JEB®

Journal of Environmental Biology

p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP
Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Triveni Enterprises
(Educational Services Set-up)

Original Research

DOI : http://doi.org/10.22438/jeb/44/4/MRN-5062

Acute exposure of Cr and Cu induces oxidative stress, genotoxicity and histopathological alterations in snakehead fish *Channa punctatus*

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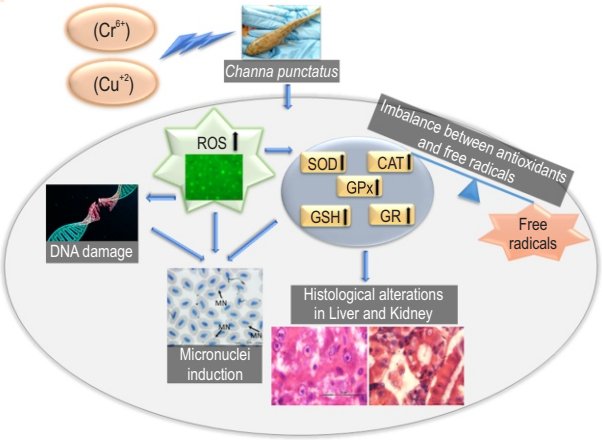
Received: 04.06.2022 *Revised*: 06.09.2022 *Accepted*: 24.12.2022

Abstract

Aim: This study aimed to investigate the oxidative stress, genotoxicity and histopathology after sub-lethal exposure of chromium (Cr^{6+}) and copper (Cu^{+2}) to common food fish *Channa punctatus*. Metal exposure to fishes of River Ganga is a very common phenomenon due to their ubiquitous nature and through discharge of domestic and industrial waste into the river water.

Methodology: An *in-vitro* experiment was performed to study ill effects of metallic stressors on fish. Fishes were divided into seven groups. Group I served as control, Group II, III, IV as 1/20th ,1/10th and 1/5th of 96 hr-LC_{50} of Cr^{6+}. Group V, VI and VII were similarly treated with Cu at 1/20th, 1/10th and 1/5th of 96 hr LC_{50} of Cu^{+2}, respectively. All the treatments were done for 15, 30 and 45 days of exposure period. Genotoxicity was evaluated by micronuclei (MN) induction. Enzymatic and non-enzymatic oxidative stress markers, including reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione reductase (GR), and glutathione peroxidase (GPx) were evaluated. Histopathological studies of liver and kidney tissue were also performed.



Results: Stress biomarkers were more profound in fishes of chromium treated groups in comparison to copper. Particularly, SOD and CAT showed a significant hike in the activity levels. ROS levels in blood cells increased significantly ($p < 0.05$) in all the treated groups (Group II, III, and IV) in a dose dependent manner as compared with control (Group I). There was significant induction in micronuclei (MN) frequency in all the treated groups for both the metals. The highest frequency of MN induction was recorded in Group IV after 96 hr of the exposure period.Significant histological alterations were observed in liver and kidney of treated fish, changes were more pronounced in chromium treated fish than copper.

Interpretation: Acute exposure of chromium and copper induces ROS and generates oxidative stress mediated genotoxicity and histopathological perturbations in the prime organs of fish.Cr and Cu induces the changes in the production and accumulation of enzymatic antioxidant system for the regulation and scavenging of ROS induced by Cr and Cu.

Key words: *Channa punctatus*, Genotoxicity, Histology, Heavy metals, Micronuclei, Oxidative stress

How to cite : Kumar, M., S. Singh, S. Dwivedi, A. Trivedi, I. Dubey and S.P. Trivedi: Acute exposure of Cr and Cu induces oxidative stress, genotoxicity and histopathological alterations in snakehead fish *Channa punctatus*. *J. Environ. Biol.*, **44**, 552-561(2023).

Introduction

Water pollution is one of the major concerns throughout the globe because of increasing industrialization, urbanization and over exploitation of natural resources. Atmospheric deposition, geological weathering, untreated discharge of effluents from industries and domestic waste are the chief sources of pollutants in the aquatic environment that have been marked ecologically significant due to their toxic nature and bioaccumulation properties (Savassi *et al.,* 2020). Consequently, they have created many environmental and public health problems across the world (Carolin *et al.,* 2017). Chemically stable or non-biodegradable metals and metalloids are dangerous pollutants because of their bioaccumulation in the tissues of aquatic organisms, causing adverse effects to the organism and its consumers, including human beings (Kumar *et al.,* 2019). Exposure of aquatic organisms to metallic stressors induce the release of free radicals that cause oxidative stress, genotoxicity, apoptosis and other disorders (Trivedi *et al.,* 2022.).

Copper (Cu) is an essential trace metal involved in various functions like cofactor of antioxidant enzymes, biosynthesis of neurotransmitters and cellular respiration. An increase in the concentration of copper adversely affects aquatic organisms, causing osmoregulatory disorders, histological damage, induces oxidative stress that causes a reduction in enzyme activity, DNA damage, lipoperoxidation, protein carbonylation and ultimately animal death (Paula *et al.,* 2021). Another metallic stressor, chromium (Cr) is a transition metal found in different oxidation states like Cr^{3+} and Cr^{6+} in the environment. The Cr^{6+} state has a strong oxidation ability and higher toxicity than the Cr^{3+} state (Diaz *et al.,* 2012). It is a harmful pollutant because of its high mobility in water and high toxicological activity (Kim and Kang, 2017) causing oxidative stress, cellular apoptosis and necrosis, enzyme inactivation deformities, mutations and cancers (Chen *et al.,* 2021).

Toxicants present in the water enter the bodies of fish through gills, skin, and digestive tract. The type, degree and duration of exposure to pollutants, water quality and characteristics of fish affects the accumulation of toxicants in an organism which varies among species (Ali and Khan, 2018). Metals generally accumulate in the metabolically active tissues of the body and affect the enzymatic and metabolic activities of the organism because of the generation of reactive oxygen species; the imbalance between the antioxidant defense system and ROS level in the body causes induction of oxidative stress leading to the oxidation of macromolecules, peroxidation of lipid membranes, genotoxicity and apoptosis (Ali and Khan, 2018). SOD catalyzes the reduction of superoxide radical into hydrogen peroxide, which in turn is converted into oxygen and water by CAT. Non-enzymatic biomarker GSH also assists in the reduction of free radicals and frequently, it gets depleted at the time of neutralization of free radicals. Stress-induced genotoxicity was evaluated bymicronucleus assay. Comparatively large nucleus of erythrocytes is most ideal and suitable for micronuclei test in fish. Micronucleus appears as a small mass of chromatin in close vicinity of nucleus having similar stain as that of the main nucleus. The MN test shows both clastogenic and eugenic effects, which ultimately generate acentric chromosomal fragments of lagging chromosomes during mitotic anaphase. Thus, the MN test is a proactive approach to study genotoxic threats to fish and other aquatic organisms, their ecosystem, and ultimately their consumers (Trivedi *et al.,* 2022). In, the present study MN assay was an effective investigative test to assess the genotoxic potential of chromium and copper in freshwater fish, *C. punctatus*. Heavy metals cause histological damage in the fish with an inflammatory defensive reaction. It is adamant that histological perturbations are the markers of pollutants in the complete health of organisms in the ecosystem (Trivedi *et al.,* 2022). Tissue histopathological evaluation of vital organs like liver and kidney gives an insight into the toxic potential of chromium and copper.

Fish play a far more important role as contributors of nutrients to marine ecosystems. They contribute more nutrients to their local ecosystems than any other source-enough to cause changes in the growth rates of the organisms at the base of the food web and maintains the ecological balance in the aquatic ecosystem (Javed *et al.,* 2015). Being a rich source of protein, minerals and omega-3 fatty acid, fish is considered as an essential part of human diet (Zeitoun and Mehana, 2014). Although, *C. punctatus* is an important food fish of Northern India and is relished in huge quantities because of its good taste and ready availability, hence, the problem of metal toxicity needs detailed investigation. This study was performed to fill this gap and investigate discrete parameters such as oxidative stress, genotoxicity and histopathological alterations induced by chromium and copper in the liver and kidney of fish *C. punctatus*.

Materials and Methods

Ethical approval for this study was obtained from Institutional Animal Ethics Committee (IAEC) of University of Lucknow (vide registration no. 1861/ GO/ Re/ S/ 16/ CPCSEA, Lucknow).

Test fish, *Channa punctatus* (35 ± 3.0 g; 14.5 ± 1.0 cm) was obtained from the natural lentic habitats of Lucknow (26°55' N; 80°59' E), India. Fish were transported to the laboratory in plastic containers and treated with 0.05% $KMnO_4$ solution for 2-5 min before transferring into the aquaria, to remove dermal infections if any. Fish acclimatized for 15 days in well aerated 160 l capacity glass aquaria (100 × 40 × 40 cm^3) filled with 100 l of 10 day-old tap water (APHA, 2017). During the acclimatization period, commercial aquarium food pellets (Perfect Companion Group Company Limited, Thailand) were fed to fish. Fish were kept on starvation for a day prior to the start of the experiment. Copper sulfate (S.D. Fine-Chem. Ltd. Mumbai) and Chromium trioxide, (Sisco Research Laboratories Pvt. Ltd., Mumbai) was used as the test chemical.

Determination of 96 hr-LC_{50} of copper and chromium for fish, *Channa punctatus*: The standard acute toxicity bioassay under

OECD guidelines for fish acute bioassays (OECD203, 92/69/EC, method C1) and the standard methods of APHA (2017) were used to determine the 96 hr LC_{50} of Cu^{+2} and Cr^{6+} for *C. punctatus*. To ascertain the approximate toxic range of the test chemicals, ten healthy, equal-sized, and well-acclimatized fish (35±3.0 g; 14.5± 1.0 cm) were exposed to definite concentrations of test chemicals on a logarithmic scale *viz.,* 0.1, 1.0, 10, 100 and 1000 mg l^{-1} up to 96 hr in a semi-static bioassay system. The toxicity range of copper was reported between 1.0 and 10 mg l^{-1} and of chromium 10 and 100 mg l^{-1} based on fish mortality. After ascertaining approximate toxic range, ten distinct logarithmic concentrations *viz.,* 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg l^{-1} for copper and 15, 25, 35, 45, 55, 65, 75, 85, 95, and 105 mg l^{-1} for chromium and control were used. Experiments were performed in triplicate to check their reproducibility. At 24, 48, 72, and 96 hr of exposure periods, percentage fish mortality was recorded and their mean values were subjected to the Trimmed Spearman-Karber method (Hamilton *et al.,* 1977) software for determining the 96 hr-LC_{50} of copper and chromium.

Experimental setup: A total of 105 duly acclimatized fish were randomly selected and divided into seven groups, each having 15 fish, the Group I served as control, Group II – 1/20th of 96 h-LC_{50} of Cr^{6+}, Group III – 1/10th of 96 hr-LC_{50} of Cr^{6+}, Group IV - 1/5th of 96 hr-LC_{50} of Cr^{6+}, Group V – 1/20th of 96 hr-LC_{50} of Cu^{+2}, Group VI – 1/10th of 96 hr-LC_{50} of Cu^{+2} and Group VII - 1/5th of 96 hr-LC_{50} of Cu^{+2}. Every group was maintained in triplicate in separate aquaria. No mortality was evinced across the experiment. Aquaria were replenished every day to avoid the accumulation of waste and excretory materials (OECD, 2019). Physico-chemical parameters (pH, Temperature, DO, Alkalinity and Hardness) were recorded in the test medium before and after completion of 24, 48, 72, and 96hr of exposure periods, both in control and treated groups. After each exposure period (24,48,72 and 96 hr), three fish from each replicate were anesthetized using 0.1% (v/w) diethyl ether, and their blood (200 µl) was drawn from cardiac heart puncture through a heparinized syringe and stored in EDTA (1.8 mg ml^{-1}) coated vials for the analysis of ROS and MN. After sacrificing the fish, liver and kidney samples were collected and kept in phosphate buffer saline for the estimation of oxidative stress.

Micronuclei test for genotoxicity assessment: On a pre-cleaned glass slide, a uniform blood was made with a drop of blood of each sampled fish from every group. Slide was prepared as per Schmid (1975), Scoring of micronuclei and their identification was done by counting a minimum of 2000 erythrocytes per slide (Fenech *et al.,* 2011). Percentage micronuclei was calculated by the following formula:

MN% = Number of cells containing micronucleus x 100/Total number of cells counted

Estimation of ROS level: To estimate the ROS generation in blood cells, a fluorescent dye 2′, 7′-dichlorodihydrofluorescein (20 µM, DCFH-DA; Sigma Aldrich, USA) was used (Saha *et al*., 2023). The concentration of intracellular ROS produced in the blood cells is directly proportional to the fluorescence intensity of DCF dye. Quantification of fluorescence intensity was measured using Image J software (version 1.50, USA) and ROS data was expressed as the fold changes of exposed groups against the control.

Activity of enzymatic and non-enzymatic biomarkers of oxidative stress: To study the biochemical parameters, phosphate buffer saline washed liver and kidney tissues were homogenized separately in homogenization buffer (HB) in ratio 1:10 (w/v). Homogenized cell suspension of each tissue was centrifuged twice at 3000 rpm for 10 min. at 4°C in a falcon tube. The pellet obtained was suspended in 500µl of lysis buffer, 10 µl of Phenylmethylsulfonyl fluoride and 10µl of Dithiothreitol was also added to it and incubated for 30 min. at 4°C, and further centrifuged at 12,000 rpm for 15 min at 4°C, and supernatant was used for the analysis of oxidative stress biomarkers. SOD activities in the liver and kidney tissues were estimated by the method of Kakkar *et al.* (1984) and was expressed as units min^{-1} mg^{-1} protein. Catalase activity was measured by the method of Aebi (1984), which is based on the decomposition of H_2O_2 and expressed as µM H_2O_2 decomposed min^{-1} mg^{-1} protein using an extinction coefficient of H_2O_2 as 0.041/µmole/cm^2. GPx activity was estimated following the protocol of Flohé and Günzler (1984) and expressed as nMGSH oxidized min^{-1} mg^{-1} protein. Carlberg and Mannervik, (1975) method was used to estimate the activity of GR. GR activity was measured by estimating the rate of NADPH oxidation at 340 nm and was expressed in terms of nM NADPH oxidized min^{-1} mg^{-1} protein. GSH, a non-enzymatic marker of oxidative stress was analyzed by the method of Ellman (1959) and expressed as µg mg^{-1} of protein.

Liver and kidney histopathological analyses: The tissues were washed in 0.9% NaCl solution to remove the impurities, debris and blood. Tissues were fixed in Bouin's fluid for two days, and washed twice a day with ethanol for 4–5 days to remove excess Bouin's fluid. Thereafter, tissues were dehydrated in graded series of ethanol, and washed with xylol and then embedded in paraffin wax to obtain. The tissue blocks. Section of liver and kidney were cut by rotary microtome (YSI062 Yorco, India). Staining and counter staining was done with haematoxylin and eosin. The tissue photographs were taken using an oil immersion microscope (Nikon Corporation K 12,432) with 10/40X magnification. Analysis of images were performed using Image J software; version.

Statistical analyses: All the data were analyzed using SPSS software (version 20.0, SPSS Company, Chicago, USA). Three replicates from each experimental group were taken for analysis and all the data were presented as mean ± standard error mean (S.E.M.). To test the significance ($p<0.05$), one-way analysis of variance (ANOVA) with Tukey's post hoc test was performed for each result.

Results and Discussion

The 96 hr-LC_{50} value of Cr^{6+} to *C. punctatus* was estimated to be 78.12 mg l^{-1} with 95% lower and upper

confidence limits of 67.72 mg l^{-1} and 92.50 mg l^{-1}, respectively. Physico-chemical parameters *viz.,* pH-7.6, temperature - (25.2ºC), dissolved oxygen (DO) – 6.78 mg l^{-1}, alkalinity – 74.21 mg l^{-1} and hardness – 72.45 as $CaCO_3$ mg l^{-1} were recorded in the test medium after completion of 24, 48, 72 and 96 hrs of exposure, both in control and treated groups. All the values were observed well within the prescribed limits for the survival of fishes (APHA, 2017). There was a significantly higher induction of MN in fish exposed to different concentrations of Cr^{6+} and Cu^{2+} than the control group (Fig. 1A). Further, the induction of MN was found to be highest in Group IV and VII after 96 hr exposure period (Fig. 1B). The frequency of MN induction was higher in chromium than in copper. Micronucleus assay test in fish is usually performed on enucleated erythrocytes, mainly because of the technical feasibility. Most studies have reported that a short-term exposure time of 24 to 96 hr is sufficient to induce micronuclei, and erythrocytes have been described as a sensitive biomarker of genotoxicity (Bücker *et al.,* 2012). Micronuclei are a mass of cytoplasmic chromatin resembling a small nucleus that arises from chromosome fragments or whole chromosomes remaining intact at the anaphase stage of cell division (Fenech *et al.,* 2011).

In our study, we observed that higher doses of chromium and copper increased the frequency of micronuclei in *Channa punctatus* erythrocytes. Chromium can cause chromosomal breakage and induces MN induction in erythrocytes of fish *C. punctatus* has been demonstrated in earlier studies (Trivedi *et al.*, 2021, 2020). Copper significantly increases the MN frequency in erythrocytes in a dose-dependent manner. MNT as a genotoxic endpoint for copper-induced clastogenic effects has been extensively studied in aquatic organisms (Trivedi *et al.,* 2021). Although the mechanism of copper genotoxicity is still poorly understood, several mechanisms of Cr^{6+} genotoxicity have been proposed. Upon contact with the cell, the chromate anion easily crosses the cell membrane and is reduced inside the cell. Reduction of Cr^{6+} results in the formation of Cr^{3+}, a stable reduced form that binds to DNA more efficiently than Cr^{6+} and is thought to play a role in chromium genotoxicity. In this study, it was observed that the Cr^{6+} intoxicated groups had a higher MN frequency than the Cu^{2+} groups, which is consistent with the reports of Nagpure *et al.* (2017) who documented higher micronuclei induction in chromium-contaminated water. The exposure of erythrocytes to Cr^{6+} and Cu^{2+} increased ROS production in the cells. This increase was much higher in Group IV and VII (Fig. 2A).

The ROS levels were significantly ($p < 0.05$) increased in a dose dependent manner in Groups II, III, IV, V, VI and VII as compared to Group I (Fig. 2B). There was an increase in ROS at each concentration of Cr^{6+} and Cu^{2+} but the highest increase was recorded at 15.62 mg l^{-1} of Cr^{6+} and 0.8 mg l^{-1} of Cu^{2+}, respectively. The current study was initiated to demonstrate the sensitivity of *Channa punctatus* to the potentially harmful heavy metals chromium and copper. Fish are confined to aquatic habitats and are most susceptible to heavy metal toxicity. Aquatic toxicity test is used to assess the potential toxicological effects of environmental pollutants on aquatic biota. There is a need to test for the presence of water-borne heavy metals for tolerance of most sensitive bioindicators of water pollution, especially fish. ROS are important for cellular signaling and various physiological processes; induction in ROS levels andoxidative stress is dangerous for body. Since Cr^{6+} and Cu^{2+} can increase ROS in the liver and kidneys (Liu *et al.,* 2020; Wakeel *et al.,* 2020), additional studies we performed on the rate of ROS generation and the activation status of various antioxidant enzymes SOD, CAT, GSH, GR and GPxon the vital organs. ROS increase was significantly accompanied with increased levels of exposure to Cr^{6+} and Cu^{2+} in this study. It has previously been reported that ROS are increased in the liver of copper-poisoned rats (Liu *et al.,* 2020). After accumulation of H_2O_2 in sufficient amount in exposed cells it increases ROS and oxidative stress, H_2O_2 gets transported into the nucleus wherein they cause DNA damage resulting in the generation of MN. Similarly, ROS increased was observed in Channa punctatus when exposed to dichlorvos (Trivedi *et al.*, 2021) and zinc (Ratn *et al.*, 2018).

Similarly, Cr^{6+} significantly stimulated ROS production in carps (Krumschnabel and Nawaz, 2004). The highest increment in ROS was observed in the liver of Cr^{6+} exposed groups compared to the Cu^{2+} exposed groups, this may be due to ROS produced during the reduction of Cr^{6+} to Cr^{3+}. The catalytic power of Cr is greater than Cu (Wakeel *et al.,* 2020). GSH level in liver and kidney of fish exposed to different concentrations of Cr^{6+} and Cu^{2+} were significantly higher ($p<0.05$) at each exposure period compared to the control (Fig. 3a). After 96hr, there was a statistically significant difference between the group exposed to the highest concentration of Cr^{6+} and Cu^{2+} and the control. The effects of exposure on GR activity in fish liver and kidney are presented in Fig. 3 b. The GR activity increased significantly in all the treated Groups (II, III,IV, V, VI and VII) as compared to Group I in liver and kidney tissues. A significant ($p < 0.05$) highest increase in the activities were observed in GSH and GR in the Cr^{6+} exposed liver than Cu^{2+} exposed kidney. GPx activity was significantly ($p< 0.05$) elevated in a concentration dependent manner in liver, and kidney tissues after Cr^{6+} and Cu^{2+} exposure of concentrations, when compared to the control group (Fig. 3 c). A significant ($p< 0.05$) increase in GPx activity in exposed tissues was observed in all treatment groups at six test concentrations. The highest GPx activity was observed in liver than kidney after 96hr of exposure in chromium exposed groups than copper. SOD activity in liver, and kidney tissues of control and intoxicated groups are presented in Fig. 3d. In these tissues, SOD activity increased in a concentration dependent manner. The highest level of SOD activity was observed in liver as compared to kidney tissue at each concentration on 28th day of exposure as compared to the control. In general, exposure of fish specimens to Cr^{6+} and Cu^{2+} resulted in an increased CAT activity in subject tissues, as compared to that of control specimens (Fig. 3e). A significant ($p<0.05$) increase in CAT activity was observed after 24hr, 48hr, 72hr and 96hr of exposure period in liver and kidney at each concentration, as compared to the control group.

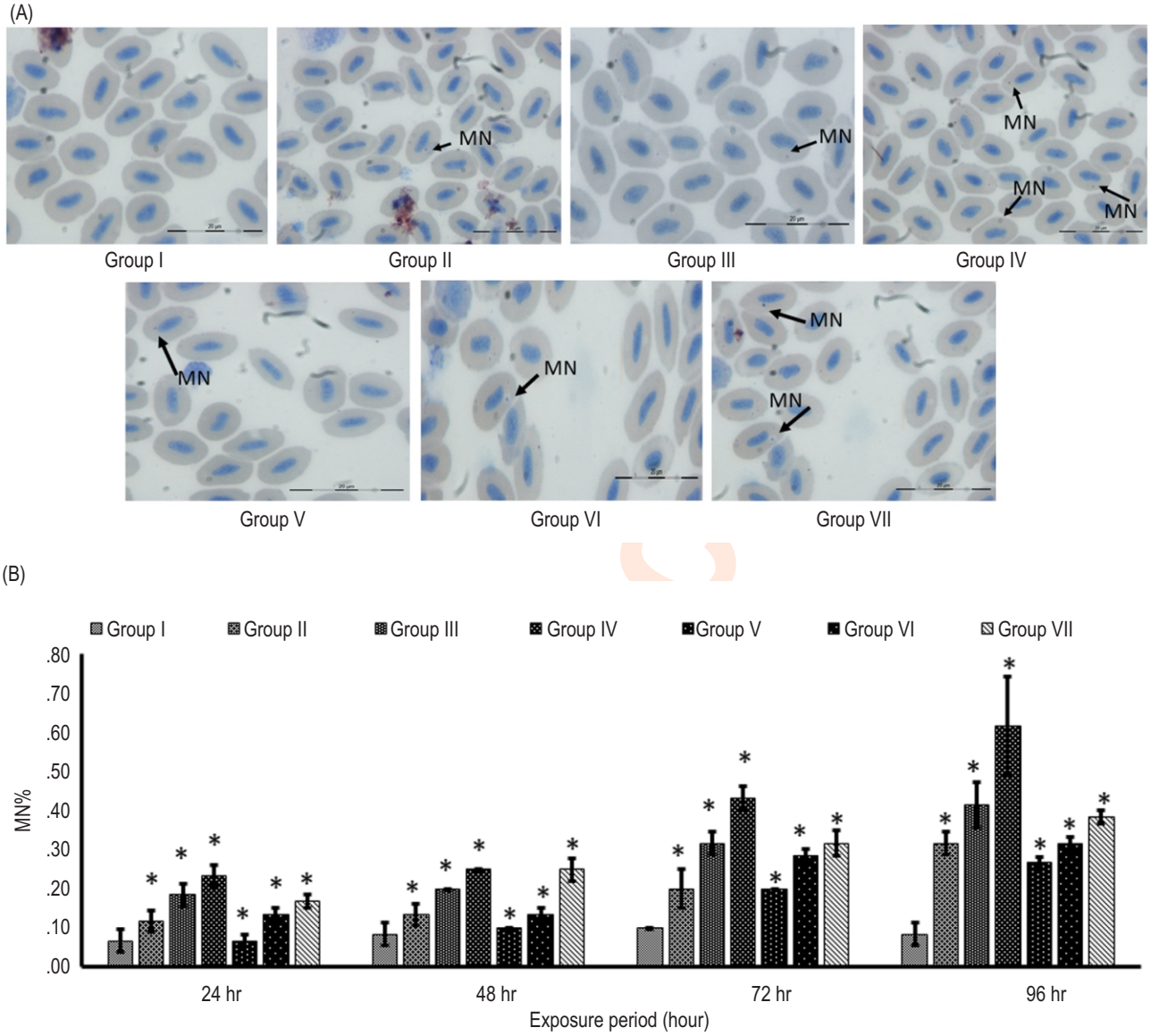


Fig. 1: (A) Cr^{6+} and Cu^{2+} induced micronuclei frequency in Groups II, III,IV, V, VI and VII as compared to Group I in erythrocytes of *Channa punctatus* after 96 hr exposure period; (B) Changes in MN frequency in Cr^{6+} and Cu^{2+} induced erythrocytes of Groups II, III, IV, V, VI, and VII as compared to Group I for 24, 48, 72 and 96 hrs of exposure period. The data are expressed as mean ± S.E.M. (n = 3 fishes) of three replicates of each group. (Mean ± SD, n = 3 fishes of three replicates of each group). (*represent the significant ($p < 0.05$) difference from control). Microphotographs of micronuclei at 100X magnification.

The maximum increase in CAT activity in liver tissues was found after 28 days of exposure period in chromium exposed groups than in copper exposed groups. Mitochondrial respiration is the main endogenous source of ROS. Increased ROS production can lead to protein and lipid oxidation, gene expression changes, and changes in the redox status of cells. Antioxidant protection mechanisms in fish include enzyme systems similar to those found in mammals. SOD, CAT, GPx and GR are the main antioxidant enzymes and important indicators of oxidative stress. GSH plays a key role in the protection of non-enzymatic antioxidants. The overproduced superoxide radicals are captured and converted to hydrogen peroxide (H_2O_2) by the SOD enzyme, and the CAT enzyme converts this released H_2O_2 into water and oxygen (Trivedi *et al.,* 2022). This study demonstrated a dose-dependent increase in SOD and CAT levels in liver and kidney tissues. Given that the SOD-CAT antioxidant defense system forms the basic protective framework against toxic substances in the body, it can be assumed that the abundance of Cr^{6+} and Cu^{2+} enhances the effects of SOD and CAT. The increase in GSH levels observed in this study after acute exposure to the liver and kidneys of *Channa punctatus* indicates of the detoxification process in the body after exposure to Cr^{6+} and

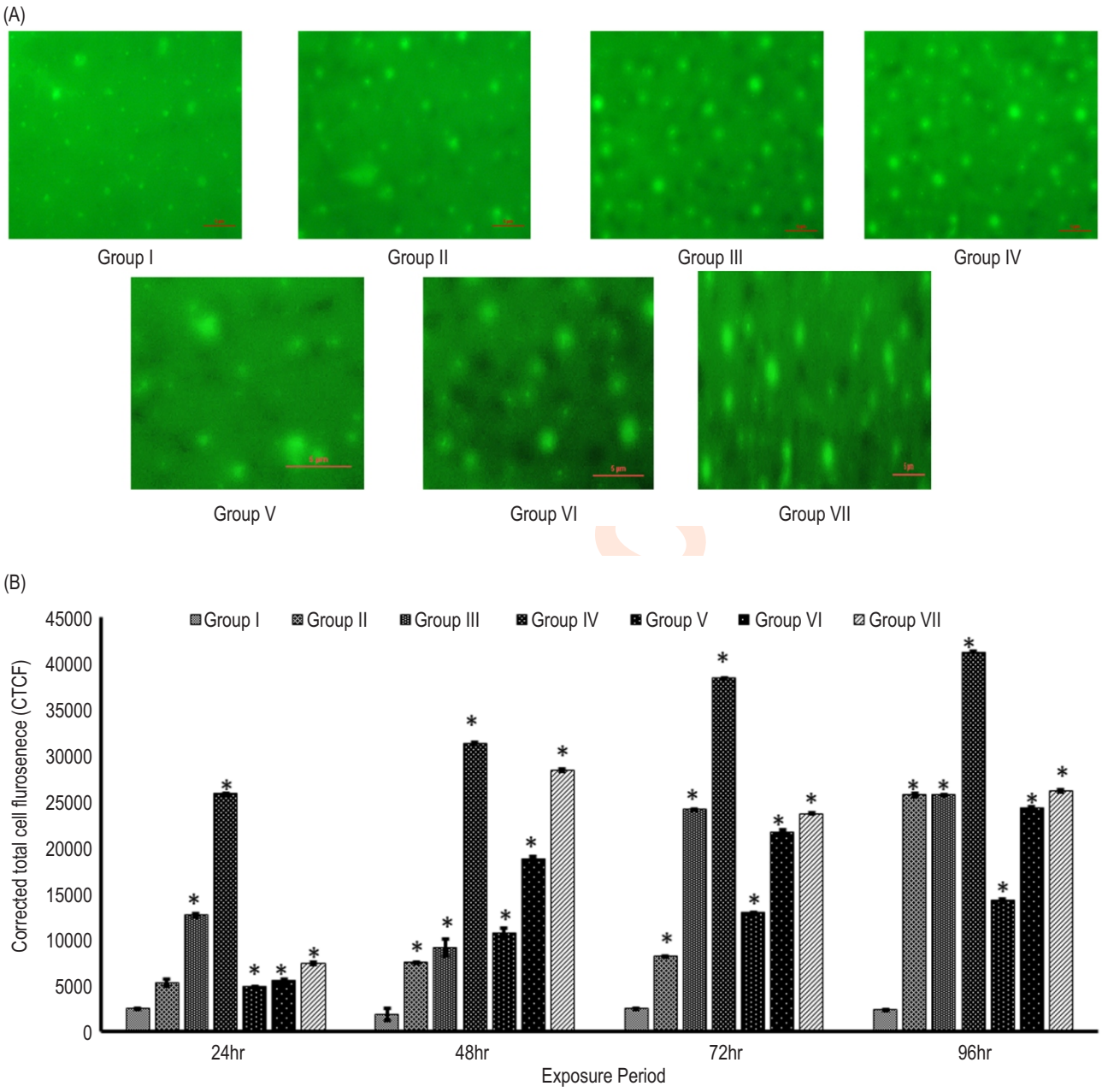


Fig. 2: (A) ROS induced by Cr^{6+} and Cu^{2+} in Groups II, III,IV, V, VI and VI after 96 hr of exposure period as compared to group I in erythrocytes of *Channa punctatus;* (B) The significant ($p < 0.05$) increase in ROS in Cr^{6+} and Cu^{2+} induced Groups II, III,IV, V, VI, and VII as compared to group I for 24h, 48, 72, and 96 hrs of exposure period. The data are expressed as mean ± S.E.M. (n = 3 fishes) of three replicates of each group. (Mean ± SD, n = 3 fishes of three replicates of each group). (*represent the significant ($p < 0.05$) difference from control).

Cu^{2+}. GPx is a protected antioxidant enzyme because it adapts to convert H_2O_2 and other peroxides into water and alcohol (Velma and Tchounwou, 2010). GR can reduce GSSG to GSH by utilizing NADPH, which effectively controls ROS production during exposure to xenobiotics. In this study, the levels of SOD, CAT, GSH, GR and GPx increased in a dose dependent manner after 24, 48, 72 and 96 hr of exposure period in liver and kidney tissues of fish exposed to chromium and copper. Similarly, Kumari *et al.* (2014) highlighted the significance of oxidative stress biomarkers in assessing Cr^{6+} toxicity in *Labeo rohita*. This study documents that oxidative stress has been considered as the main contributor to Cr^{6+} and Cu^{2+} toxicity in fishes, which is in accordance with the study of Quamar *et al.* (2019) who evaluated the toxicity of Cu^{2+} in terms of oxidative stress in liver and kidney of rats. This study also

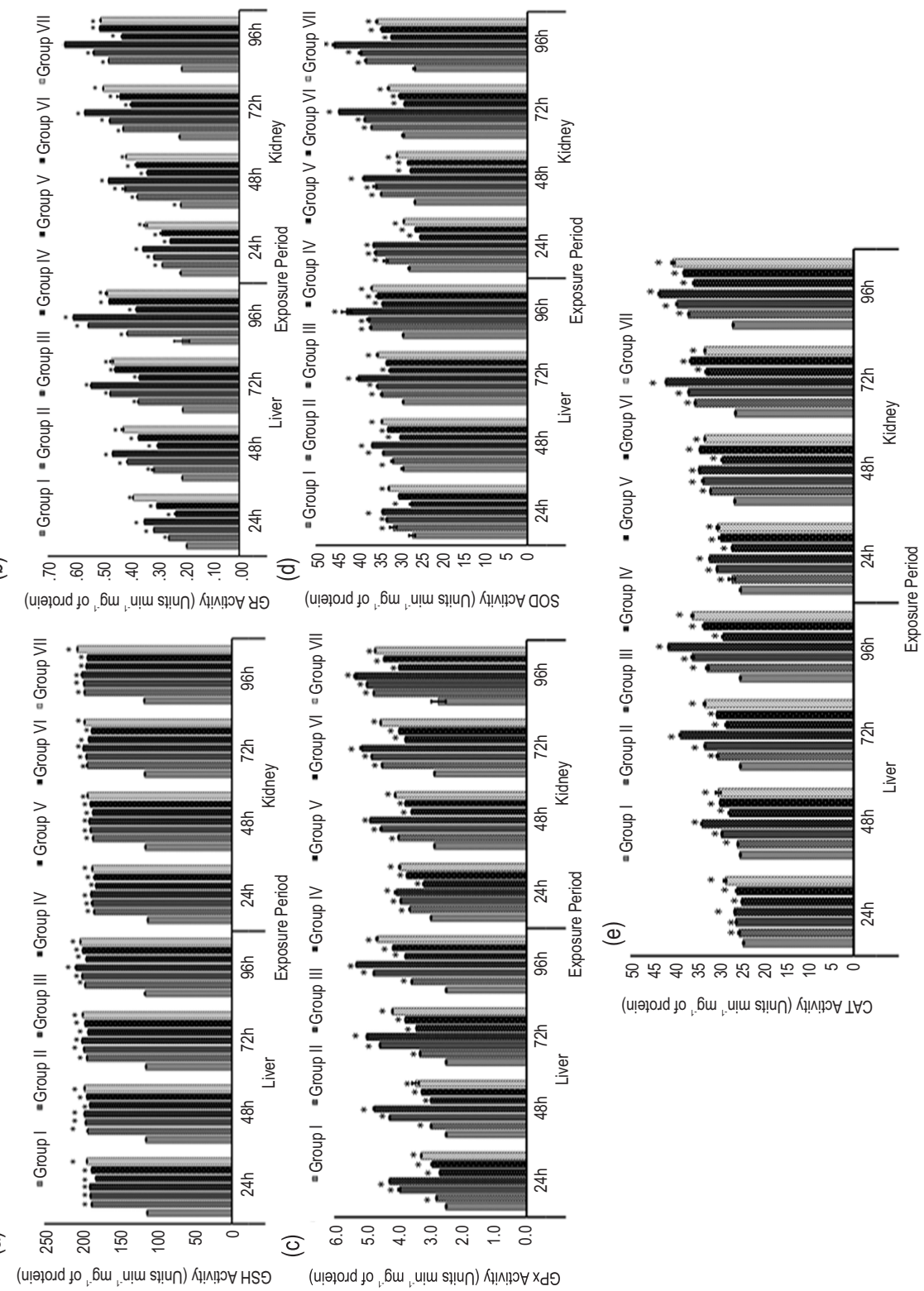


Fig. 3: Alterations in (a) GSH, (b) GR, (c) GPx, (d) SOD and (e) CAT activities in control (Group I) and intoxicated groups (Group II- 1/20th 96h-LC_{50} of Cr^{6+}; Group III- 1/10th 96h-LC_{50} of Cr^{6+}; Group IV- 1/5th 96h-LC^{50} of Cr^{6+}; Group V- 1/20th 96h-LC_{50} of Cu^{2+}; Group VI- 1/10th 96h-LC_{50} of Cu^{2+} and Group VII- 1/5th 96h-LC_{50} of Cu^{2+}) in liver and kidney tissues for 24h, 48h, 72h and 96h of exposure period in fish *C. punctatus*. (Mean ± SD, n=3 fishes of three replicates of each group). (*represent the significant ($p < 0.05$) difference from control).

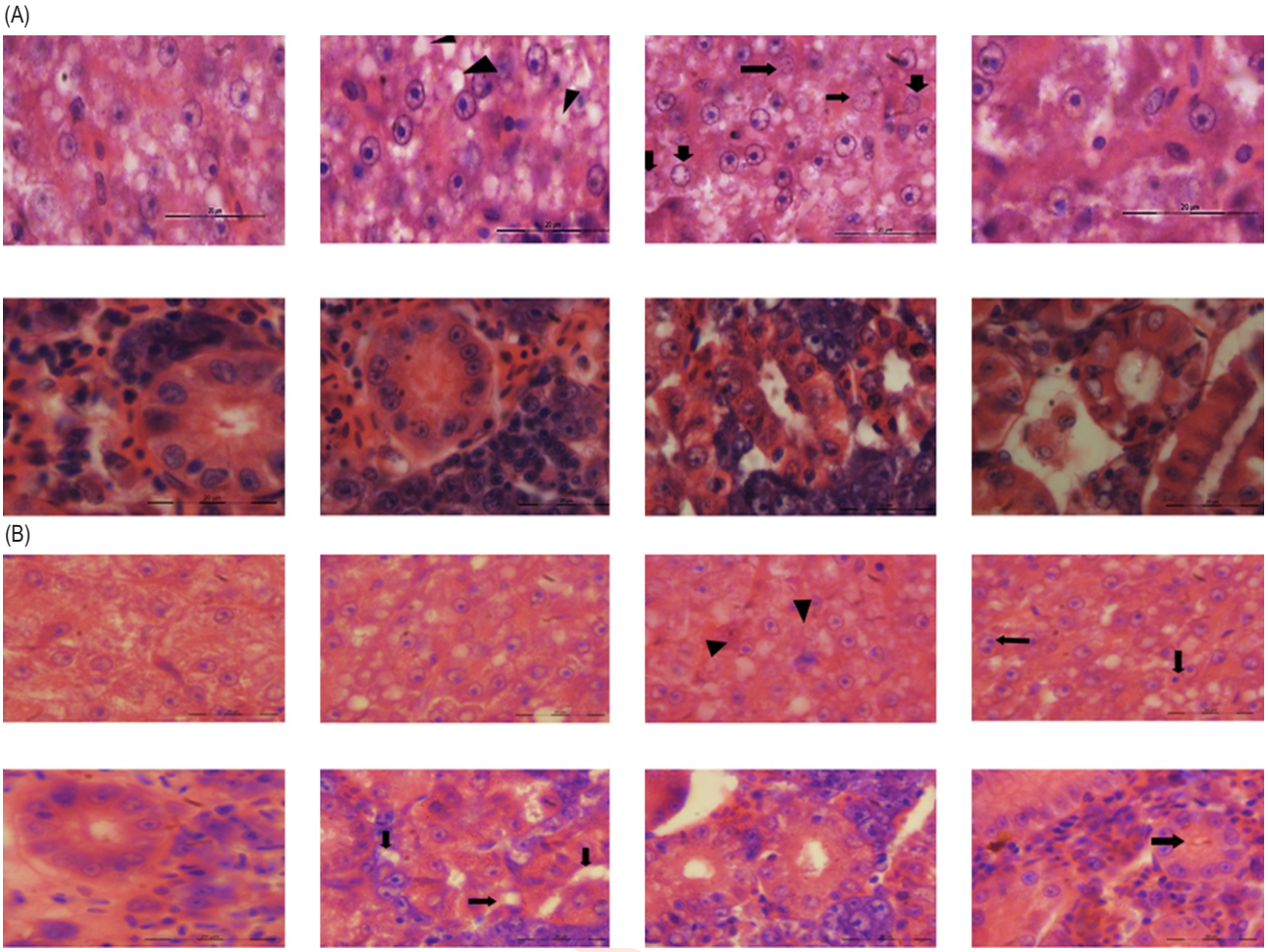


Fig. 4: (A) Microphotographs of section of the liver and kidney of *Channa punctatus* showing normal hepatocytes and well-spaced sinusoids, vacuolization (arrow head), nuclear degeneration (arrow), Pyknotic nuclei (first panel) and section of the kidney from control showing normal renal tubules, cavity reduction in renal tubule (CRRT), vacuolization and hypertrophy (second panel)induced by chromium in Group IV- 1/5th 96h-LC_{50} after 96 hr of exposure; (B) Microphotographs of section of the liver of *Channa punctatus* showing vacuolization, nuclear degeneration (arrow head), Pyknotic nuclei (third panel) and section of the kidney showing vacuolization hypertrophy and cavity reduction in renal tubule (CRRT)(fourth panel) induced by copper in Group VII- 1/5th 96 hr-LC_{50} after 96 hr of exposure (H & E X 100).

highlights the potential of Cr^{6+} to induce oxidative stress in liver and kidney of *Channa punctatus*.

Chromium and copper showed significant histological alterations in liver and kidney of *Channa punctatus* after 96hr of exposure period (Fig. 4 second and fourth panel) respectively. The incessant exposure of chromium and copper damage the cells of liver and kidney. The most crucial alteration induced by chromium and copper was observed as hypertrophy (35.34%) in the renal tubule of *C. punctatus* in Group VII and the highest percentage of hypertrophy (40.56%) was observed in Group IV. In the tubules, the most common alterations were: vacuolization 34.23 % and cavity reduction in renal tubule (CRRT) 30.21 % in group IV and (38.21 %) vacuolization and CRRT (34.78 %) in group VI. The quantitative histological alterations induced by Cr and Cu in the kidney and liver of *Channa punctatus* were more definite in Group IV and VII. The liver histology exhibited hepatocytes and sinusoids. Histological findings of liver tissue of *Channa punctatus* for control and exposed groups with chromium and copper, after 96 hr are shown Fig. 4 (first and third panel). Nuclear degeneration was evinced in the liver of Cr exposed Group IV (24.45 %) and Cu exposed Group VII (20.64 %). Vacuolization while in Group IV (15.28 %) and in Group VII (14.23 %), was observed in pyknotic nuclei in Group IV (18.10 %) and VII (15.45 %) were also common when compared with control group. In Groups II, III, V and VI, all the above-mentioned alterations were recorded in increasing trend. Relatively, the maximum damage was observed in Groups IV and VII. Abundant data are available on metal-induced histopathological changes in fish species. Similarly, chromium induced histopathological alterations

were observed by Awasthi *et al.* (2019) in liver of *Channa punctatus*. The results of this study showed histopathological lesions such as increased vacuolation (V), pyknosis (Py) and nuclear degeneration in the liver and decreased renal tubular cavity (CRRT), vacuolation (V) and hypertrophy (Hy) in the kidneys of exposed fish for all the groups. Similarly, Kaur *et al.* (2018) have documented the histopathological changes in liver, muscle and kidney of *Labeo rohita* intoxicated with different doses of heavy metals. Bioaccumulation of the heavy metals leads to many toxic effects on a variety of body tissues and organs (Balali-Mood *et al*., 2021). Heavy metals disrupt cellular events including growth, proliferation, differentiation, damage-repairing processes, and apoptosis which leads to various alterations in tissues of organism (Ameur *et al*., 2015; Jaishankar *et al*., 2014; Mela *et al*., 2007) .

This study was designed to investigate the toxic potential of Cr^{6+} and Cu^{2+} in *Channa punctatus*. The results of this study shows that *Channa punctatus* is more sensitive for DNA damage and oxidative stress in chromium contaminated aquatic habitats. Moreover, these findings may be helpful in establishing the best practical technologies and regulatory levels of chromium and copper in industrial effluents.

Acknowledgments

We record our sincere thanks to the Department of Higher Education, Uttar Pradesh Government, U.P. India, for providing the grant under Research and Development Scheme 2020-21. We are also grateful to the Head, Department of Zoology, University of Lucknow, Lucknow for providing laboratory facilities.

Authors' contribution: M. Kumar: Supervision, conceptualization, review and editing of the manuscript; **S. Singh:** Experimental design, writing of the original MS; **S. Dwivedi:** Raw data collection, research and management of the references; **A. Trivedi:** Validation of the experiment and methodologies; **I. Dubey:** Statistical analysis of the data **and S.P. Trivedi:** Editing of the manuscript.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Institutional Animal Ethics Committee (IAEC) of University of Lucknow (vide registration no 1861/GO/Re/S/16/ CPCSEA, Lucknow).

Conflict of interest: The authors declare that there is no conflict of interest.

Data availability: All data generated or analyzed during this study are included in this manuscript.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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