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Copper toxicity investigated at the biochemical, genotoxicological and histopathological levels under environmentally relevant concentration on food fish *Channa punctatus*

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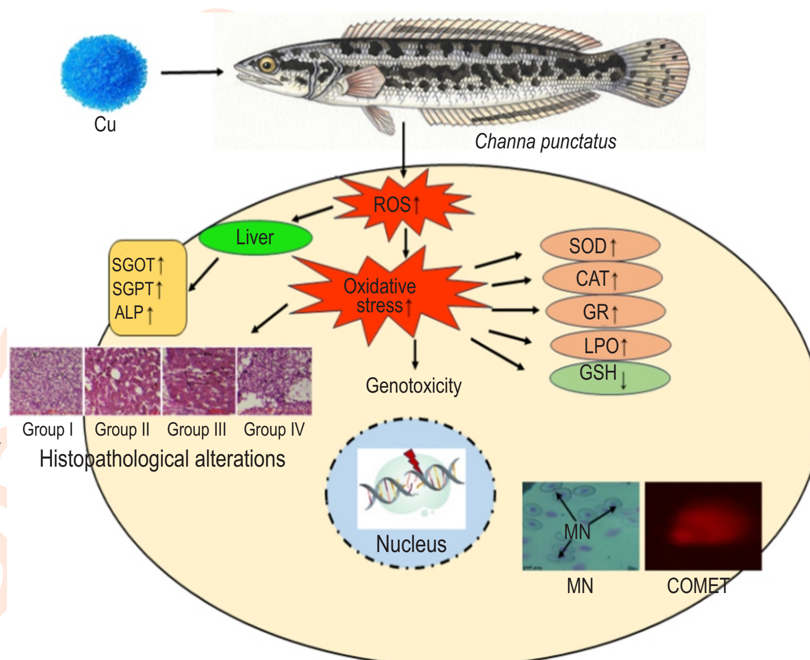
Abstract

Aim: The aim of this study was to investigate the effects of Environmentally Relevant Concentration of Copper (ERCC) on anti-oxidant defense machinery and genotoxicity as well as their ultimate threats on the liver of *Channa punctatus.*

Methodology: After acclimatization, fish were categorized into four separate groups. Group I served as control Group, while II, III and IV were exposed to ERCC, 10% and 20% increase in ERCC (0.85 mg l^{-1}, 0.935 mg l^{-1} and 1.02mg l^{-1}, respectively) for 15, 30, 45 and 60 days. Post-completion of stipulated exposure period, oxidative stress, DNA damages and hepato-architectural modifications were assessed.

Results: The activities of Superoxide dismutase, catalase, glutathione reductase, and lipid peroxidation were enhanced, while glutathione level decreased significantly ($p<0.05$) with elevated levels of reactive oxygen species in a dose-dependent manner. Micronuclei induction and COMET assay confirmed the genotoxic potential of ERCC. Elevated levels of serum glutamic oxaloaceticacid transaminase, glutamic pyruvate transaminase and alkaline phosphatase along with the distorted liver architecture validated hepato-toxicity.

Interpretation: Fish are prone to toxicity at ERCC. Further increase by 10% or 20%, would be hazardous to fish, and eventually to humans at various tropic levels.



Key words: Comet assay, Copper, Fish, Histopathology, Micronucleus

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Introduction

Copper (Cu) is a requisite redox active trace element essential for all biological systems (Zhong *et al.,* 2023). It occupies a significant place among micronutrients, and is notable and pertinacious in aquatic environments, but poses environmental risk if present in higher concentrations (Kumar *et al.,* 2022). As a consequence of anthropogenic (agriculture, industry) and natural (chemical and physiological modification of rocks) activities, Cu^{2+} is present in various aquatic bodies and interacts with the delicately balanced fabric of biological systems. Recently, industrial and economic build-out in Moradabad region (Uttar Pradesh, India), a renowned city for brass work, has put up the environment to endangerment due to excessive discharge of metal-containing effluents in River Ramganga (Pathak and Alam, 2022).

Many aquatic ecosystems near industrial zones in Moradabad are contaminated with metals like copper, zinc, chromium, arsenic and cadmium (Sarah *et al.,* 2019). Cu is one of the important ingredients of brass. The waste stream generated from different steps of brass industries contains copper in large amount. Apart from this, Cu compounds are also used as algicidal, herbicidal and molluscicidal (Hasan, (2010). Besides being toxic, copper also serves as a stressor agent for fish, altering several biological functions (Hsiao *et al.,* 2024) and also affecting histopathology of vital organs of fish (Sabullah *et al.,* 2014). These effects jeopardize humans as well as aquatic flora and fauna. Among all known salts of copper, the most extensively used salt is copper sulphate which is termed as blue-vitriol or blue-stone. The most noxious ionic form of copper is Cu^{2+} (Malhotra *et al.,* 2020). The sensitivity of aquatic organisms such as crustaceans and fish towards copper toxicity is 10 to 100 folds more than that of mammals (Malhotra *et al.,* 2020). Fishes are one of the most sensitive bio-indicators and are able to accumulate copper in their tissues, which depends on, the metal concentration, duration of exposure, method of exposure, environmental factors (water temperature, pH) and some intrinsic factors (fish age, size). Heavy metal contaminants get access in fish body through various routes like particulate matter suspended in water, ion-exchange through gills and adsorption through skin. Liver is a major site for detoxification and biotransformation and is one of the most vulnerable organ for damage caused by several toxicants. Its accumulation results in an increased synthesis of glutathione and metallothionein in fish liver which plays protective role in detoxification of metals (López-Alonso *et al.,* 2005). Moreover, it has been evidenced by several studies that the augmentation of heavy metals in the fish liver can cause serious hepato-architectural changes and ultimately death (Saxena *et al.,* 2007; Varanka *et al.,* 2001).

Histological modifications are considered as biomarkers for the assessment of complete health of ERCC-exposed fish as it provides better understanding about the potential of copper induced toxicity. (Kumar *et al.,* 2023). To analyze the genotoxic menaces to fish and other aquatic fauna, their ecological community, and eventually their consumers; the micronucleus test and comet assay is proved to be a dynamic approach. The copper-induced toxicity on fish has been explored by many researchers but till now scanty information about the toxic effects of copper at environmentally relevant concentration is available. The term "Environmentally relevant concentration (ERC)" is referred to the concentration/ dose which is relatable to the extents that organisms are literally "exposed to" in their natural environment.

Keeping all the above facts in mind, estimation of copper-toxicity at ERC was done to substantiate whether the copper in the river Ramganga, even at ERC, is dangerous for the food fish *C. punctatus* and if the concentration of Cu^{+2} increases by 10% or 20% due to increasing industrialization, what negative impacts it will cause to test fish?

Materials and Methods

Study area: The present study was conducted in the district Moradabad (28°-21' to 28°-16' North latitude and 78°- 4' to 79° East Longitude) which is located on the bank of river Ramganga in Uttar Pradesh, India. In the present study three sampling sites i.e., S1, S2 and S3 as Nawabpura (upstream), Katghar (midstream) and Pitalnagri (downstream), respectively were chosen due to the presence of numerous metal (especially brass) industries which discharge their effluents in the river Ramganga.

Collection and preparation of water sample for copper analysis: Water samples were collected in one litre sterilized plastic bottles. The samples were immediately acidified with 6N HNO_3 and taken to the laboratory for the estimation of heavy metals. In the laboratory, water samples were digested with conc. HNO_3 with continuous heating to obtain a clear solution, thereafter, cooled and filtered through 'Whatman No.42' filter paper. Metal-free distilled water was added to digested water sample to make up its volume to 100 ml (APHA, 2017).

Determination of metal concentration in water samples: Water samples were analysed for determining Cu-concentration by Atomic Absorption Spectrophotometer (AAS) (Model No, Varian AAS 240-FS, India) using acetylene-air flame. Operating parameters were set according to the manual. The results were expressed in mg l^{-1}.

Determination of ERCC: To determine the ERCC, the concentration of Cu^{+2} in water was estimated at all the three selected sites and thereafter, the mean value of those evaluated Cu^{+2} concentrations was calculated to obtain ERCC (Zhang *et al.,* 2010).

Fish acclimatization and experimental setup: Test fish *C. punctatus* (35 ± 3.0 g; 14.5 ± 1.0 cm, 8-12 months old), were caught by local fishermen from natural aquatic habitats and transferred to the laboratory in well aerated wide mouthed containers. The fish were washed rigorously with tap water, then

with 0.05% $KMnO_4$ to eliminate external infections, if any. Further, after dip washing in clean water, they were acclimated for 15 days in 1000 l high graded plastic aquaria pre-filled with de-chlorinated water. The aquaria water was assessed for water physico-chemical parameters following the standard methods (APHA, 2017). The fish were fed twice a day with fish food produced by Perfect Companion Group Ltd., Thailand. They were not fed 24 hr before the initiation of experimental study (OECD, 2019). Well-familiarised fish were categorised into four groups in triplicates that confined 10 specimens each. Group I (GI) served as control. Group II (GII), Group III (GIII) and Group IV (GIV) were exposed to the ERCC (0.85 mg l^{-1}), 10% increase in ERCC (0.935 mg l^{-1}) and 20% increase in ERCC (1.02 mg l^{-1}), respectively, for the period of 15, 30, 45 and 60 days. Fish from each test group were euthanized with 0.01% diethyl-ether after the completion of stipulated exposure period. Fish blood and liver was collected for further investigation.

Test chemicals: The test chemical used in the research were of analytical grade. Cupric Sulphate ($CuSO_4.5H_2O$), of Qualigens fine chemicals, Bombay was procured through a local dealer of Bareilly.

Determination of ROS: To examine the ROS in blood samples of fish, a fluorescent dye, 2´, 7´-dichlorodihydrofluorescein (20µM, DCFH-DA; Sigma Aldrich, USA) was applied. All blood samples with dye were incubated for 30 min and slides were prepared. The measurement of the intracellular fluorescence was carried out by using Fluorescence microscope (Floid™ cell imaging station, Life Technologies) at excitation and emission wavelength of 482 nm and 532 nm, respectively with 10/40X magnification. Image J software was used (version 1.50, USA) to quantify the fluorescent intensity (Saha *et al*., 2023).

Estimation of biochemical parameters in the liver

Formation of cell lysate: After the completion of exposure, fish liver was removed, weighed and washed with phosphate buffer saline. Thereafter, tissue homogenization was carried out with the homogenization buffer (HB) in the ratio of 1:10 (w/v) and for further procedure, Ratn *et al.* (2018) was followed. Cell lysate was preserved for the estimation of protein content, GSH and antioxidant enzymes.

Estimation of reduced glutathione (GSH): With some alterations, the methodology of Moron *et al.* (1979) was followed to determine the GSH. The process of assessing GSH, in the liver includes the reaction of tissues with DTNB (5,5'dithio 2-nitrobenzoic acid), also known as Ellman's Reagent. UV-VIS spectrophotometer (Shimadzu, UV-1900i) was used to measure the absorbance at 412 nm.

Assessment antioxidant enzymes: The method of Kakkar *et al.* (1984) was followed for the estimation of SOD in the liver and absorbance was read at 560 nm. CAT was evaluated by the method of Aebi (1984) at 240 nm. The assessment of GR activity was done following the method of Carlberg and Mannervik (1985) at 340nm. UV-VIS spectrophotometer (Shimadzu, UV-1900i) was used to measure the absorbance.

Estimation of LPO activity: LPO activity was estimated by the method of Ohkawa *et al*., (1979). It was performed by reacting malondialdehyde (MDA) with thiobarbituric acid reactive substance (TBARS). The absorbance of the sample was taken at 532 nm with UV-VIS spectrophotometer.

Estimation of liver biomarkers: SGOT and SGPT were estimated by the method of Reitman and Frankel (1957), while alkaline phosphatase was estimated by the procedure of Kind and King (1954) with some modifications.

Assessment of Genotoxicity

Micronucleus test: From each group a sample of blood was taken to make a uniform blood smear on a pre-cleaned glass slide Schmid, (1975). Evaluation and identification of MN% was done by counting 2000 erythrocytes per slide Fenech *et al*. (2011). Following formula was applied to calculate MN frequency:

MN% = (Number of cells containing MN / Total number of cells counted) X 100

Single-cell gel electrophoresis: The technique of comet assay consists of a three-layer procedure Singh *et al*., (1988). Frosted glass slides were firstly coated with 1% normal melting agarose (NMA) and left overnight. The mixture of 20µl cell suspension and 80µl 0.5% low melting point agarose (NMA) was spread on NMA-coated slides to form the second layer. The third layering was done with 100µl LMPA. Thereafter, the slides were laid in lysing solution overnight at 4°C, then in electrophoresis buffer for 20 min at 4°C for unwinding. Subsequently, electrophoresis was executed for 20 min at 15V (0.8V/cm) and 300 mA at 4°C. Slides were neutralized with 0.4M neutralizing buffer at pH 7.5 and stained with 75µl ethidium bromide, then randomly observed under a fluorescence microscope (Floid™ cell imaging station, Life Technologies). Twenty five cells per slide were scored for each variable. For the quantification of DNA damage, % tail length was determined with the help of Image J software version 1.8.0_172.

Histopathology of liver: Fish were dissected in normal saline (0.9 % NaCl). Liver was extracted in 10% neutral buffered formalin solution for 48 hr. After dehydration procedure, tissues were inserted in blocks of paraffin wax. Sections measuring 3 µm were obtained with the help of Microtome (YSI062 Yorco Precision Rotary Microtome, India), and stained with haematoxylin and eosin for 1 and 2 min, respectively. The sections were mounted in DPX after they were processed properly. Later, an oil-immersion microscope (Nikon Corporation K-12,432) with 10/40X magnification was used for capturing the photographs.

Statistical analyses: Three replicates of each experimental group were analysed, and all were presented as mean ± standard error mean (S.E.M.). The significance ($p<0.05$) of each result was analyzed with One-way ANOVA followed by Tukey's post hoc test. The analysis of the whole data-set was carried out with SPSS software (version 20.0, SPSS Company, Chicago, USA). In order

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to demonstrate clear interrelationships between numerous physiological indicators of copper-induced toxicity, regression and correlation studies were carried out.

Results and Discussion

Copper is an indispensable micronutrient required for normal physiological functions and methodical growth of life-forms, however, it can also bring noxious outcomes on reaching the concentration that surpasses the acquiescence limit of organisms. With ever-increasing industrialisation, various types of pollution have also been escalated and aquatic pollution is of utmost concern. As aquatic bodies serve as the eventual receptor for commercial and municipal wastewater, different anthropogenic activities and other atmospheric depositions, etc., ultimately become intoxicated with elevated concentrations of copper. To cope with this challenge present study has been performed to evaluate the Cu-viciousness in the creatures inhabiting aquatic environments that have vulnerability against Cu-pollution Malhotra *et al.* (2020).

The perusal of data revealed that the concentration of copper in the water samples from all three sites S1, S2, and S3 was 0.72 mg l^{-1}, 0.86 mg l^{-1} and 0.97 mg l^{-1}, respectively (Bakhasha *et al*., 2024). ERCC (0.85±0.02 mg l^{-1}) was found within the permissible limit 2 mg l^{-1}, 1.5 mg l^{-1} and 1.3 mg l^{-1} as per WHO, BIS, 2012; NRC (US) Committe on Copper in Drinking Water, 2000). These values have not yet, but will surely surpass the permisible limit eventually due to the ever-expanding population and escalating contamination of aquatic bodies. Therefore, the present investigations were carried-out by evaluating the ERCC and its 10% and 20% increased concentrations for 15, 30, 45 and 60 days. The physico-chemical parameters viz. total dissolved solids (TDS) 182.34 ± 3.2 mg l^{-1}, hardness 188.62 ± 4.0 as $CaCO_3$ mg l^{-1}, dissolved oxygen (DO) 6.9 ± 0.3 mg l^{-1}, temperature (T) 26.0 ± 1.5°C and pH 7.1 ± 0.3 were recorded in the test medium of all groups after the completion of exposure period. To elucidate the cytotoxicity generated due to Cu-intoxication, numerous mechanisms have been suggested. Amongst the most acceptable is the generation of reactive oxygen species, which culminates into oxidative stress (Guo *et al.,* 2017; Takemura Mariano *et al.,* 2024). ROS are paramount for physiological operations and cellular signalling. In this study, as depicted in Fig. 1, significantly ($p<0.05$) higher levels of ROS were recorded in all ERCC-exposed groups. The % increase in the ROS of GII, GIII and GIV as compared to control was calculated as 155.18, 207.92 and 338.14, respectively after 15d; as 170.80, 279.65 and 388.36, respectively after 30d; as 239.29, 327.40 and 446.46, respectively after 45d; while as 291.17, 371.53 and 524.72, respectively after 60d. The highest elevations were observed after 60 days as illustrated in Fig. 1a, b. Elevated ROS generation and eventually developed oxidative stress are like foes to the standard physiological functions. The perusal of data showed significant ($p<0.05$) increased levels of ROS in a time- and dose-dependent manner. It has been reported that copper intoxication boosts ROS production in the liver of mice (Liu *et al.,* 2020). Similarly, however levels of ROS were found in the liver of

Table 1: Antioxidant enzyme activities after 15, 30, 45 and 60 days

Liver					
Antioxidant enzymes	**Groups**	**Days 15**	**Days 30**	**Days 45**	**Days 60**
	GI	2.37±0.02	2.39±0.02	2.42±0.02	2.35±0.02
SOD	GII	2.79±0.03*	3.03±0.05*	3.35±0.03*	3.35±0.07*
	GIII	3.01±0.10*	3.23±0.06*	3.61±0.04*	3.69±0.06*
	GIV	3.29±0.04*	3.51±0.08*	3.85±0.03*	3.85±0.10*
	GI	24.67±0.23	24.68±0.23	24.68±0.26	24.62±0.28
CAT	GII	26.31±0.27*	27.42±0.23*	28.99±0.28*	29.89±0.21*
	GIII	27.15±0.10*	28.78±0.28*	29.92±0.20*	31.67±0.25*
	GIV	29.06±0.32*	29.68±0.20*	31.70±028*	34.01±0.21*
	GI	17.72±0.32	17.69±0.39	17.69±0.69	17.72±0.44
GSH	GII	15.85±0.18*	15.26±0.31*	14.77±0.22*	14.00±0.67*
	GIII	15.21±0.44*	13.97±0.34*	12.49±0.35*	10.50±0.67*
	GIV	14.28±0.27*	12.97±0.40*	11.33±0.51*	8.96±0.66*
	GI	0.028±0.000	0.028±0.001	0.029±0.000	0.029±0.001
GR	GII	0.035±0.000*	0.040±0.001*	0.044±0.001*	0.047±0.002*
	GIII	0.039±0.001*	0.042±0.001*	0.047±0.001*	0.050±0.001*
	GIV	0.041±0.001*	0.048±0.001*	0.053±0.001*	0.056±0.001*
	GI	0.60±0.018	0.59±0.022	0.60±0.040	0.60±0.029
LPO	GII	0.80±0.025*	0.94±0.025*	1.10±0.031*	1.29±0.037*
	GIII	1.044±0.024*	1.19±0.029*	1.37±0.035*	1.77±0.035*
	GIV	1.32±0.025*	1.38±0.020*	1.49±0.037*	2.09±0.037*

Values are mean of three replicates ± S.E.M.; * represent significant ($p<0.05$) comparison with Group I.

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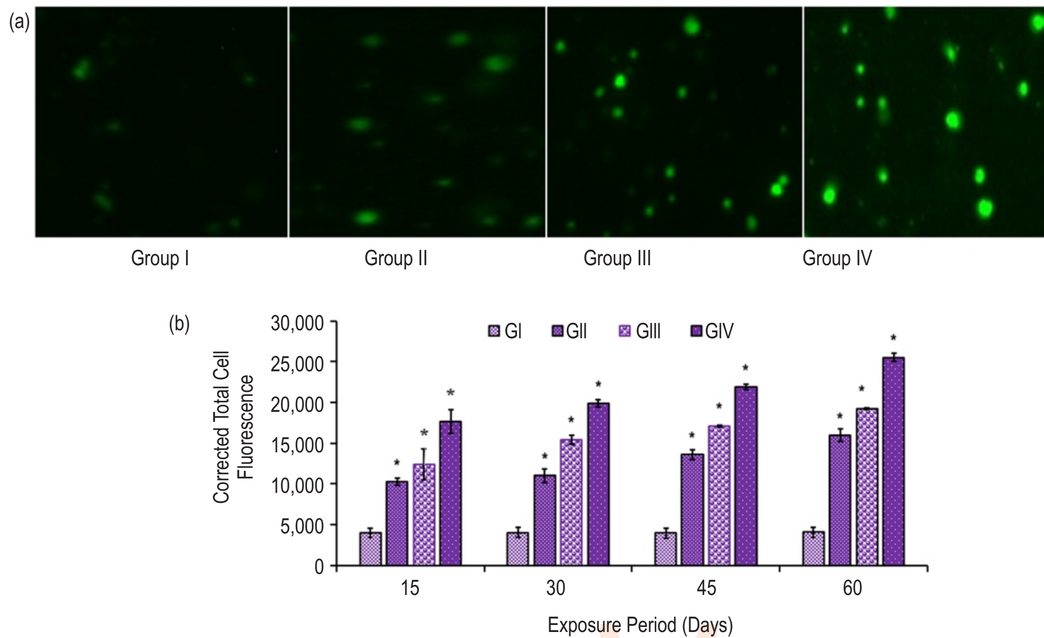

Fig.1: (a) Microphotographs showing fluorescent intensity of ROS level measured with DCFH-DA dye; (b) CTCF induced by Cu exposed as compared to Group I at 15, 30, 45 and 60 days. (Values are mean of three replicates ±S.E.M.). *symbol represents the significant ($p<0.05$) difference from the control).

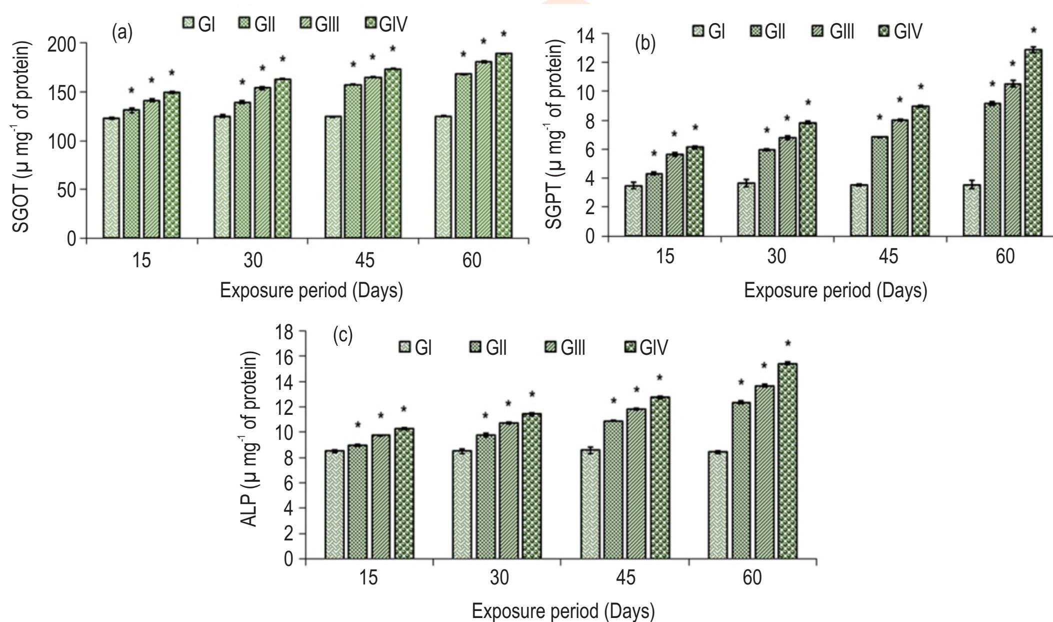

Fig. 2: Activity of SGOT (a), SGPT (b) and ALP (c) in *C. punctatus* for 15, 30, 45 and 60 days. (Values are mean of three replicates ±S.E.M.). *symbol represents the significant ($p<0.05$) difference from the control.

C. punctatus, when treated with Zinc (Trivedi *et al.,* 2023) and dichlorvos (Trivedi *et al.,* 2021). Cu-exposure has also induced the developmental deformities in the intestine of Zebrafish through ROS production (Zhao *et al.,* 2020). In the course of time, ROS generation in excessive manner can lead to the oxidation of cellular elements like protein, resulting in protein

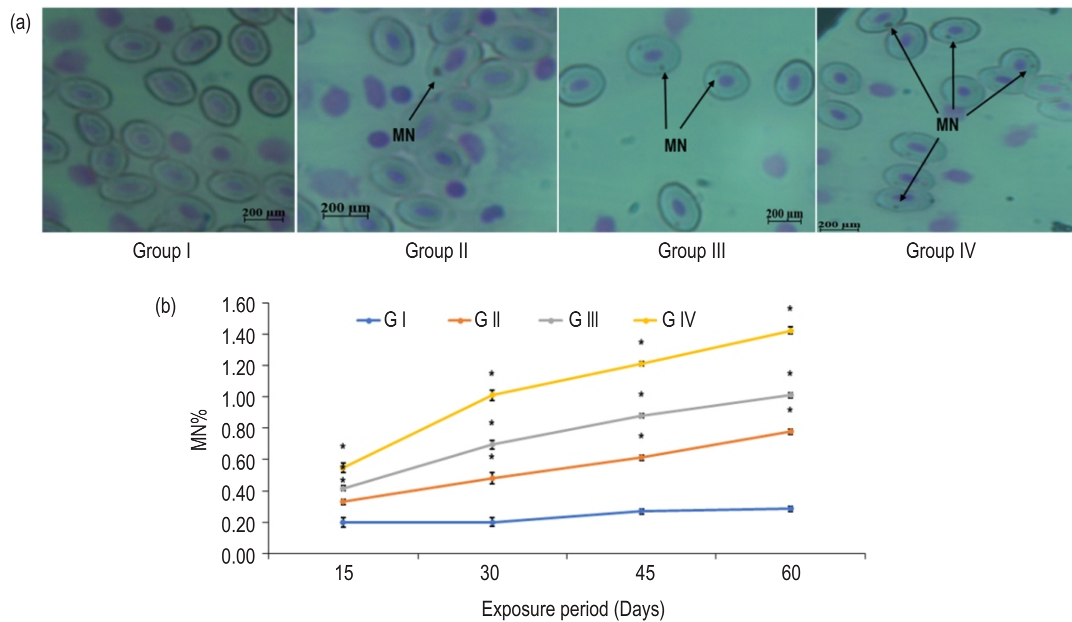

Fig. 3: MN frequency induced by Cu in GII, GIII and GIV as compared to GI for 15, 30, 45 and 60 days. (Values are mean of three replicates ±S.E.M.). *symbol represents significant ($p<0.05$) difference from the control.

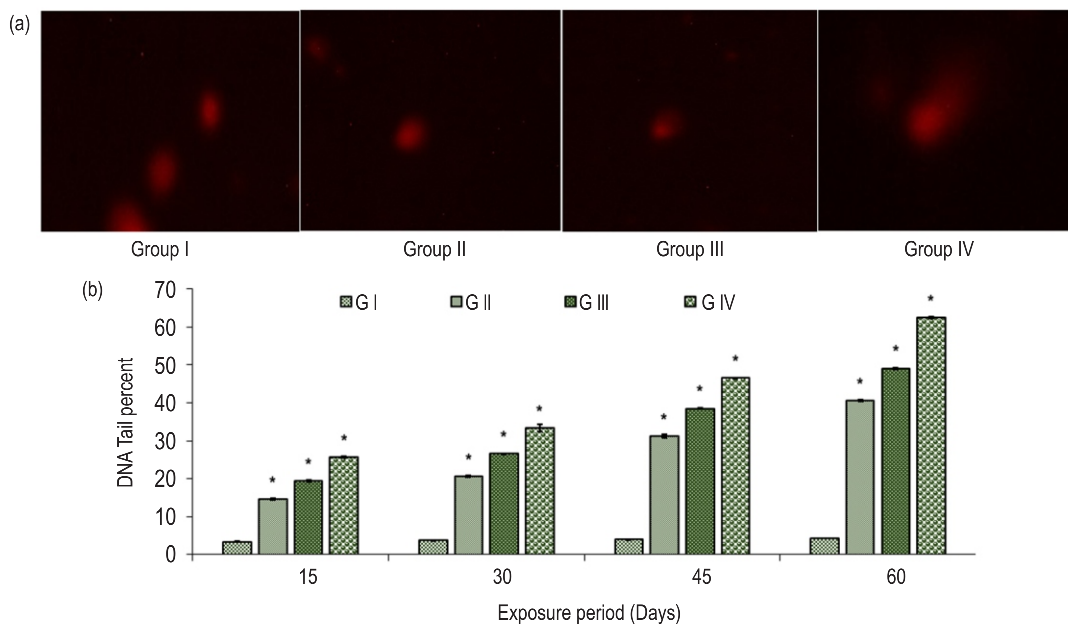

Fig. 4: DNA tail length % induced by Cu in GII, GIII and GIV) as compared to GI for 15, 30, 45 and 60 days. (Values are mean of three replicates ±S.E.M.). *symbol represents significant ($p<0.05$) difference from the control.

oxidation (Coccimiglio *et al.*, 2016).

Antioxidant enzyme's function is the ammunition against high ROS production. The lop-sidedness between the antioxidant combatants and ROS effectuates the oxidative stress. SOD and CAT are the main baseline of defence against ROS. SOD converts superoxide radicals into H_2O_2 and CAT catalyzes this H_2O_2 into H_2O and O_2. SOD-CAT have been regarded as the

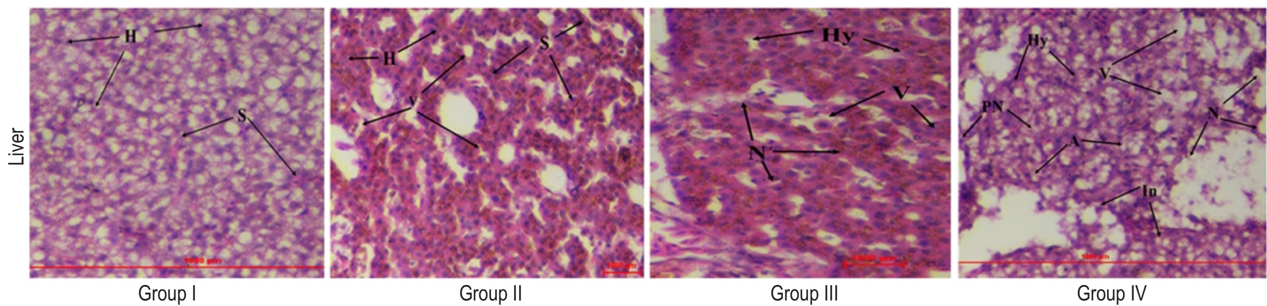

Fig. 5: Microphotographs of GI showing normal hepatocytes (H) and sinusoids (S) and exposed groups showing vacuolization (V), pyknosis (PN), Inflammation (In), necrosis (N), apoptosis (A) and hypertrophy (Hy) in the liver.

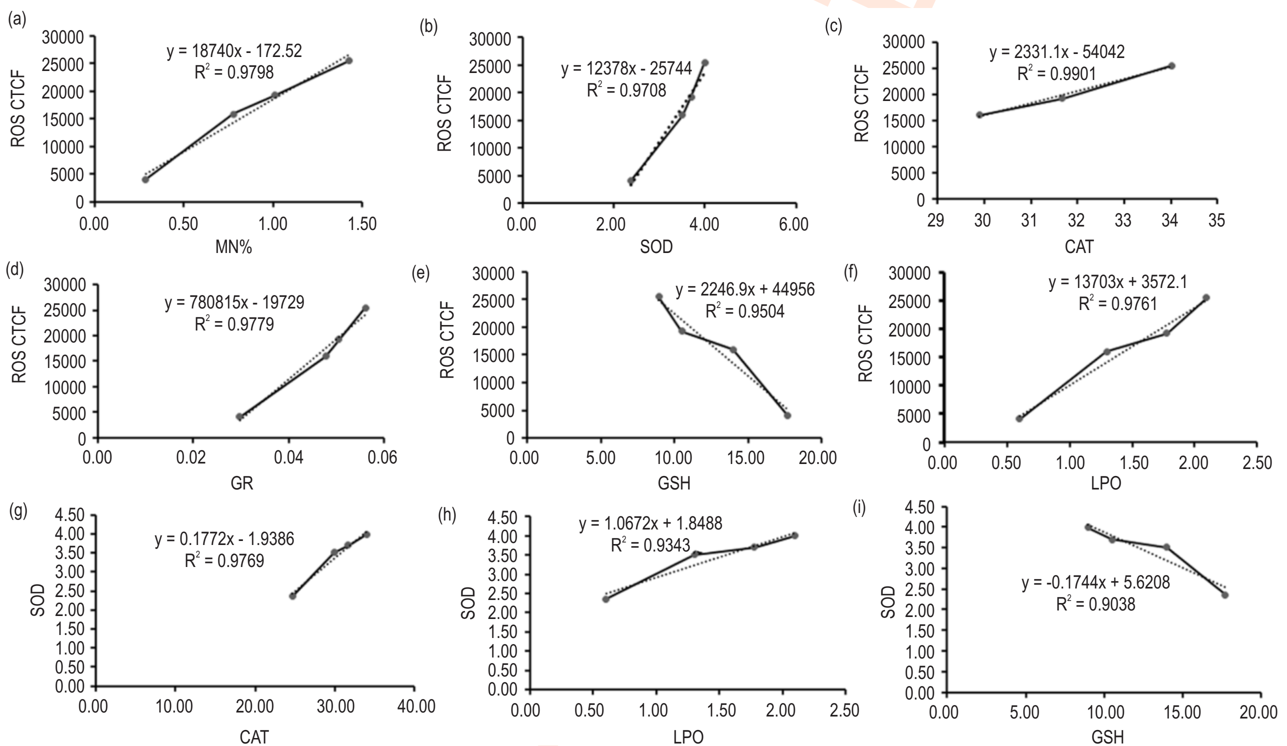

Fig. 6: a, b, c, d, f, g and h show the positive correlation between ROS-CTCF and MN%, ROS-CTCF and SOD, ROS-CTCF and CAT, ROS-CTCF and GR, ROS-CTCF and LPO, SOD and CAT, SOD and LPO; while Fig.6e and Fig.6i represent the negative correlation between ROS and GSH, and between SOD and GSH in the liver of *C. punctatus*.

crucial enzymes for stress tolerance and an increment in SOD-CAT activities is an indication of enhanced oxidative stress. In present explorations, impact of ERCC on SOD, CAT, GSH and GR activities in the fish liver were estimated and are presented in Table 1. Present findings observed significant ($p<0.05$) increased activities of SOD and CAT in a time- and dose-sensitive way in all exposed groups after 15, 30, 45 and 60d. The % intensification in SOD levels of GII, GIII and GIV as compared to GI was estimated after 15d as 17.72, 27 and 38.81, respectively; after 30d as 26.72, 35.14 and 46.86, respectively; after 45d as 38.42, 49.17 and 59.09, respectively; while after 60d as 42.55, 57.02 and 63.82, respectively. Similarly, the % rise in CAT levels of GII, GIII and GIV as compared to control was assessed as 6.64, 10.05 and 17.79, respectively after 15d; as 11.10, 16.61 and 20.25, respectively after 30d; as 17.46, 21.23 and 28.44, respectively after 45d; while as 21.40, 28.63 and 38.14, respectively after 60d. The maximum elevation of SOD and CAT activities were recorded in Group IV. An expansion in SOD-CAT activities in *C. punctatus* (Kumar *et al.*, 2022) and three-spined stickleback (Sanchez *et al.*, 2005) under Cu-stressed environment was also observed in previous studies. The reduction of GSSG into GSH occurs by GR via using NADPH, which employs an efficacious regulation against the generation of

ROS amidst the duration of subjection to xenobiotics. The activity of GR was found to be significantly ($p<0.05$) intensified upon exposure of Cu^{2+} in present studies. The % increase in GR extents of GII, GIII and GIV as compared to GI was evaluated after 15d as 25, 39.28 and 46.42, respectively; after 30d as 42.85, 50 and 71.42, respectively; after 45d as 51.72, 62.06 and 82.75, respectively; while after 60d as 62.06, 72.41 and 93.1, respectively. GIV reported the maximum elevations in GR activity. GSH is majorly involved in the safeguarding of antioxidants that are non-enzymatic. In present exploration, the levels of GSH were significantly ($p<0.0.5$) declined at each exposure duration in all treated groups. The % reduction in GSH activities of GII, GIII and GIV as compared to control was calculated as 10.53, 14.16 and 19.41, respectively after 15d; as 13.73, 21.02 and 26.68, respectively after 30d; as 16.5, 29.39 and 35.95, respectively after 45d; while as 20.99, 40.74 and 49.43, respectively after 60d. The highest decrease in GSH extents was noted in GIV at 60d. Therefore, the deviated extents of enzymatic and non-enzymatic anti-oxidants proclaim the increment in oxidative stress in Cu-stressed organs of the test fish. Similar outcomes i.e., escalations in the activities of SOD, CAT and GR were found in the test fish *C. punctatus* intoxicated with the variable doses of Cu^{2+} and Cr^{6+} (Kumar *et al.,* 2023). In another related experiments, the importance of stress biomarkers was documented in evaluating the chromium intoxication in *Labeo rohita (*Kumari *et al*., 2014) and $HgCl_2$ - induced toxicity in *C. punctatus* (Trivedi *et al.*, 2022). Another research highlighted the assessment of oxidative stress induced by Cu-treatment in liver of rats (Quamar *et al.*, 2019). It has been acclaimed that not only ROS but also the oxidative stress can perform the function of stimulator of programmed cell death (Aghvami *et al*., 2018).

As the antioxidant production is overstepped by the ROS generation, biomolecules can be devastated by exaggerated ROS through the process of attachment of free radicals to PUFA (Polyunsaturated Fatty Acid) side chains present in the plasmalemma and subsequently LPO is elicited. LPO is a cataclysmic process that is to be blamed for the deterioration of fluidity and strengthening of rigidity of cell membranes (Ma'rifah *et al*., 2019). In present findings, activity of LPO was significantly ($p<0.05$) increased in all treated groups as compared to control which is shown in Table.1. The % increment in LPO quantities of GII, GIII and GIV as compared to control was estimated after 15d as 33.33, 74 and 120, respectively; after 30d as 59.32, 101.69 and 133.89, respectively; after 45d as 83.33, 128.33 and 148.33, respectively; while after 60d as 115, 195 and 248.33, respectively. The activity LPO significantly ($p<0.05$) increased in all treated groups as compared to control (Table 1). In a dose and time-dependent manners. The highest activity was recorded in Group IV after 60 days.

Present study pointed out important alterations in different levels of TBARS production in liver which is in line with the results of Ballesteros *et al.* (2009); Li *et al.* (2003) who have recorded increased TBARS levels, which may be due to enhanced ROS generation in Cu-exposed fish (Samanta *et al.,* 2014). Lipid peroxidation caused due to free radicals may lead to the destruction of plasma membrane structure and consequently to the hepatic disruption which is signified by the elevated levels of liver biomarkers (Husen *et al.,* 2019). The assessment of liver biomarkers *viz*. SGOT, SGPT and ALP activities was carried-out to explicate the biochemical alterations that were generated due to Cu-induced cytotoxic stress. The activities of SGOT, SGPT and ALP in all copper-exposed groups are displayed in Fig. 2 a-c. The % increment in SGOT levels of GII, GIII and GIV as compared to GI was calculated after 15d as 6.9, 15.27 and 21.92, respectively; after 30d as 11.46, 22.87 and 30.41, respectively; after 45d as 26.46, 33.33 and 39.39, respectively; while after 60d as 34.62, 44.84 and 51.35 respectively. Similarly, the % rise in SGPT levels of GII, GIII and GIV as compared to control was estimated as 23.78, 61.6 and 75.64, respectively after 15d; as 64.36, 87.01 and 116.29, respectively after 30d; as 94.03, 127.84 and 154.26, respectively after 45d; while as 156.74, 195.22 and 260.95, respectively after 60d. Likewise, the % increase in ALP levels of GII, GIII and GIV as compared to GI was estimated after 15d as 5.77, 14.95 and 21.08, respectively; after 30d as 14.95, 26.26 and 34.51, respectively; after 45d as 26.77, 37.60 and 48.54, respectively; while after 60d as 46.62, 62.27 and 83.15 respectively. Present outcomes validated a significant ($p<0.05$) enhancement in the activities of SGOT, SGPT and ALP in test fish under Cu^{2+}-stressed environment which is in assent with the findings of Trivedi *et al.* (2021). Similar increasing trends were reported in previous researches performed, on chromium-poisoned *C. punctatus* Trivedi *et al.* (2021) and on Zinc sulphate-exposed *Clarias batrachus* Srivastava and Prakash (2018).

It has been well-confirmed that copper induces oxidative stress Wang *et al.* (2023) and can invade DNA, as a result of which clastogenic and molecular damages are caused Mandil *et al.* (2020). The induction of MN formation has been outlined as a responsive biomarker of genotoxic stress in aquatic life-forms (Trivedi *et al*., 2021). Micronuclei are cytoplasmic chromatin structures that have appearance like small-nuclei and they are formed from the bits and pieces of chromosome or from the complete chromosome that persists unbroken at anaphase of cellular division (Kumar *et al*., 2022). An escalation in the frequency of micronuclei in copper-exposed fish is an indication of chromosomal destruction. In the present study, after being exposed to various ERCC, significant ($p<0.05$) increase in the induction of micronuclei was evinced in the blood of *C. punctatus*. MN frequency in GII, GIII and GIV as compared to control was recorded as 0.33% ± 0.01, 0.41% ± 0.01 and 0.54% ± 0.02, respectively after 15d; as 0.47% ± 0.03, 0.69% ± 0.02 and 1.01% ± 0.03, respectively after 30d; as 0.61% ± 0.01, 0.87% ± 0.01 and 1.21% ± 0.01, respectively after 45d; while as 0.77% ± 0.01, 1.00% ± 0.01 and 1.42% ± 0.02, respectively after 60d. The highest frequency was recorded in GIV as compared to GI (Fig. 3). Present findings depicted that the intoxication of copper has accelerated the prevalence of MN in model fish *C. punctatus* in a dose- and time-dependent manner. Parallel inferences were drawn in former investigations, when *C. punctatus* was treated with chromium (Trivedi *et al.,* 2021). Oxidative stress-mediated

genotoxicity was also described by Awasthi *et al*., (2019) in *C. punctatus.* Besides micronuclei test, SCGE is another reliable technique to evaluate the genotoxicity in terms of DNA disruption upon exposure to xenobiotics Khan *et al.* (2020). In SCGE, the damaging of DNA is observed as the formation of a tail like structure that appears similar to a comet.

In the present study, the DNA damage was evaluated as percentage tail length of DNA in fish blood cells and a significant ($p<0.05$) increment was witnessed in treated groups as represented in Fig.4. The DNA tail length % in GII, GIII and GIV as compared to GI was noted as 14.56 ± 0.18, 19.42 ± 0.13 and 25.68 ± 0.13, respectively after 15d; as 20.64 ± 0.18, 26.58 ± 0.17 and 33.29 ± 0.96, respectively after 30d; as 31.19 ± 0.47, 38.56 ± 0.19 and 46.58 ± 0.15, respectively after 45d; while as 40.59 ± 0.15, 49.12 ± 0.25 and 62.49 ± 0.21, respectively after 60d. The maximum DNA damage was recorded after 60 days in Group IV fish. The DNA damage was recorded to be dose and time-dependent. These findings were in accordance with the reports of Khan *et al*., (2020) and Bhat *et al*., (2023) who had conducted research on heavy metals-stressed *Oreochromis niloticus* and *Oncorhynchus mykiss*. Vishwakarma and Singh, (2023) also reported the copper-induced genotoxicity in the major carp *Labeo rohita*. Earlier investigations have also recorded the excessive damage in the DNA of various fish species viz. *C. punctatus, O. niloticus, Clarias gariepinus, Catla catla* which were intoxicated with different heavy metals (Javed *et al.,* 2016; Kehinde *et al.,* 2016; Latif *et al*., (2022). Furthermore, the histological alterations have been depicted as the end-point markers for assessing the potential of heavy metals to produce deleterious effects in the important organs of *C. punctatus* (Awasthi *et al.,* 2019). The effect of ERCC on the histology of fish liver after 60 days is shown in Fig. 5. The histology of normal liver showed polygonal cells which were found to be arranged in liver parenchyma with round nuclei, hepatocytes, bile ducts and blood vessels (G1). Notable changes that occurred in liver fish exposed to copper after 60 days of exposure were pyknosis (PN), vacuolization (V), inflammation (In), apoptosis (A) and necrosis (N) and the maximum deformities were found in GIV fish as compared to control. Likewise, Kumar *et al.* (2023) observed nuclear degeneration and vacuolization in liver of Cu-exposed *C. punctatus.* In another related research conducted on *Labeo rohita* stressed under various doses of heavy metals, histopathological modifications were documented in various vital tissues (Kaur *et al*., 2018). Histopathological lesions were also observed in the liver of mouse intoxicated with Cu^{2+} (Liu *et al*., 2020). Numerous ruinous effects are caused when heavy metals are accumulated in varied tissues of living creatures (Balali-Mood *et al*., 2021).

Close relationship of physiological and molecular perturbations with higher value of the coefficient of correlation (R) in tissues of experimental fish is delineated in Fig. 6. Markedly, copper-stressed groups had the overproduction of ROS which led to the DNA damage, increase inthe MN% induction. In present experimentation, positive correlation data was analyzed between ROS-CTCF (Corrected Total Cell Fluorescence) and MN% as +0.979 (Fig. 6a); ROS-CTCF and SOD as +0.970 (Fig. 6b); ROS-CTCF and CAT as +0.990 (Fig. 6c); ROS-CTCF and GR as +0.977 (Fig. 6d); ROS-CTCF and LPO as +0.976 (Fig. 6f); CAT and SOD as +0.976 (Fig. 6g); and also between SOD and LPO as +0.934 (Fig. 6h) while the negative correlation between ROS-CTCF and GSH as +0.950 (Fig. 6e) and between SOD and GSH as +0.903 (Fig. 6i). Thus, a highly positive correlation (R= +0.990) was observed between ROS-CTCF and CAT activity, while a highly negative correlation (R= +0.903) between the SOD and GSH activity was studied in present outcomes. This research was planned to outline the virulent repercussions of ERCC and other % of ERCC intoxication in *C. punctatus.* Findings of this study, surprisingly demonstrate that fish living in the river Ramganga are experiencing severe toxic effects even at ERCC. Repercussions in terms of biochemical end-points, genotoxicity and histopathology were evaluated in the present investigation but still, there remains many questions to be explored in the future researches, such as, (i) Whether ERCC cause impairment in the gene expression of *C. punctatus* ? (ii) Whether ERCC-induced toxicity provokes any cell-death pathway in the test fish? (iii) Do other aquatic organisms also get negatively impacted by ERCC-exposure?

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