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Original Article

Studies on bioaccumulation patterns, biochemical and genotoxic effects of copper on freshwater fish, *Catla catla*: an *in vivo* analysis

Estudos sobre padrões de bioacumulação, efeitos bioquímicos e genotóxicos do cobre em peixes de água doce, *Catla catla*: uma análise *in vivo*

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Abstract

During present study, the copper (Cu) mediated oxidative stress was measured that induced DNA damage by concentrating in the tissues of fish, *Catla catla* (14.45±1.24g; 84.68±1.45mm) (Hamilton,1822). Fish fingerlings were retained in 5 groups for 14, 28, 42, 56, 70 and 84 days of the exposure period. They were treated with 2/3, 1/3, 1/4 and 1/5 (T1-T4) of 96h lethal concentration of copper. Controls were run along with all the treatments for the same durations. A significant ($p < 0.05$) dose and time dependent concentration of Cu was observed in the gills, liver, kidney, muscles, and brain of *C. catla.* Among organs, the liver showed a significantly higher concentration of Cu followed by gills, kidney, brain, and muscles. Copper accumulation in these organs caused a significant variation in the activities of enzymes *viz*. superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). The SOD activity varied significantly in response to the exposure time of Cu as 56 > 70 > 42 > 84 > 28 > 14 days while CAT activity exhibited an inverse relationship with the increase in Cu concentration. POD activity showed a significant rise with an increase in Cu exposure duration. Comet assay exhibited significant DNA damage in the peripheral erythrocytes of Cu exposed C. *catla.* Among four exposure concentrations, 2/3rd of LC_{50} (T1) caused significantly higher damage to the nuclei compared to control. Increased POD and SOD activity, as well as a decrease in CAT activity in response to Cu, demonstrates the involvement of a protective mechanism against reactive oxygen species (ROS), whereas increased ROS resulted in higher DNA damage. These above-mentioned molecular markers can be efficiently used for the biomonitoring of aquatic environments and conservation of edible fish fauna.

Keywords: copper bioaccumulation, antioxidant enzymes, genotoxicity, comet assay, *Catla catla.*

Resumo

Durante o presente estudo, o estresse oxidativo mediado pelo cobre (Cu) foi medido que induziu danos ao DNA por concentração nos tecidos de peixes, *Catla catla* (14,45 ± 1,24g; 84,68 ± 1,45mm) (Hamilton, 1822). Os alevinos foram retidos em 5 grupos por 14, 28, 42, 56, 70 e 84 dias do período de exposição. Eles foram tratados com 2/3, 1/3, 1/4 e 1/5 (T1-T4) de 96h de concentração letal de cobre. Os controles foram executados junto com todos os tratamentos para as mesmas durações. Uma significativa ($p < 0,05$) concentração dependente do tempo e da dose de Cu foi observada nas brânquias, fígado, rim, músculos e cérebro de *C. catla*. Entre os órgãos, o fígado apresentou uma concentração significativamente maior de cobre, seguido por guelras, rins, cérebro e músculos. O acúmulo de cobre nesses órgãos causou uma variação significativa nas atividades das enzimas viz. superóxido dismutase (SOD), catalase (CAT) e peroxidase (POD). A atividade de SOD variou significativamente em resposta ao tempo de exposição de Cu como 56> 70> 42> 84> 28> 14 dias, enquanto a atividade de CAT exibiu uma relação inversa com o aumento na concentração de Cu. A atividade POD mostrou um aumento significativo com um aumento na duração da exposição ao Cu. O ensaio do cometa exibiu dano significativo ao DNA induzido por Cu nos eritrócitos periféricos de *C. catla*. Entre as quatro concentrações de exposição, 2/3 do LC50 (T1) causou danos significativamente maiores aos núcleos em comparação com o controle. O aumento da atividade de POD e SOD, bem como uma diminuição na atividade de CAT em resposta ao Cu, demonstra o envolvimento de um mecanismo protetor contra espécies reativas de oxigênio (ROS), enquanto o aumento de ROS resultou em maior dano ao DNA. Esses marcadores moleculares mencionados acima podem ser usados de forma eficiente para o biomonitoramento de ambientes aquáticos e conservação da ictiofauna comestível.

Palavras-chave: bioacumulação de cobre, enzimas antioxidantes. genotoxicidade, ensaio do cometa, *Catla catla*.

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1. Introduction

Copper is found throughout the world as one of the potential environmental toxicants due to its easier bioavailability and high oxidizing potential (Kováčik, 2017). Its widespread presence in aquatic ecosystems as a result of excessive use in various industrial activities, as well as the subsequent disposal of untreated effluents directly into running freshwaters, has alarmed eco-toxicologists. The natural aquatic ecosystems in Pakistan have been heavily polluted with toxicants that are generated through various natural and anthropogenic activities. Intense contamination of freshwater resources, particularly in developing countries, has been reported to contribute serious health hazards and environmental issues (Kutty and Al-Mahaqeri, 2016). Prominant anthropogenic sources of aquatic contamination include mining operations, untreated industrial effluents, domestic sewage, waste dump leachates and combustion emissions (Al-Ghanim et al., 2016; Shahid et al., 2021). Many studies have strived to link the morphological and physiological effects of chronic contaminant exposures to biochemical responses and functional traits that influence the well-being and survival of aquatic organisms (Sloman and McNeil, 2012; Barbee et al., 2014;). Copper occurs naturally in soil and water. Its bioavailability in water and toxicity to fish vary with physico-chemical properties of water like pH, suspended solids, organic compound content, alkalinity, and hardness (Di Giulio and Meyer, 2008).

Metallic ions toxicity in an aquatic environment is influenced by its speciation, solubility, bioavailability, toxic kinetic and the interactions when present in the form of metals mixture (Mahboob, 2013; Sfakianakis et al., 2015). The extent of metal's accumulation in the fish depends upon various biological and environmental factors including species, age, tissue types, season and duration of exposure (Georgieva et al., 2014; Masouleh et al., 2017). Another mechanism of metallic ions toxicity is through the induction of oxidative stress, a condition of transiently enhanced production of reactive oxygen species (ROS) and malfunctioned antioxidant machinery (Birnie-Gauvin et al., 2017) that may lead to DNA damage (Kousar and Javed, 2015). Copper is a transitional metal that can alter the antioxidant enzyme activities (Atli and Canli, 2010). Fish have a well-developed endogenous antioxidative system of enzymatic (catalase, glutathione-s-transferase, peroxidase, superoxide dismutase) and non-enzymatic (glutathione, urate, vitamin E, b-carotene and ascorbate) pathways (Pizzino et al., 2017). It is an inevitable phenomenon of aerobically respiring organism's life because various by-products including reactive nitrogen and oxygen species are normally associated with mitochondrial respiration. These reactive species are highly toxic as they possess unpaired electrons (Halliwell and Gutteridge, 2015). Fish tissues are more vulnerable to oxidative damage due to the presence of polyunsaturated fatty acids (PUFAs) containing double carbon-carbon bonds; an easy target of radical oxidations (Jin et al., 2017; Welker and Congleton, 2005). The activity index of antioxidant enzymes may be used as potential biomarkers to assess pollutant-mediated oxidative stress in different aquatic organisms (Poletta et al., 2016). The ROS attacks on the back-bone of DNA and nucleobases result in various lesions (8-hydroxy-20-deoxyguanosine), apurinic sites (without any base), single strands fragmentation and sugar oxidations (Remya, 2010). Fish are sensitive to metallic ions pollutants, damaging the DNA, and may lead to mutations and cancer. DNA damage in aquatic organisms can cause abnormal development, poor growth and reduced surviving rate of embryos and adults (Ginebreda et al., 2014; Lee and Steinert, 2003). Therefore, different genotoxic biomarkers have been established to evaluate DNA damage in various cells of the fish. Comet assay is a versatile, well-established, rapid and most extensively used tool to measure DNA damage, both qualitatively and quantitatively, in single cells (Olive and Banáth, 2006). During the present studies, the chronic effects of copper at different exposure dose and duration were observed in the freshwater fish, *Catla catla*.

2. Methodology

2.1. Fish specimens and in vivo setup

Fingerlings of *C. catla* (14.45±1.24g, 84.68±1.45mm) were procured from rearing ponds of Fisheries Research Farms (FRF), University of Agriculture, Faisalabad. They were acclimatized to laboratory conditions at water temperature (30 °C), 7.5 pH and total hardness (250mgL^{-1}) for 15 days in glass aquaria and fed with the pelleted feed of 30% DP and 3.0kcalg^{-1} DE, twice daily. For *in-vivo* trials, fish were divided into 4 experimental groups, a negative control (unstressed) and a positive control group (exposed to cyclophosphamide), each containing 15 specimens. Each research group was maintained in triplets in other aquaria and fish were exposed continuously to $CuCl_2.6H_2O$ for 84 days. For the estimation of metals accumulation, biochemical and genotoxic effects, fish were exposed to four sub-lethal concentrations as 33.42mgL^{-1} (66% of 96h LC_{50}, as T1), 16.71mgL^{-1} (33% of 96h LC_{50} as T2), 12.66mgL^{-1} (25% of 96h LC_{50} as T3), and 10.13mgL-1 (20% of 96h LC_{50} asT4) (Table 1). Tissues and blood sampling were done fortnightly on 14, 28, 42, 56, 70 and 84th day of post exposure intervals. On each day of sampling, two specimens from each group were anaesthetized with diethyl ether and a blood was taken out of the caudal vein and stored in a heparin sodium chloride for stabilization. After the collection of blood, fish were euthanized for

Table 1. Treatments and Sub-lethal Concentrations of Cu.

	Treatments	Concentrations (mgL^{-1})
	96-hr LC_{50}	50.64
T1	2/3rd of LC_{50}	33.76
T2	1/3rd of LC_{50}	16.88
T3	1/4th of LC_{50}	12.66
T4	1/5th of LC_{50}	10.13

Replicates n = 10.

the removal of organs viz. kidney, liver, gills, brain and skeletal muscles. This study has taken into account the concentration of Cu in the fish organs, oxidative stress by evaluating the activities of enzymes peroxidase, catalase and superoxide dismutase along with the DNA damage through comet assay in the peripheral erythrocytes of Cu exposed fish.

2.2. Determination of median lethal concentration (96h LC_{50}) of Cu

Bioassays were performed to determine the median lethal concentration at 96h of Cu exposure for *C. catla* by following the standard methods as described earlier (APHA, 2012). Five range-finding tests were conducted in triplicate by exposing ten fishes in each set to specific concentrations on a logarithmic scale, started from zero with an increment of 0.05 and 5.00mgL^{-1} for low and high doses, respectively, up to 96 h. The toxicity range of Cu to *C. catla*, based on mortality, was found between 20 and 70 mgL^{-1}. Ten different conc. of Cu as 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70mgL^{-1} and control were used for tests in triplets. On the basis of these tests, ten fishes in each glass tank were exposed to above mentioned conc. of Cu exposure. Fish mortality (%) was noted at 96h intervals of Cu exposure. For the measurement of 96h LC_{50} of Cu, mean values of percentage mortalities were subjected to the MINITAB[17] software for the Probit Analysis (Hamilton et al., 1977).

2.3. Analysis of Cu concentration in fish tissues

After the completion of each exposure period (fortnightly), the liver, kidney, gills, brain, and skeletal muscles were extracted from the treated and control fishes and rinsed properly with phosphate buffer solution. These tissue samples were autolyzed and digested by transferring it in 30 mL glass beakers containing HNO_3 and $HCLO_4$ (3:1 v/v), and heated at 100 °C on hot plate until the solution got clear (APHA, 2012). At the end, these samples were diluted with double distilled (DD) water. Cu concentration was estimated in these digested samples by using Perkin Elmer AA-400 Atomic Absorption Spectrophotometer.

2.4. Biochemical analyses

The tissue samples (liver, kidney, gills, brain, and skeletal muscles) were homogenized (1:4 W/V) in 0.2 M potassium phosphate buffer (pH 6.5). Homogenate tissues were centrifuged at 10,000 rpm for 15 minutes at 4 °C. The supernatant fraction was preserved as enzyme extract at -4 °C for further enzyme assays.

2.4.1. Peroxidase (POD)

Peroxidase activity in the selected organs was estimated by following Zia et al. (2011). Reaction mixture contained 47 mL phosphate buffer (0.2M), 0.7 mL guaiacol and 0.32 mL H_2O_2 and 0.06 mL enzyme extract. Samples were incubated for 3 minutes, after that the absorbance was measured at 470 nm through a spectrophotometer (Optima SP-300) against blank solution (phosphate buffer+ guaiacol). Peroxidase activity was calculated by using the following Formula 1:

$$\text{Peroxidase Activity U / mL} = \frac{\Delta A}{26.6 \times 0.06 / 3} \quad (1)$$

were: ΔA = Absorbance at 470 nm; 26.6 = Extinction Coefficient for tetraguaiacol (mM^{-1}cm^{-1}); 0.06 = Volume of enzyme Extract (mL); 3.0 = Volume of phosphate buffer (mL).

2.4.2. Catalase (CAT)

The catalase activity was measured by "it's ability to reduce H_2O_2 at 240 nm" (Maehly and Chance, 1954) with slight modifications. A 0.1 mL enzyme extract was added to 3 mL of phosphate buffer (50 mM, pH 7) and 0.1 mL hydrogen peroxide (30 mM). Absorbance was measured through a spectrophotometer at 240 nm after 3 minute of reaction time. Catalase activity was calculated by using the Formula 2:

$$\text{Catalase Activity U / mL} = \frac{\Delta A / 3}{0.04 \times 0.1} \times 3 \quad (2)$$

were: ΔA/3 = Absorbance at 3 minutes interval at 240 nm; 0.04 = Extinction Coefficient for H_2O_2 (M^{-1} cm^{-1}); 0.1 = Volume of Enzyme Extract (mL); 3.0 = Volume of phosphate buffer used (mL).

2.4.3. Superoxide dismutase (SOD)

This assay was performed by following the protocol of Worthington (1988) with few modifications. The SOD activity was measured by its rate of inhibition of the photoreduction of nitro blue tetrazolium (NBT). Reaction mixture contained 1 mL potassium phosphate buffer (0.0067 M, 7.8 pH), 0.0067 mL riboflavin (0.12 mM) and 0.05 mL enzyme extract. This solution was incubated in the lightbox (30V fluorescent bulb) for 12 minutes. After incubation, 0.067 mL of EDTA/NaCN solution (0.1M) and 0.033 mL nitroblue tetrazolium solution (1.5 mM) were added in the mixture. Absorbance was measured after 30 seconds of reaction time at 560 nm through spectrophotometer (Optima SP-300) against blank (phosphate buffer solution). The activity of SOD was calculated through Formula 3:

$$\text{Percent Inhibition} = \frac{\Delta A\left(\text{Blank}\right) - \Delta A\left(\text{sample}\right)}{\Delta A\left(\text{Blank}\right)} \times 100 \quad (3)$$

were: ΔA Absorbance at 560 nm.

2.5. Evaluation of genotoxicity by comet assay

During chronic exposure of Cu, for 84 days, the fish peripheral erythrocytes were collected fortnightly to observe the dose dependent and time dependent DNA damage in the fish through comet assay by following Singh et al. (1988). The positive control fish were injected intraperitoneally with 20 µgg-1 cyclophosphamide in 4% saline solution, while the negative control fish group was unstressed. The heparinized blood samples that were collected after each fortnight were centrifuged at 10,000rpm for 2 minutes to get erythrocytes separated.

a. Encapsulation

Then these erythrocytes were diluted by adding 1 mL phosphate buffer saline, out of which 60 µL were and mixed with 110 µL of low melting point agarose (1.7%). This solution was then evenly layered on the glass slides, pre-coated with normal melting point agarose (0.5%) and covered with glass slip, let it solidify for 15 minutes in the refrigerator. After that, the coverslips were gently removed, and slides were coated evenly with 75 µL of low melting point agarose (0.8%) and were covered again with glass cover slips.

b. Lysis

After solidification, cover slips were removed and slides dipped well in a chilled lysing solution comprising of Na_2-EDTA (100mM), NaCl (2.5M), Tris (100mM; pH12), Triton X (1%) and DMSO (10%) and stabilized for 60 minutes at 4°C.

c. Alkaline unwinding

For alkaline unwinding of DNA strands, the slides were washed with deionized water and placed for 20 minutes in comet tank (CS-300v, Cleaver Science, UK) containing freshly prepared electrophoresis solution (13.5 pH) that was comprised of NaOH (1mM), EDTA (1mM) for 20 minutes.

d. Electrophoresis

After 20 minutes of slides immersion in the solution, electrophoresis was performed for 25 minutes at 300mA and 25V in the same solution.

e. Neutralization

The slides were then neutralized by immersing them in Tris buffer (0.4M; 7.5 pH) and stained carefully with 20 µgmL^{-1} ethidium bromide.

f. Slide analysis

Each slide was examined under Epi-Flourescence microscope (N-400M, American scope; USA) with light source of mercury short arc reflector lamp filters for ethidium bromide at 400X magnification and low lux camera (MD-800, American scope; USA; Figure 3) was used to take the images of cells. DNA damage was estimated by the length of DNA migration in the comet tail.

g. Measurement of DNA damage

DNA damage was measured in terms of genetic damage index (GDI) using formula, Comet tail lengths of damaged cells were measured by using TriTek CometScore™ (Summerduck, USA) software Jose et al. (2011), Nassour et al. (2016) and Kousar and Javed (2018) whereas, the cumulative tail length of comets (CTL) (µm) was calculated by adding the comet tail length of all the cells examined.

2.6. Statistical analyses

The acute toxicity of Cu the fish was determined by using Probit analysis (Hamilton et al., 1977). Mortality data of fish were analyzed by using MINITAB 17 software. For statistical similarities and differences among variable means, the values were analyzed through ANOVA and Tukey's Student Newman-Keul test Steel and Torrie (1982) by using Statistix 8.1 computer package. Mean values of DNA damage in the fish erythrocytes were compared by performing non-parametric Mann-Whitney U-test.

3. Results

3.1. LC_{50} of Cu

Acute toxicity tests were conducted to measure the 96h LC_{50} of Cu for *C. catla* in semi-static water system. Acute toxicity of the test chemical was determined as 50.64±2.33 mgL^{-1} with 95% confidence limits (lower and upper) of 45.29 and 54.99 mgL^{-1}, respectively, while its lethal concentration was 78.76±4.46mgL^{-1}.

3.2. Physico-chemical analyses of the test water

The physico-chemical variables *viz.* water temperature, total hardness, pH, dissolved oxygen, electrical conductivity; carbon dioxide, sodium, ammonia, calcium, potassium and magnesium were measured on daily basis during sub-lethal exposure of Cu to each fish species for 84 days. The mean values of physico-chemical variables are presented in Table 2. All the recorded values of the

Table 2. Physico-chemical Parameters of test media.

Parameters	T1	T2	T3	T4
Temperature (°C)	30.06	29.00	30.41	30.09
pH	7.49	7.51	7.50	7.49
Total Hardness	252.01	253.01	252.01	250.01
Dissolved Oxygen	4.58	4.84	5.49	5.50
E. C. (MScm^{-1})	2.49	2.37	2.49	2.42
Carbon Dioxide (mgL^{-1})	1.82	1.68	1.54	1.31
Sodium (mgL^{-1})	302.16	301.80	301.97	301.53
Potassium (mgL^{-1})	7.18	8.55	8.57	8.38
Ammonia(mgL^{-1})	1.26	1.25	1.26	1.24
Calcium (mgL^{-1})	23.18	22.10	22.69	24.14
Magnesium (mgL^{-1})	48.02	48.33	48.02	47.41

physico-chemical parameters were observed suitable within the recommended limits for the survival of these fishes (APHA, 2012).

3.3. Concentration of Cu in the fish organs

The data on dose and time dependent accumulation of Cu in the fish organs are presented in Table 3. All the selected organs of fish showed significantly variable tendency to amass Cu and exhibiting significantly linear correlation with both dose and duration of Cu exposure. Fish liver exhibited significantly higher tendency to amass Cu while accumulation in other organs was in the order: gills > kidney > brain > muscles. Overall accumulation of Cu in the organs of the fish appeared significantly maximum due to metal exposure at T1 (67.87±14.45 μgg^{-1}), followed by T2 (60.77±15.80 μgg^{-1}), T3 (49.73±11.11 μgg^{-1}) and T4 (42.75±9.90 μgg^{-1}) as $1/5^{th}$ of LC_{50} exposures. At different exposure durations, Cu concentration in organs showed significant increase with concomitant increase in exposure duration.

3.4. Estimation of activities of antioxidant enzymes

3.4.1. Superoxide dismutase assay (SOD)

The dose and time dependent changes in SOD activity in the fish organs varied significantly due to Cu exposure (Table 4). Fish showed significant variations for SOD activity that changed with exposure concentrations as $2/3^{rd}$ > $1/3^{rd}$ > $1/4^{th}$ > $1/5^{th}$ of LC_{50}. A significantly direct relationship existed between enzyme activity and Cu exposure concentration in all the organs of fish. In *C. catla*, the SOD activity varied significantly due to exposure time as 56 > 70 > 42 > 84 > 28 > 14 days of Cu exposure. The fish liver exhibited significantly maximum activity of SOD with value of 106.73±22.65 UmL^{-1}, followed by that of gills (91.40±19.38 UmL^{-1}), kidney (85.54±19.55 UmL^{-1}), brain (80.51±18.73 UmL^{-1}) and muscles (71.83±17.06UmL^{-1}).

3.4.2. Catalase assay

The dose and time dependent changes in CAT activity in the fish organs varied significantly due to Cu exposure (Table 5). Fish showed significant variations for CAT activity that changed with exposure concentrations as T1 (503.41±65.34UmL^{-1}) < T2 (530.27±67.89 UmL^{-1}) < T3 (569.87±76.53UmL^{-1}) < T4 (632.17±89.21UmL^{-1}) of LC_{50}. Significantly inverse relationship existed between enzyme activity and Cu exposure concentration in all the organs of fish. Catalase activity varied significantly with exposure time followed the order: 14 > 28 > 42 > 56 > 70 > 84 days of Cu exposure. The fish liver exhibited significantly maximum activity of CAT with a mean value of 643.35±59.56 UmL^{-1}, followed by that of gills (627.80±57.67 UmL^{-1}), kidney (607.81±55.80 UmL^{-1}), brain (587.48±58.47 UmL^{-1}) and muscles (474.65±36.54 UmL^{-1}).

3.4.3. Peroxidase assay

The peroxidase activity in the fish organs varied significantly when exposed to different concentrations of Cu. Activity of POD was significantly higher for fish exposed to T1 (0.323±0.094UmL^{-1}) while it was significantly lower for control fish (0.210±0.102UmL^{-1}). However, the POD activity in all the three species of fish varied significantly between 0.168±0.065 and 0.347±0.133UmL^{-1} during the whole experimental period of 84 days (Table 6). The liver of all three species of fish showed significantly maximum POD activity, followed by kidneys, gills, brain and muscles with the mean values of 0.388±0.106, 0.287±0.075, 0.317±0.081, 0.199±0.037 and 0.138±0.041 UmL^{-1}, respectively.

3.5. Evaluation of genotoxicity using comet assay

Table 7 shows significant variability in the nuclear damage of *C. catla* due to various exposure periods and doses of Cu. Among six exposure concentrations, $2/3^{rd}$ of LC_{50} caused significantly higher damage to the nuclei, GDI and CTL of comets with the mean values of 74.48±6.43%,

Table 3. Accumulation of copper (μgg^{-1}) in the selected organs of *C. catla*.

	Gills	Liver	Kidney	Muscle	Brain	Overall Means
Treatments						**Means±SD**
T1	76.79±33.14	82.87±34.60	71.21±33.02	45.64±24.26	62.83±30.70	**67.87±14.45** [a]
T2	67.84±31.61	74.70±32.08	63.81±30.87	41.23±22.10	56.29±27.62	**60.77±15.80** [b]
T3	56.85±25.79	61.20±27.25	51.89±25.88	32.59±16.21	46.14±21.66	**49.73±11.11** [c]
T4	49.11±23.10	52.38±25.88	45.73±22.20	27.35±14.14	39.18±19.01	**42.75±9.90** [d]
Control	4.67±0.42	5.40±0.43	2.79±0.24	0.45±0.12	1.76±0.29	**3.01±2.04** [e]
Exposure Period						
14-day	20.22±6.54	24.74±6.24	18.94±5.64	9.50±2.16	16.13±3.22	**17.91±5.63** [f]
28-day	38.82±7.32	41.25±10.91	32.21±6.01	18.62±3.19	26.93±5.64	**31.57±9.16** [e]
42-day	62.11±11.12	67.43±15.23	55.43±11.24	37.74±7.48	51.25±10.22	**54.79±11.37** [d]
56-day	75.07±15.55	79.39±15.99	71.45±15.18	44.08±11.24	63.23±12.25	**66.64±13.94** [c]
70-day	86.37±16.41	90.43±16.50	82.19±15.71	51.08±12.44	73.28±15.25	**76.67±15.65** [b]
84-day	93.29±16.89	103.48±16.95	88.74±16.76	59.20±13.32	75.84±15.92	**84.11±17.10** [a]

Table 4. Superoxide dismutase activity (UmL^{-1}) in the fish exposed to copper for 84 days.

	Gills	Liver	Kidney	Muscle	Brain	Means±SD
Treatments						
T1	130.66±19.86	146.37±27.09	120.92±20.22	106.24±17.78	117.10±23.12	**124.26±15.10** [a]
T2	111.80±25.47	128.41±27.95	103.26±23.14	87.61±20.85	98.20±22.77	**105.86±15.35** [b]
T3	95.00±28.13	112.57±36.21	91.95±25.04	75.22±24.88	82.95±24.26	**91.54±14.16** [c]
T4	80.20±21.14	96.84±23.83	74.54±24.50	60.50±19.95	68.86±21.92	**76.19±13.66** [d]
Control	39.35±8.45	49.48±8.08	37.04±11.46	29.56±8.39	35.44±9.14	**38.17±7.26** [e]
Overall Mean	**91.40±19.38** [b]	**106.73±22.65** [a]	**85.54±19.55** [c]	**71.83±17.06** [e]	**80.51±18.73** [d]	
Exposure Period						
14-day	68.86±33.57	82.60±37.39	57.68±31.92	51.10±27.03	56.67±28.32	**63.38±12.13** [f]
28-day	79.84±34.23	95.41±33.67	71.84±31.85	58.75±28.29	73.64±33.54	**75.90±13.34** [e]
42-day	98.40±37.93	123.71±47.07	89.86±35.74	76.85±33.68	84.87±32.58	**94.74±17.99** [c]
56-day	124.69±49.54	140.75±55.35	111.43±47.01	97.41±40.37	109.30±45.11	**116.72±16.41** [a]
70-day	93.80±31.81	110.96±31.63	101.03±32.96	82.64±29.19	91.26±30.91	**95.94±11.71** [b]
84-day	82.82±24.45	86.97±22.02	81.40±28.52	64.20±19.25	67.31±18.03	**76.54±13.06** [d]

SD = Standard Deviation. Means having similar letters in the row/column are statistically at par ($p<0.05$).

Table 5. Catalase activity (UmL^{-1}) in the fish exposed to copper for 84 days.

	Gills	Liver	Kidney	Muscle	Brain	Means±SD
Treatments						
T1	537.01±89.50	561.01±84.72	521.31±66.16	392.48±37.97	505.25±69.14	**503.41±65.34** [e]
T2	570.94±81.27	590.74±81.33	546.05±73.28	416.85±37.40	526.77±59.91	**530.27±67.89** [d]
T3	623.56±66.57	635.17±77.34	583.80±72.65	443.20±28.31	563.62±75.56	**569.87±76.53** [c]
T4	680.18±34.48	695.33±38.41	669.64±55.82	476.94±24.88	638.75±74.47	**632.17±89.21** [b]
Control	727.32±18.35	734.53±20.61	718.24±20.86	643.76±55.52	703.04±21.28	**705.38±36.40** [a]
Overall Means	**627.80±57.67** [b]	**643.35±59.56** [a]	**607.81±55.80** [c]	**474.65±36.54** [e]	**587.48±58.47** [d]	
Exposure Period						
14-day	706.72±36.82	721.78±39.97	682.12±53.22	524.48±111.19	661.40±53.99	**659.30±78.83** [a]
28-day	675.92±56.75	696.96±37.86	654.93±74.24	501.82±104.63	638.98±74.48	**633.72±76.90** [b]
42-day	640.82±66.88	659.30±69.73	621.29±81.27	489.37±104.21	604.65±79.38	**603.08±66.80** [c]
56-day	605.53±87.41	618.59±84.29	590.91±91.62	458.88±96.25	572.19±99.89	**569.22±64.05** [d]
70-day	584.47±101.07	592.95±100.36	562.79±106.54	443.87±95.71	537.09±101.99	**544.24±60.13** [e]
84-day	553.35±119.31	570.54±103.91	534.79±104.41	429.45±88.11	510.60±95.33	**519.75±55.16** [f]

SD = Standard Deviation. Means having similar letters in the row/column are statistically at par ($p<0.05$).

2.45±0.17 and 216.77±25.03 µm, respectively, while the same were significantly ($p<0.05$) lower due to negative control as 1.22±0.78%, 0.05±0.02 and 3.44±0.03 µm, respectively. Cu exposure caused time dependent nuclear damage to the fish erythrocytes. Nuclear damage was significantly higher in *C. catla* (55.11±29.30%) after 56 days of exposure while GDI and CTL values remained at 1.77±0.92 and 152.03±85.75 µm, respectively. However, all the genetic index variables (damaged nuclei, GDI and comet tail lengths) were significantly low at 14th day of Cu exposure.

4. Discussion

Metallic ion pollution is widespread and has become a major concern because metallic ions are discharged indiscriminately into freshwaters, degrading their quality and ultimately affecting the fish fauna (Bhatnagar et al., 2016). During the exposure of toxicants in the fish, metallic ions cross all the biological barriers either through the epithelium of skin and gills or by crossing the wall of the digestive system and get amassed in the metabolically active organs like kidney, liver and gills (Firat et al., 2009; Mzimela et al., 2003). Acute toxicity assays play a significant

Table 6. Peroxidase activity (UmL^{-1}) in the fish exposed to copper for 84 days.

	Gills	Liver	Kidney	Muscle	Brain	Means±SD
Treatments						
T1	0.344±0.074	0.436±0.102	0.372±0.079	0.197±0.035	0.264±0.045	**0.323±0.094** [a]
T2	0.315±0.076	0.409±0.100	0.345±0.082	0.166±0.039	0.227±0.039	**0.293±0.096** [b]
T3	0.287±0.080	0.388±0.109	0.313±0.082	0.135±0.045	0.192±0.032	**0.263±0.100** [c]
T4	0.259±0.075	0.365±0.108	0.290±0.080	0.112±0.040	0.167±0.034	**0.239±0.100** [d]
Control	0.231±0.076	0.339±0.111	0.261±0.086	0.077±0.046	0.142±0.041	**0.210±0.102** [e]
Overall Means	**0.287±0.075** [b]	**0.388±0.106** [a]	**0.317±0.081** [c]	**0.138±0.041** [e]	**0.199±0.037** [d]	
Exposure Period						
14-day	0.182±0.050	0.234±0.049	0.210±0.051	0.069±0.049	0.143±0.047	**0.168±0.065** [f]
28-day	0.226±0.036	0.304±0.041	0.248±0.047	0.123±0.054	0.175±0.044	**0.215±0.069** [e]
42-day	0.275±0.047	0.379±0.034	0.286±0.041	0.129±0.052	0.191±0.047	**0.252±0.096** [d]
56-day	0.311±0.034	0.411±0.028	0.351±0.034	0.154±0.047	0.209±0.046	**0.287±0.105** [c]
70-day	0.348±0.067	0.487±0.044	0.388±0.043	0.172±0.040	0.226±0.060	**0.324±0.126** [b]
84-day	0.382±0.036	0.510±0.034	0.416±0.049	0.180±0.040	0.248±0.048	**0.347±0.133** [a]

SD = Standard Deviation. Means having similar letters in the row/column are statistically at par ($p<0.05$).

Table 7. Copper exposure induced DNA damage in the peripheral erythrocytes of *C. catla.*

	Undamaged Nuclei (%)	Damaged Nuclei (%)	GDI	CTL (μm)
Dose Dependent				
NC	95.89±1.29 [a]	1.22±0.78 [e]	0.05±0.02 [f]	3.44±0.03 [f]
PC	24.33±8.93 [c]	54.00±1.58 [c]	1.86±0.09 [c]	135.03±2.38 [d]
T1	11.89±4.18 [f]	74.78±6.43 [a]	2.45±0.17 [a]	216.77±25.03 [a]
T2	18.33±5.06 [e]	57.00±6.25 [b]	1.96±0.25 [b]	161.48±29.97 [b]
T3	22.11±7.61 [d]	56.22±12.33 [b]	1.79±1.29 [d]	156.21±38.02 [c]
T4	28.89±8.29 [b]	35.22±8.36 [d]	1.32±0.22 [e]	104.92±11.63 [e]
Time Dependent				
14 days	35.33±30.43 [b]	39.44±23.60 [d]	1.41±0.77 [e]	107.81±59.61 [e]
28 days	36.22±30.60 [a]	46.00±24.91 [bc]	1.60±0.82 [c]	120.95±71.06 [d]
42 days	30.44±32.59 [d]	48.78±26.90 [b]	1.68±0.90 [b]	142.21±82.21 [b]
56 days	31.22±33.12 [e]	55.11±29.30 [a]	1.77±0.92 [a]	152.03±85.75 [a]
70 days	33.00±32.66 [c]	47.11±26.45 [bc]	1.55±0.84 [d]	132.91±75.10 [c]
84 days	35.22±33.55 [b]	42.00±25.61 [c]	1.38±0.85 [e]	121.94±70.60 [d]

GDI = Genetic Damage Index; CTL = Comet tail length. Means having similar letters in the row/column are statistically at par ($p<0.05$).

role in the hazard classification and risk evaluation as they estimate the relative toxicity of various toxicants in different fish species (Wedekind et al., 2007). Significantly variable tolerance limit (96-h LC_{50} and lethal concentrations) of *Tilapia nilotica* for Cu and Co was described by Rai et al. (2015). Mahboob et al. (2016) found a higher concentration of Cr and Cu in the liver followed by the gills, kidney, and body muscles of *Wallago attu* and *Cyprinus carpio*.

The toxicity of metals is generally depending upon the potency of toxicants, their relative affinity and speciation. Additionally, their toxicity and bioavailability are subsequently altered by a number of physicochemical factors including water temperature, hardness, pH, dissolved oxygen, and total alkalinity (Smith et al., 2015) as observed during the present investigation. Aquatic organisms usually accumulate low doses of metals without any immediate negative effects on them, but this may gradually increase the toxicity with increasing exposure duration (Teryila Tyokumbur and Grace Okorie, 2013). However, when the metals uptake process exceeded the regulatory and excretory processes would initiate the metals bioaccumulation process in the fish (Luoma and

Rainbow, 2008). In the present studies, in all the organs of fish, the accumulation of Cu exhibited a significantly positive correlation with both dose and time of exposure. Moreover, metal's exposure time, concentration, uptake mechanism, intrinsic factors and water quality parameters can also effect the metal's accumulation in the fish (Pereira et al., 2013). Metals and other toxicants are taken up differentially by the organs due to the variable uptake affinity of the fish tissues (Yancheva et al., 2015). Among all organs, the liver receives the blood that carries food contents from the intestine through the portal hepatic vein and the blood that transports oxygen to the hepatocytes through the liver artery. Therefore, the liver accumulates all the blood transported metals due to the presence of non-saturable ion channels and cytosolic metallothioneins in the hepatocytes.

Fish tissues are often rich in (PUFA) polyunsaturated fatty acids, which renders them more susceptible to free radical oxidation (Welker and Congleton, 2005), thus the fish possess the antioxidant defense system as a biochemical pathway to deal with the harmful effects of endogenous as well as exogenous factors to cause oxidative stress. The antioxidant enzyme system in the fish can be affected by both; intrinsic (age, feeding habit and phylogenetic position) and extrinsic factors (contaminants in water, dissolved oxygen level, temperature fluctuations and pathogens/parasites). The activity of CAT inhibited by all exposure concentrations of Cu as compared to control in the gills, spleen, kidney and liver of Crucian carp (Jiang et al., 2016). They observed this decline was due to the inactivation of CAT by superoxide radicals or due to increased activity of POD. Latif et al. (2020) also reported decrease in the catalase activity in the tissues of metal exposed *Cirrhina mrigala.* Increased levels of SOD, CAT, GSH and GST were observed in the fish collected from highly polluted site while all of these enzymes showed significantly decreased activity in the control fish group. The SOD activity was observed to increase significantly in the brain of Cu exposed *Catla catla*. However, the CAT activity was inhibited in the fish heart and gills after sub-lethal exposure to Cu (Rajasekar and Venkatakrishnaiah, 2016). Copper exposure caused a significant decline in CAT activity in the gills of *Sparus aurata* after 28 days of the exposure period (Isani et al., 2012). This decrease was might be due to the fact that Cu replace the essential metals in amylase or it may make bond with the functional groups like peptidyl, hydroxyl group or hydrosulfide groups of the enzymes and hence lower enzymatic activity (Borell, 2000; Muhlia-Almazán and García-Carreño, 2002). Since the pollutant level and its exposure time, and functional capacity of the organs, determine the kind of enzymatic mechanisms, therefore, the responses of CAT and POD in the fish may be considered as biomarkers of metallic ions pollution in the natural aquatic habitats (Latif and Javed, 2019).

In the natural environment, exposure to metallic ions may lead to abnormal histological and physiological responses and induce adverse effects on the reproduction, development, behavior and ultimately growth of aquatic organisms (Ginebreda et al., 2014). Metallic ions toxicity may cause DNA damage by oxidative stress, competition for ligand binding and molecular mimicry in the cells (Varotto et al., 2013). The negative effects of heavy metals are not limited to acute and chronic toxicity exposures rather they get accumulated in various fish tissues which can cause genotoxicity as well (Hussein Kehinde et al., 2016). Heavy metals cause genotoxicity in the fish either by direct damage to the DNA or indirectly through oxidative stress/damage, inhibiting DNA repair mechanisms and interacting with tumor suppressor proteins (Bolognesi and Cirillo, 2014; Tchounwou et al., 2012). The genotoxic effects of metals can be monitored by using various biomarker assays but during the present studies, comet assay technique was used to detect DNA damage in the fish peripheral erythrocytes. Variations in immunity, DNA repair mechanisms and metabolic rate of various fish species are responsible for the differences in inter-specific and intra-specific variations in the fish to induce genotoxicity/mutagenicity (Chairi et al., 2010). The polyanionic nature of DNA makes it susceptible to the adherence of metal cations and the production of hydroxyl radicals that attack invariably on the nucleobases and sugar-phosphate backbone of DNA (Manoj and Padhy, 2013). The toxic properties of transition metals is due to their higher potential to act as catalysts in the production of ROS through Haber-Weiss/Fenton reactions, resulting in potentially damaging DNA modifications (Aboul-Ela et al., 2011; Sultana et al., 2020). Fedeli et al. (2010) reported increased comet parameters (tail moment, tail intensity) in the RBC's of *Onchorhyncus mykiss* exposed to 50 µM Cu. Exposure of Cu caused an increase in the tail moment, length and intensity in the erythrocytes of *Sparus auratus* (Gabbianelli et al., 2003). Copper makes bond to PO_4^- groups of DNA strands and bases specifically cytosine and guanine by competing with hydrogen ions. This binding disrupts the linkage between nitrogenous bases of DNA, thereby unwinding the double helix (Govindaraju et al., 2013). Bagdonas and Vosyliene (2006) reported that the genotoxicity of Cu was due to the production of ROS and inhibition of DNA repair mechanisms caused by non-specific binding of Cu to the essential sites of enzymes. As the DNA damage increases in the cell, more DNA moves towards the tail that is quantified by the amount of fluorescence of comet tail length (CTL). The CTL and tail intensity has frequently been used to quantify the DNA strand breakage in genotoxic studies (Cok et al., 2011). Kousar and Javed (2015) reported significant increase in the CTL with increasing exposure concentration of Cu.

Thus, based on our results, we explained the interrelationships among these described factors of physiological processes as accumulation, oxidative stress and DNA damage in the Cu exposed fish. Exposure to Cu caused significant dose and duration dependent damage in the *C. catla.*

5. Conclusion

The present study investigates the molecular biomarkers which cause changes in multiple routes and mechanisms related to oxidative stress, DNA damage in the Cu treated fish, *C. catla*. The increase in the activity of POD and SOD, two most effective antioxidant enzymes, and decreased

in CAT activity shows the presence of a protective system against reactive oxygen species production due to the accumulation of Cu in the fish. Furthermore, higher DNA damage was also evident by increased ROS as infer by comet assay. Thus, these above-mentioned molecular markers can be efficiently used for the biomonitoring of aquatic enviro ents and conservation of edible fish fauna.

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