level, all the test mixtures were altered after 24 hours.

Table 1: Physico-chemical parameters used for the experiments

Parameters	Unit	Mean	Analysis Method
Total Hardness	mg/L	250	Titration method
PH		7.5	pH meter
Temperature	°C	30	Temperature meter
Dissolved Oxygen	mg/L	5.8	Oxygen meter
Electrical Conductivity	µSiemens/cm	545.78	Conductivity meter

Determination of LC_{50}: Under the controlled conditions, the acute toxicity for *H. nobilis* was investigated. Ten fish were exposed to 0, 20, 50, 80, 110, 140, 170, 200, 230, 260, 290, 320, 350, 380, 410, 440 and 470 mg/L for 96 h in triplets. The fish were not fed during testing durations to reduce the sorption of the nanoparticles in solid feed and feces. The LC_{50} and lethal concentration of CuO-NPs for *H. nobilis* was 309.617and 462.929 mg/L, respectively (Fig. 1).

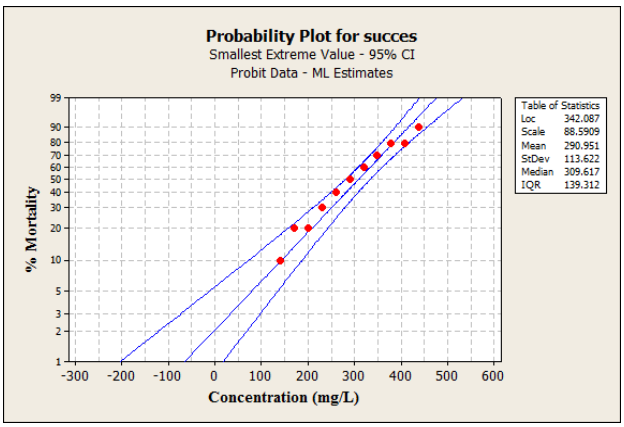


Fig. 1: Probability plot for Success at 95 % Confidence interval.

DNA damage: Fish erythrocytes were processed for the comet assay to examine dose and duration dependent DNA damage by the protocol of Singh *et al.* (1988) after exposure to both sub-lethal concentrations ($1/3^{rd}$ and $1/5^{th}$ of 96-h LC_{50}) of CuO-NPs along with negative and positive control group for 45 days. The positive control fish were injected intraperitoneally with $20\mu gg^{-1}$ cyclophosphamide in a 4% saline solution, while the negative control fish group was unstressed. Sampling was done on 15-30 and 45 days. The heparinized blood samples were centrifuged after collection from the caudal vein (n=3) of fish at 10,000rpm for 2 minutes to get separate erythrocytes. Slides were prepared by applying three different layers of agarose gel and in second gel layer blood samples were present that used to be tested. The prepared slides were dipped in lysing solution (10mMTris, Triton X-100, 2.5 M NaCl, 100mMNa_2 - EDTA, pH 12 with 1 and 10% DMSO) and refrigerated at 4°C. After the lysis step, slides were settled in Comet tanks (CSL-COM20, Cleaver, UK) which were filled with

freshly prepared electrophoresis buffer (pH 13) for 20 min to unwind DNA. For electrophoresis, similar solutions were used at 24 V, adjusting 300 mA current for 30 min. Then slides were placed in neutralizing solution and after staining of slides with ethidium bromide (20ug/ml), they were examined under Epi-Flourescence microscope (N-400M, American scope; USA). DNA damage was checked in terms of % damaged nuclei and genetic damage index (GDI) by following Kousar *et al*. (2018).

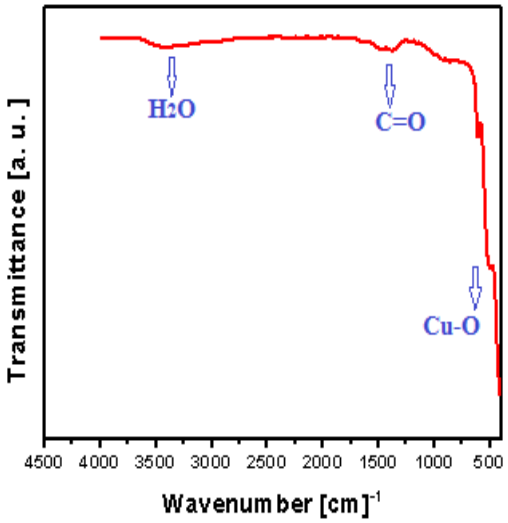
Oxidative stress: *H. nobilis* was exposed to sub lethal dose (1/3rd and 1/5th of 96-h LC_{50}) for 45 days. After every 15 days oxidative stress in terms of SOD, GST, CAT and LPO was assessed in the gills, liver, heart and muscle of the fish. All organs of bighead carp were separately homogenized by using the chilled phosphate buffer saline in a 1/4 ratio (weight/volume) by Homogenizer. After that, the mixture was centrifuged at 10,000 rpm, 4°C for 15 min. For analysis of antioxidant enzymes, the supernatant was used. SOD activity was measured by its potency to reduce the photo-reduction of NBT (nitroblue tetrazolium) (Worthington 1988). The activity of GST was observed by measuring the CDNB (1-chloro, 2,4-di-nitrobenzene) conjugation with GSH that reduces glutathione (Mannervik, 1985). The activity of CAT was determined by measuring its capacity to lessen hydrogen peroxide at 240nm (wavelength) (Chance and Maehly, 1955). The TBARS in tissues was calculated as the index of lipid peroxidation (LPO) as described by Gatta *et al*. (2000).

Statistical Analysis: The probit analysis method was used with the 95% confidence interval to measure the 96-h LC_{50} and lethal concentration. All experiments were conducted in three replicates. Analysis of Variance was used to check statistical differences and a comparison of means was done Tukey's/Student Newnan-Keul tests.

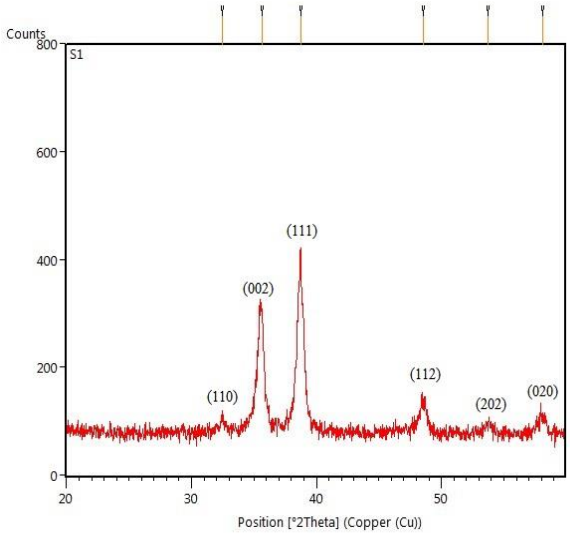
RESULTS

Characterization of NPs: Fig. 2 (A) confirmed the functional groups in samples of synthesized copper oxide nanoparticles. For determining the size, X-ray diffraction technique (XRD) was used. The mean observed size was 32.84 nm for the nanoparticles and their recorded peak values; (110), (002), (111), (112), (202) and (020) were located at 2θ = 32.47°, 35.63°, 38.73°, 48.54°, 53.76° and 58.12°, respectively (Fig. 2B). And Fig. 2C showed a peak of absorbance that confirmed the reduction of particles.

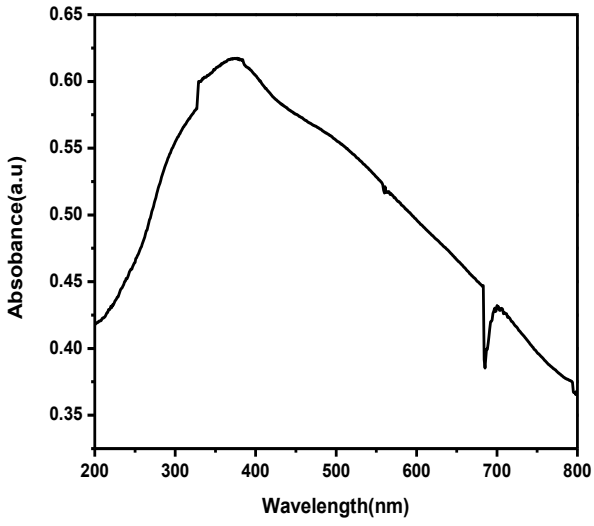
Analysis of DNA Damage: Regarding the overall performance of bighead carp towards DNA damage, under the exposure of CuO-NPs, both doses showed a significantly higher frequency of damaged cells (Fig. 3A) and genetic damaged index (GDI) (Fig. 3B). The overall 1/3rd of LC_{50} caused maximum % of DNA damage and GDI significantly than 1/5th of LC_{50} exposure. The induction of DNA damage and genetically damaged index after CuO-NPs exposure increased consistently in fish with a rise in exposure time and dose, indicating time and dose-dependent induction of DNA damage in bighead carp.



(A): FTIR spectra of CuO-NPs.



(B): X-Ray diffraction pattern of CuO-NPs.



(C) UV-spectroscopy of copper oxide nanoparticles.

Fig 2: Characterization of copper oxide nanoparticles (A) FTIR spectra of CuO-NPs. (B) X-Ray diffraction pattern (C) UV-spectrocopy of copper oxide nanoparticles.

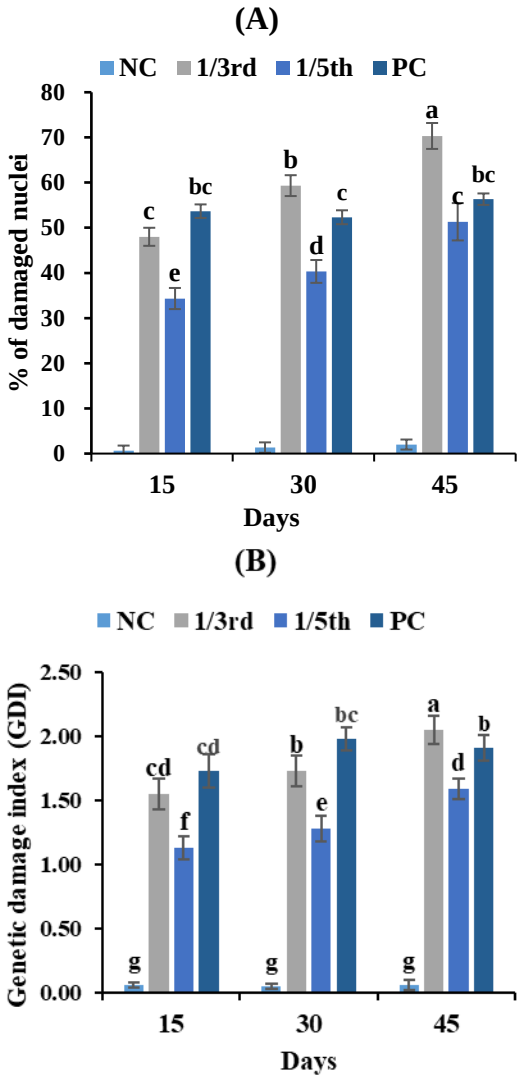


Fig 3: % of damaged nuclei (A) and genetic damage index (B) in the peripheral erythrocytes of Bighead carp exposed to 1/3rd and 1/5thof LC_{50} of CuO-NPs for the 15th, 30th and 45th day. NC=Negative Control, PC= Positive Control. Values are means of three replications.

Oxidative stress analysis

Superoxide dismutase (SOD) assay: Sub-lethal exposure of CuO-NPs caused significant variations regarding the SOD activity in different organs of bighead carp (*H. nobilis*) (Fig. 4 A-D). Carp organs showed a significant increase in SOD activity after 15th and 30th days, while serious decline occurred on the 45th-day than the controlled group. In this present research work, the level of superoxide dismutase was in the following trend: liver > gills > heart > muscle.

Glutathione S-transferase (GST) assay: CuO-NPs exposure showed significant variations in GST activity of selected organs of bighead carp (Fig. 5 A-D). The GST activity increased consistently up to 30 days by the elevation of the CuO-NPs exposure duration while it decreased with further increase in exposure time. The fish organs indicated significantly different ability to induce GST activity under CuO-NPs exposure, stress as liver > gills > heart > muscle with the obtained mean values of 560.71±21.26UmL^{-1}, 535.52±17.37UmL^{-1}, 453.97±23.66 UmL^{-1} and 432.82±22.60UmL^{-1}, respectively.

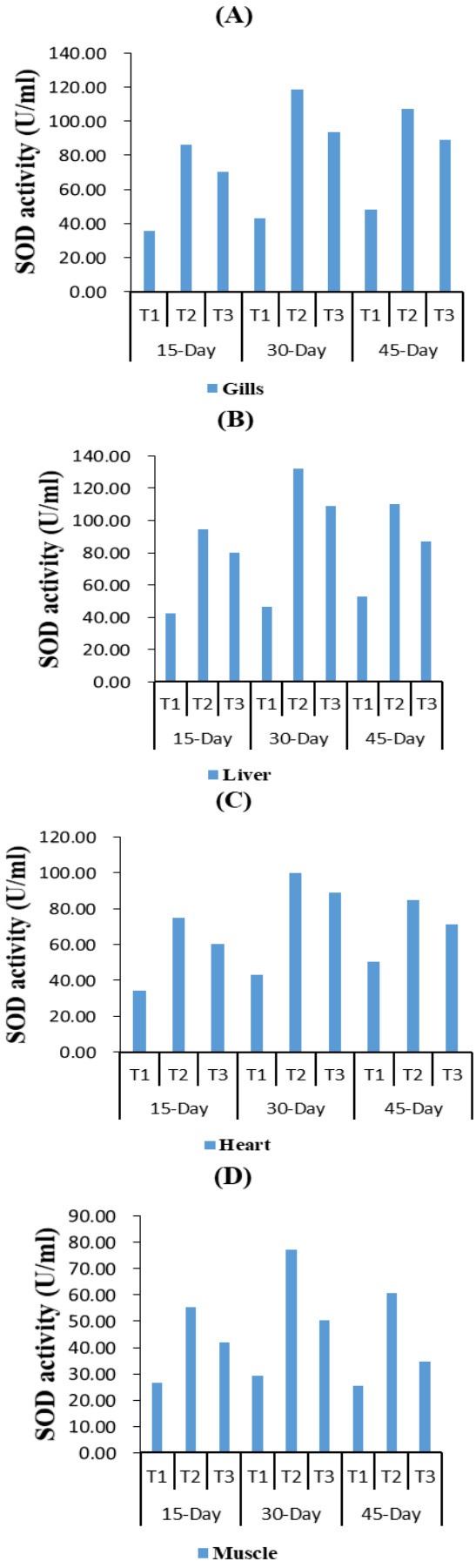


Fig. 4: Changes in SOD activity (A) Gills (B) Liver (C) Heart (D) Muscle of Bighead carp for the 15th, 30th, and 45th days. T1=control, T2=1/3rd of 96-h LC_{50}, T2= 1/5th of 96-h LC_{50}. Values are means of three replications.

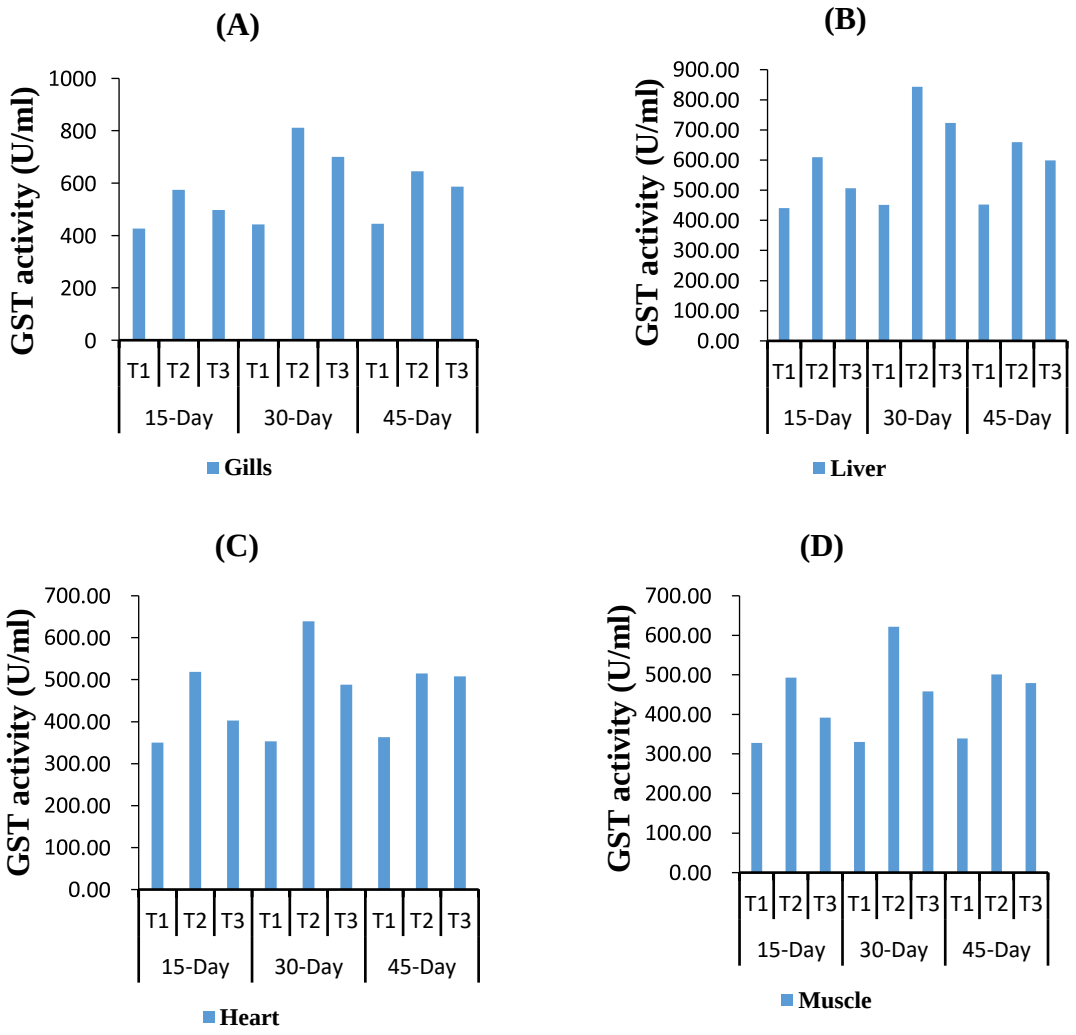
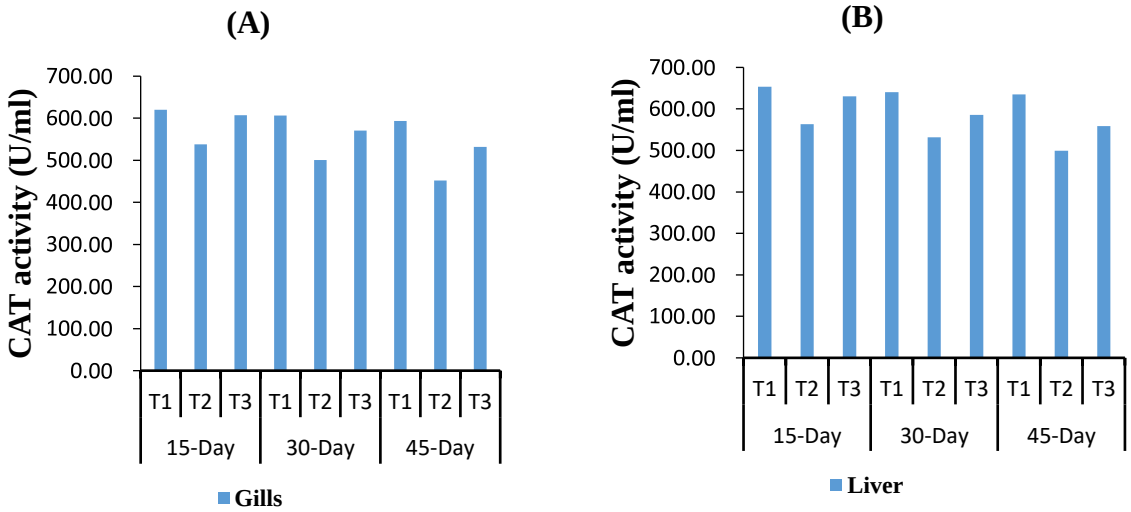


Fig. 5: Changes in GST activity (A) Gills (B) Liver (C) Heart (D) Muscle of Bighead carp for the 15th, 30th, and 45th days. T1=control, T2=1/3rd of 96-h LC_{50}, T2= 1/5th of 96-h LC_{50}. Values are means of three replications.



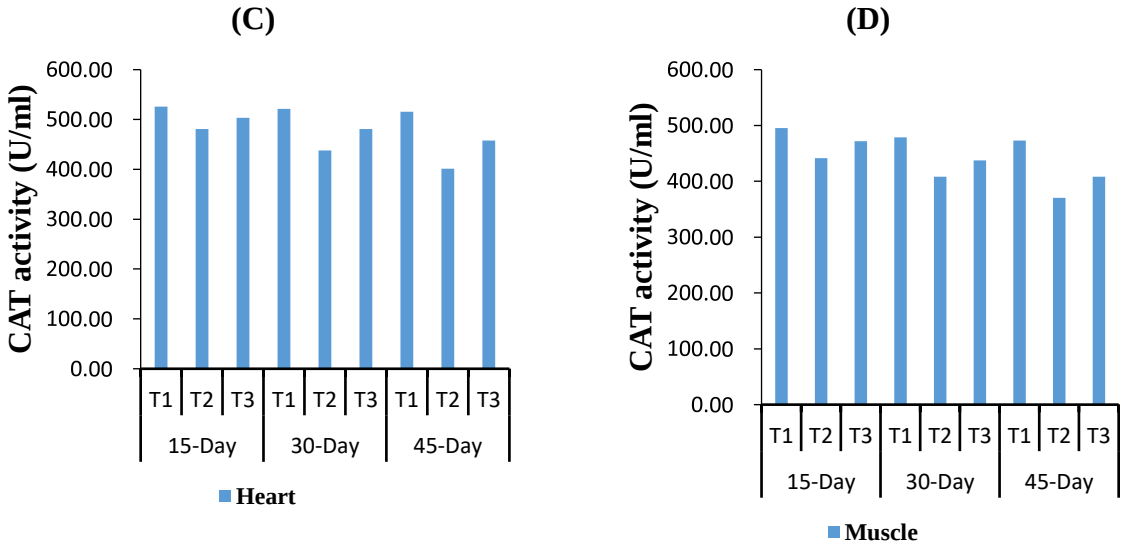


Fig. 6: Changes in CAT activity (A) Gills (B) Liver (C) Heart (D) Muscle of Bighead carp for the 15th, 30th, and 45th days. T1=control, T2=1/3rd of 96-h LC_{50}, T2= 1/5th of 96-h LC_{50}. Values are means of three replications.

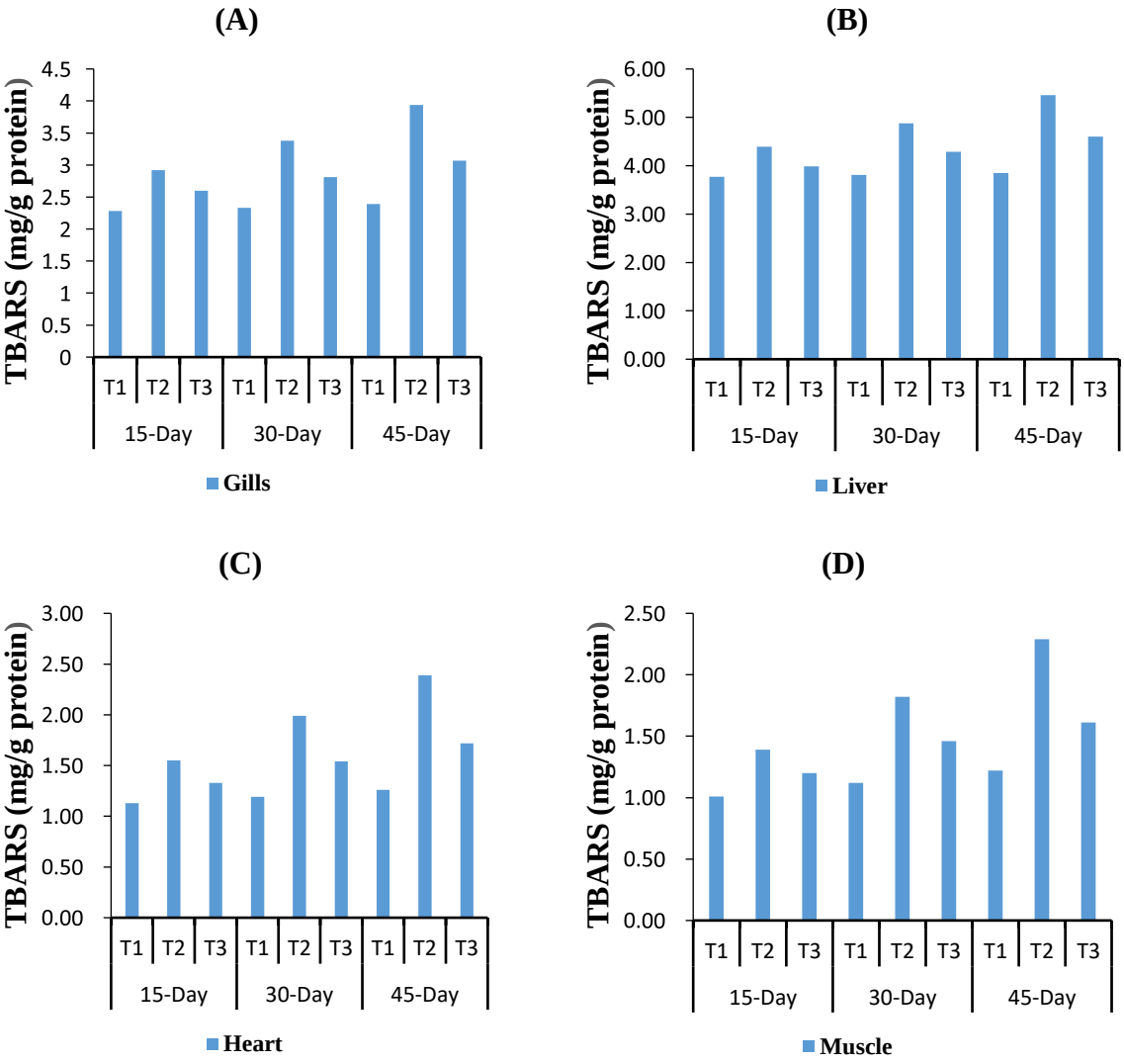


Fig. 7: Changes in TBARS level (A) Gills (B) Liver (C) Heart (D) Muscle of Bighead carp for the 15th, 30th, and 45th days. T1=control, T2=1/3rd of 96-h LC_{50}, T2= 1/5th of 96-h LC_{50}. Values are means of three replications.

Catalase (CAT) assay: CAT activity for sampled organs of *H. nobilis* was assessed after exposing the fish to sub-lethal doses for a period of 45days (Fig. 6 A-D). Fish showed a downward trend significantly in response to the increased days and resulted in inactivation in catalase assay. High concentration showed more decline in catalase activity as compared to low sub-lethal.

Thiobarbituric acid reactive substances (TBARS) assay: It serves an important signal for the Lipid peroxidation level which was incorporated in the present work. Chronic exposure of the CuO-NPs triggered significant variability in TBARS levels in fish organs. The level of TBARS increased significantly with increased dose and exposure duration than the control group (Fig. 7 A-D).

DISCUSSION

Fish are important in the food chain owing to their ability to metabolize and accumulate metals in aquatic mediums. Lethal concentration is the mortality of experimental organisms within a stipulated time. It is mainly done to monitor the relationships for a specific effect of a dose and chemical substance, and, therefore, has biological and ecological significance (Kumar *et al*., 2018). Results of the present investigations determined 309.617mg/L as LC_{50}-96h of the CuO-NPs for the Bighead carp. No mortality occurred in the controlled group. Kaviani *et al*. (2019) observed 286.47 mg/l as 96-h LC_{50}() While the results of Vajargah *et al*. (2018) have reported 124.9 mg/L as 96-h LC_{50} NPs for the common carp (*Cyprinus carpio).* Moreover, research works on the acute toxicity of CuO-NPs on the rainbow trout (*Oncorhynchus mykiss*) at 1, 5, 20 and 100 mg/l concentrations indicated no mortality (Khabbazi *et al*., 2015). Present data also showed that copper oxide nanoparticles had low acute toxicity to fish. The level of toxicity of nanoparticles depends mainly on the type of test animal, concentration and aggregation of nanoparticles (Morgaleva *et al*., 2015). Reduced NPs toxicity was perhaps due to the accumulation of nanoparticles despite frequent aeration utilized in the toxicity test could reduce aggregation and sedimentation of the nanoparticles. Due to the aggregation of nanoparticles, the exposure dose of carp might be reduced than the added nominal level, and therefore, for the present study, the tested solutions were exchanged daily to reduce the aggregation of NPs.

The earliest ecotoxicological information has suggested that metal oxide-based nanoparticles caused toxic effects after acute and chronic exposure (Zhao *et al*., 2011). Therefore, it was evident in this that the period 45-days of sub-lethal toxicity test was done to check genotoxicity and oxidative stress at $1/3^{rd}$ and $1/5^{th}$ of 96-h LC_{50} of nano-CuO exposure. CuO-NPs could be entered into the nucleus through a nuclear pore or physical wound in the membrane and directly interact with nuclear DNA (Wang *et al*., 2012). Alarifi *et al.* (2013) also reported genotoxic effects in human skin keratinocytes cells after CuO-NPs exposure. Copper develops bonds with the phosphate group and bases of DNA, especially guanine (G) and cytosine (C) by competing with hydrogen ions. Such binding breaks links between bases of DNA and unwinds double-helical structure. The SOD and CAT system supplies the first line of protection against the toxicity of oxygen and is normally used as a biomarker of ROS (reactive oxygen species) creation. Noureen *et al*. (2018) reported high lipid peroxidation levels and reduced glutathione activity in the livers of *C. carpio* after exposure to Cu-NPs than CuO treated groups, depending upon the dose concentration. Shahzad *et al*. (2018) evaluated oxidative stress in tilapia (*Oreochromis mossambicus*) after waterborne exposure to CuO-NPs. Higher SOD was observed in gill and liver tissues but higher LPO and CAT were observed in gills than the liver. Tunçsoy *et al*. (2017) reported decreased SOD, CAT and increased GPx activity in tissues (gill and liver) after exposure to CuO-NPs of *Oreochromis niloticus.* Wang *et al*. (2016) observed a high level of malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) in *Trachidermus fasciatus* after exposure to 20 and 100 µg/L CuNPs.

Results in the present investigation further indicated that CuO-NPs significantly change the activity of antioxidants and lipid peroxidation, both at low and high concentrations. Activities of SOD and GST became accelerated for the first 30 days and declined subsequently. Present increased and decreased value the biological biomarkers (enzymatic and non-enzymatic) was due to peroxidation and carbonization by the formation of free radicals and ROS because of metal toxicity. Similar findings were also reported by Tabrez and Ahmad (2011). According to Jiang *et al*. (2016) the different response of antioxidants in fish organs was due to different metal oxide nanoparticles concentration, type and structure of enzymes, the interaction between enzyme and metal nanoparticles, the accumulation tendency of metal's and physiological functions of different organs of fish.

In the present study CAT activity declined due to increased days at both dose levels. Possibly such reduction of the CAT activity was due to the accumulation of hydrogen peroxide and other oxygen radicals. A high level of hydrogen peroxide (H_2O_2) which had resulted from CAT reduction would finally further inhibit the activity of SOD. MDA is an end product of lipid peroxidation and hence is a robust index of oxidative stress. Various organs of bighead carp indicated significantly high lipid peroxidation levels after sublethal exposure. Work done by Aziz *et al*. (2020, 2021) measured the high lipid peroxidation level in rohu, than the control fish group ($P>0.05$) after nanoparticles exposure.

Conclusions: It was concluded that sub-lethal concentrations of CuO-NPs induced significant DNA damage in blood erythrocytes of the test fish. The damage % increases as dose and time of exposure increases. Further, CuONPs also produce oxidative stress. These findings of the present study are mainly focused on the toxicological effects of CuO-NPs.

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