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Chromium induced alterations to *Cyprinus carpio* L. and its amelioration using ascorbic acid

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Abstract
Heavy metal pollution is a major concern due to the non-biodegradable nature and bioaccumulation of metals like chromium, which often exceeds permissible water limits and harms aquatic life. This study assessed the ameliorative effects of ascorbic acid on chromium-induced toxicity in *Cyprinus carpio* over 28 days. The 96-hour LC_{50} for chromium was 176.97 ppm; two sublethal concentrations (1/10th and 1/5th LC_{50}) were used for chronic exposure, with and without ascorbic acid. Chromium exposure led to reduced TEC, Hb, and Ht, and increased TLC, MCV, MCH, and MCHC. Activities of oxidative stress enzymes SOD, CAT, GR, GST, GPx, and LPO were significantly elevated. Histological changes included liver vacuolization, pyknosis, kidney tubule and glomerular damage, and gill lamellae destruction. Ascorbic acid supplementation showed partial recovery of physiological and tissue parameters. The study demonstrates that ascorbic acid effectively reduces chromium toxicity in *C. carpio*, indicating its potential role as a protective agent against heavy metal stress.

Keywords: Toxicity, amelioration, ascorbic acid, chromium, oxidative stress

1. Introduction
Metal pollution is a great threat to aquatic life; unlike other pollutants, heavy metals are non-biodegradable and can only be converted into less toxic forms (Ayangbenro and Babalola 2017). Industries related to chemicals, fertilizers, pharmaceuticals and even steel, petroleum and others use and discharge heavy metals as waste more than their permissible limits. Among these heavy metals, chromium is a ubiquitously known heavy metal pollutant of aquatic ecosystems. In India, approximately 2000 to 3200 tons of elemental Cr are drained into the environment by industries every year (Zayed and Terry 2003) [59], and it enters as an aquatic pollutant through effluents from textiles, tanneries, mining, electroplating, dyeing, printing, pharmaceuticals, stainless steel manufacturing and rubber manufacturing industries (Sridhar *et al.* 2000 [34], Bagchi *et al.* 2001, Ahmed *et al.* 2013) [5]. Cr is a biologically important trace metal, however, concentrations greater than those required for body metabolism may cause toxicity, mutations and teratogenesis (Shaheen and Akhtar 2012) [31, 49]. In nature, Cr is found in two oxidation states, i.e., Cr(III) and Cr(VI). Cr(VI) is 700 times more toxic than Cr(III) because of its higher solubility and greater cell membrane permeability (Shaheen and Akhtar 2012) [31, 49]. The adverse effects of Cr toxicity have been studied in various fish species, including *Channa punctatus, C. carpio, Carassius auratus* and *Labeo rohita* (Mishra and Mohanty 2008 [24]; Velma and Tchnounwou 2010 [35, 55]; Praveena *et al.* 2013 [27, 41]; Rajeshkumar *et al.* 2017) [28, 42], and reported that Cr in both acute and chronic exposures induces toxicity at several physiological levels, i.e., hematological, biochemical and histomorphological alterations, in fishes.
Among the various parameters, hematological indices are considered pathophysiological indicators of the whole body and are important for detecting and diagnosing the structural and functional status of fish exposed to toxicants (Adhikari *et al.* 2004) [4]. Since blood (circulatory) is closely related to the external environment, it is considered a good indicator of environmental toxicity (Kim and Kang 2017) [19]. Any abnormal changes in the blood indices of fish could indicate the status of fish health and the aquatic environment inducing toxicity at the cellular and genetic levels (Awasthi *et al.* 2018) [8].

ROS such as hydrogen peroxide, hydroxyl radicals and superoxide are produced as a result of elevated lipid peroxidation levels along with changes in the enzymatic levels of catalase, superoxide dismutase and glutathione peroxidase in response to exposure to Cr (Kumar *et al.* 2013) [20]. Changes in the activities of these antioxidant enzymes indicate the extent of oxidative stress caused to fish by the toxicant. The oxidative stress caused by toxicants induces cellular and tissue damage; for example, gills in direct contact with water undergo several histomorphological changes in response to toxicity and deformation (Mishra and Mohanty 2008) [24]. The liver, the main detoxifying organ that performs xenobiotic metabolism, has several histopathological effects (Mishra and Mohanty 2008) [24]. However, the kidney plays a vital role in the excretion of metabolic waste and osmoregulation in fishes, which undergo several histomorphological changes due to metal toxicity (Rana *et al.* 2015) [29, 43]. However, some compounds or elements (e.g., ascorbic acid, ginseng, taurine, etc.) attenuate the effects of toxicity caused by various toxicants (Jiraungkoorskul *et al.* 2006, Rehman *et al.* 2016) [44]. One such compound is ascorbic acid (vitamin C), a micronutrient that plays a vital role in aquatic animals, as it helps in proper metabolic functioning and immunity along with proper growth and development (Wang *et al.* 2003) [38, 58].

Ascorbic acid is a strong antioxidant that helps in the detoxification of contaminants caused by various toxic metals (Kim and Kang 2015) [17] and is able to attenuate not only the toxicity caused by heavy metals (Jiraungkoorskul *et al.* 2006, Abdul *et al.* 2012 [1]) but also that caused by agrochemicals (Datta and Kaviraj 2003 [12], Saha and Kaviraj 2009 [30, 46], Rehman *et al.* 2016) [44]. These ameliorative effects of ascorbic acid on dietary Cr toxicity were observed in *Sebastes schlegelii* (Kim and Kang 2017) [19]. However, *C. carpio* cannot synthesize ascorbic acid because of the lack of the microsomal enzyme L-gulonolactone oxidase, which is responsible for its production (Vélez-Alavez *et al.* 2014) [54]. To date, no studies have attempted to reduce Cr toxicity in *C. carpio* via the use of ascorbic acid. Therefore, the present study investigated the protective role of ascorbic acid in the amelioration of Cr-induced toxicity in the widely cultured cosmopolitan carp *C. carpio*.

2. Materials and Methods

2.1 Chromium stock solution

The stock solution of chromium was prepared with 174.84 g of potassium dichromate ($K_2Cr_2O_7$) (Nice Chemicals Pvt. Ltd.) in one liter of distilled water. Aliquots were prepared from stock solutions at the concentrations required for acute and chronic exposure.

2.2 Fish and maintenance

Fingerlings of *C. carpio* (length 11.22±0.14 cm and weight 19.8 g±0.6) were procured from Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. The fingerlings were acclimatized to the laboratory conditions for fifteen days in glass aquaria (50 liters capacity) in chlorine-free water. The fingerlings were fed *ad libitum* with a basal diet (rice bran and mustard oil cake at a ratio of 1:1) throughout the acclimatization period, and the water was continuously aerated during maintenance. The exposure experimental protocols were performed according to the OECD guidelines (1992).

2.3 Feed Preparation

The basal feed was prepared from rice bran and mustard oil cake in a 1:1 ratio, while ascorbic acid (ascorbyl palmitate, Sigma–Aldrich, CAS #. 137-66-6) was supplemented with feed @1 g/kg basal feed for testing amelioration. The fish were fed twice daily *ad libitum.*

2.4 Acute toxicity test

The acute toxicity test was conducted with different concentrations of Cr (80, 120, 160, or 200 ppm) for a period of 96 h under laboratory conditions to determine the LC_{50}. These concentrations were chosen on the basis of earlier studies on Cr (Al-Akel and Shamsi 1996 [6]; Abedi *et al.* 2012 [2]; Shaukat and Javed 2013) [32, 50]. Fingerlings were starved for 24 hrs before the initiation of the bioassay and were not given feed during the exposure period. The mortality of the fish was recorded every 24, 48, 72 and 96 hrs of exposure to caLCulate the LC_{50}. Simultaneously, another group was maintained as a control that received no Cr. The 96 hr LC_{50} value of Cr to *C. carpio* was caLCulated via POLO software (Robertson *et al.* 1980) [45].

2.5 Chronic toxicity

Chronic toxicity was assessed via two sublethal concentrations of Cr, i.e., the $1/5^{th}$ and $1/10^{th}$ 96 hr LC_{50}. The fishes were divided into 6 groups, with triplicates for each group. The exposure design was conducted according to the following setup.

The fish were exposed to Cr for 28 days, and from each treatment, the fish were selected for sample collection at intervals of 14 and 28 days. Before dissection, the fish were anesthetized with clove oil, and blood samples were subsequently collected from the caudal vein. Tissues, including the liver, kidney and gills, were subsequently preserved in 10% neutral buffered formaldehyde (NBF) solution for histomorphological analysis. Furthermore, some of the liver samples were separately collected for biochemical parameter analysis.

Groups	Treatments
T_1	Basal feed (Rice bran: Mustard cake)-Negative control
T_2	1 g ascorbic acid/kg basal feed-Positive control
T_3	$1/10^{th}$ 96 hr LC_{50} of Cr+ Basal feed
T_4	$1/10^{th}$ 96 hr LC_{50} of Cr+ 1 g ascorbic acid/kg basal feed
T_5	$1/5^{th}$ 96 hr LC_{50} of Cr+ Basal feed
T_6	$1/5^{th}$ 96 hr LC_{50} of Cr+1 g ascorbic acid/kg basal feed

2.6 Blood collection and hematological parameters

Blood samples were collected from the caudal vein of anesthetized fish via a syringe with a 24-gauge needle. The collected blood was transferred to vacutainer tubes (anticoagulant vials) containing EDTA, and the samples were analyzed as soon as possible. The blood samples were used to caLCulate the total erythrocyte count (TEC) and total leucocyte count (TLC) via a Neubauer hemocytometer, hemoglobin via the acid hematin method (Sahli 1962) [47] and hematocrit via Wintrobe's tube. In addition, the mean corpuscular volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) were caLCulated via standard formulas as described previously (Sarma 1990) [48].

2.7 Biochemical parameters

2.7.1 Liver: For the estimation of biochemical parameters,

0.5 g of liver tissue was homogenized in 2 mL of phosphate buffer (0.1 M, pH 7.4) and centrifuged at 3000 rpm for 10 mins. The supernatant was subsequently removed and stored at -20°C for the estimation of total soluble protein content, lipid peroxidation (Stocks and Dormandy 1971) [53], superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase according to the standard protocols described earlier.

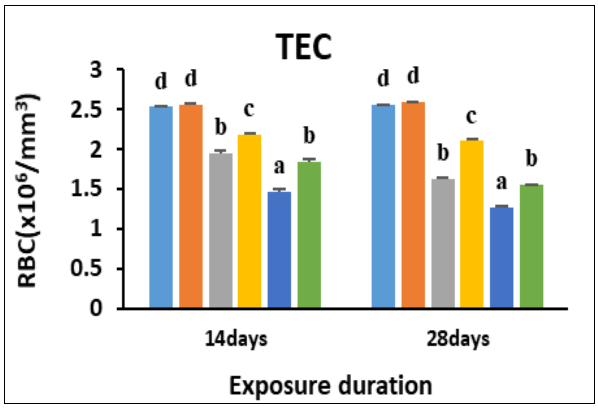
2.8 Histomorphology
For histological studies, gill, liver and kidney tissues were collected from all groups in 10% NBF. The tissues preserved in NBF were further processed for dehydration via a series of methanol (70, 80, 95 & 100%) and chloroform. The tissues were subsequently embedded and fixed in paraffin wax and sectioned via a microtome (Leica RM 2125). The tissue sections were stained according to the procedure described by Humason (1972), observed under a microscope and photographed via a camera (Nikon eclipse 80i) at 40x magnification.

2.9 Statistical analysis
The statistical analysis was performed via SPSS 16.0. The data on the haematological and biochemical parameters of the fish in the different treatment groups and the control group were subjected to Duncan's multiple range test to determine the significance of the differences with respect to the different treatments. The difference in significance with respect to duration was tested via a t test.

3. Results and Discussion
3.1 Acute toxicity: The acute toxicity bioassay results revealed 176.97 ppm for the 96 hr. LC_{50} for Cr, with 95% upper and lower confidence limits of 261.24 ppm and 149.24 ppm, respectively, with heterogeneity and slopes of 1.88 and 6.018±0.681, respectively. Various values of the LC_{50} for Cr have also been reported previously for *C. carpio* (Al-Akel and Shamsi 1996 [6]; Abedi *et al.* 2012 [2]; Shaukat and Javed 2013) [32, 50]. These differences in the LC_{50} could be due to differences in chemical form, physiological conditions, age of the fish, experimental conditions or synergism with other stressors. During the exposure experiment, behavioral changes, including lethargy, erratic swimming, loss of equilibrium, changes in the rate of opercular movement, increased mucus secretion and discoloration, were observed in Cr-exposed fish but not in the control groups. The alterations in the behavioral patterns of the fish observed in the present study were similar to those reported previously in *Ctenopharyngodon idella* (Handa and Jindal 2019) [15], *Clarias gariepinus* (Ekeh *et al.*, 2018) [13], and *Cirrhinus mrigala* (Mallesh *et al.* (2015) [23]. These behavioral changes observed during exposure could be due to respiratory impairment caused by hypoxic conditions as a result of Cr. The increase in mucus secretion observed might have caused the epithelium to be impermeable to Cr, and this lethargic behavior could be attributed to Cr-induced neurotoxicity (Handa and Jindal 2019) [15]. The presence of toxicants in water may also induce neurotoxic effects, cause irritation to the perceptive system of fish, and result in abnormal behavioral responses (Ahmed *et al.* 2013) [5].

3.2 Hematological parameters: Hematological parameters are used to assess the health status of fish, as blood is a sensitive tissue that is affected by environmental changes. Changes in fish blood prior to the onset of notable morphological and physiological changes could be indicators of unfavorable aquatic environments. Blood also has an integrated role in the immune system and can be used to assess the health status of fishes exposed to xenobiotics (Bhatkar 2011) [10]. In the present study, Cr toxicity resulted in a significant (p<0.05) reduction in TEC, Hb and Ht in *C. carpio* exposed to two sublethal concentrations of Cr compared with those in the control groups (Figure 1), with a maximum decrease at higher concentrations ($1/5^{th}$ LC_{50}). However, the reduction was greater after 28 days than after 14 days of exposure in all the treated groups. Hence, the reduction in TEC, Hb and Ht was found to be dependent on the concentration as well as the duration of exposure. Furthermore, dietary ascorbic acid had ameliorative effects, as partial recovery was observed in the TEC, Hb and Ht values with ascorbic acid supplementation (T_4 and T_6) after 14 and 28 days of exposure. However, no significant difference was recorded among the control groups, i.e., those with or without ascorbic acid supplementation.

Similar observations with reduced TEC, Hb and Ht were recorded in *Cirrhinus mrigala, Labeo rohita and C. carpio* following exposure to Cr (Mallesh *et al.* 2015 [23], Praveena *et al.* 2013 [27, 41], Abedi *et al.* 2013 [3], Shaheen and Akhtar 2012 [31, 49], Bhatkar 2011) [10]. Kim and Kang (2017) [19] also reported decreased TEC, Hb and Ht due to Cr toxicity in *Sebastes schlegelli*, with a reduced negative impact from ascorbic acid supplementation. The decrease in TEC observed could be attributed to decreased hemopoiesis or increased hemolysis as a result of toxicity (Shaheen and Akhtar 2012) [31, 49]. The increased destruction of circulating erythrocytes likely caused a decrease in the cell count as a result of the decreased affinity of hemoglobin for oxygen binding due to its altered properties, leading to increased fragility and permeability in erythrocytes and causing damage (Mallesh *et al.* 2015) [23]. The reduction in the Hb content of Cr-exposed fish was attributed to the inhibition of hemoglobin-synthesizing enzymes as a result of the inhibitory effect of toxicity (Abedi *et al.* 2013) [3]. In addition, some alterations in the heme structure might have also occurred, causing disturbances in hemoglobin synthesis. An impaired intestinal absorption of iron could also be one of the reasons behind decreased levels of Hb (Shaheen and Akhtar 2012) [31, 49]. However, earlier reports have reported increased hematocrit in fishes exposed to Cr, which was probably due to increased functioning of erythropoeitic tissue for increased production of cells to compensate for the stressed conditions (Al-Akel and Shamsi 1996 [6], Nuray *et al.* 2010) [25].



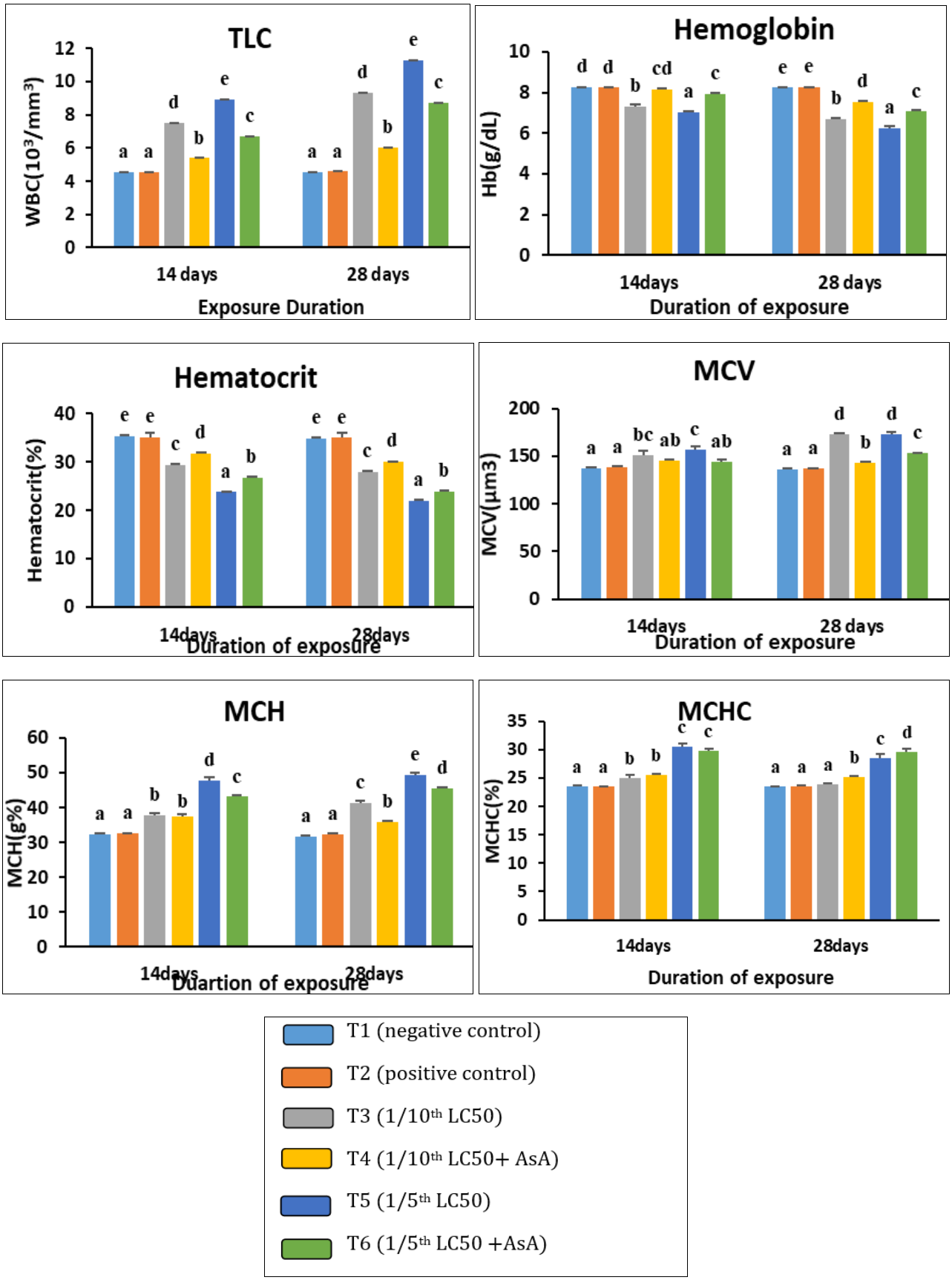


Fig 1: Effects of dietary ascorbic acid supplementation on the hematological parameters of Cr-exposed *C. carpio* after 14 and 28 days of exposure

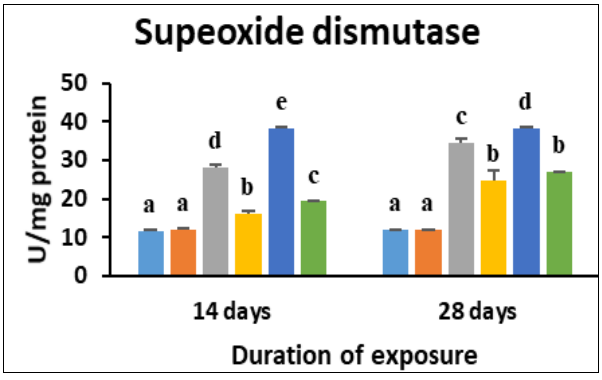
In contrast, increases in TLC, MCV, MCH and MCHC were observed in *C. carpio* as a result of exposure to Cr in the present investigation. The increase in TLC was found to be dependent on the Cr concentration, with the maximum level occurring at higher concentrations. This was revealed by the total leucocyte count recorded at T_5 ($1/5^{th}$ LC_{50}), which was greater than that recorded at T_3 ($1/10^{th}$ LC_{50}) after 14 and 28 days of exposure. The increase in all the Cr-treated groups was found to be dependent on exposure duration since TLC was greater after 28 days than after 14 days of exposure.

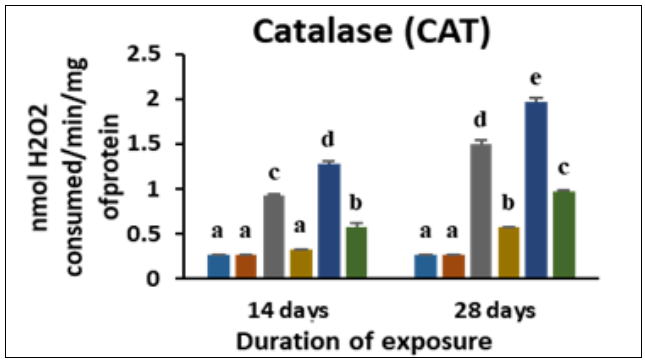
Ascorbic acid had an attenuating effect on Cr toxicity since the increase in TLC was lower in the ascorbic acid-supplemented groups (T_4 and T_6) than in the T_3 and T_5 groups, in which the fishes were fed a basal di*et al.*one. The present and earlier studies revealed that increased leucocytes are an indication of adaptation by animals to meet stressed conditions (Mallesh *et al.* 2015) [23]. Therefore, the high TLC levels recorded in the present study could be due to Cr-induced stress to the fish, which might have occurred because of the removal of the cell debris produced as a result of the damage to the tissues observed via microscopic examination. Similar observations were also made by Shaheen and Akhtar (2012) [31, 49] in *C. carpio* (Mallesh *et al.* 2015) [23], *C. mrigala* (Bhatkar 2011) [10] and *L. rohita* (Praveena *et al.* 2013) [27, 41].
In the present study, increased MCV, MCH and MCHC were recorded throughout the exposure period, although no regular trend was observed. However, the positive and negative control groups did not reveal any significant differences in these indices after 14 and 28 days of exposure. The groups treated with ascorbic acid supplement (T_4 and T_6) presented less of an increase than the groups (T_3 and T_5) fed a basal di*et al.*one did. The results also revealed a concentration-dependent increase in MCH. However, MCH values were not affected by duration of exposure, as no significant difference was found between 14 and 28 days of exposure within the treated groups. The MCHC values in all the treated groups were slightly elevated as a result of Cr exposure. A similar increasing trend for MCV, MCH and MCHC was reported in *L. rohita* exposed to trivalent Cr. This increase in blood parameters could be associated with macrocyte anemia, which could reflect possible chronic liver damage in fish (Bhatkar 2011) [10]. In contrast, a reduction in the MCV, MCH and MCHC values was reported in *C. mrigala* exposed to hexavalent Cr, which might be due to a reduction in the level of iron in the blood, leading to a reduced oxygen-carrying capacity of the blood and eventually stimulating erythropoiesis (Mallesh *et al.* 2015) [23].

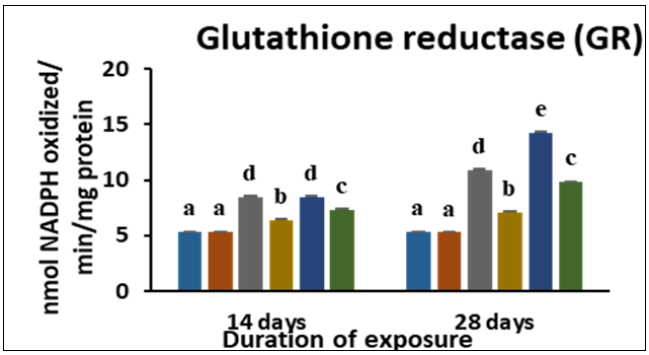
3.3 Biochemical studies

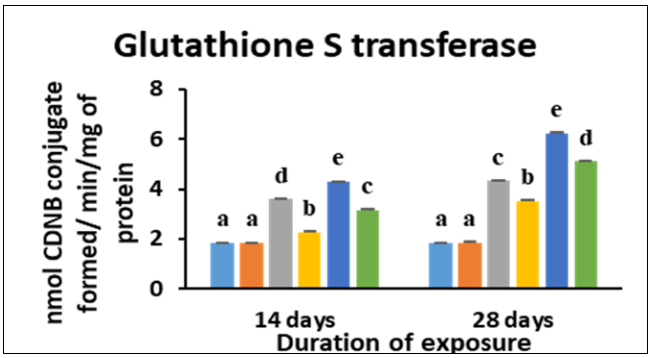
Oxidative stress enzyme analysis

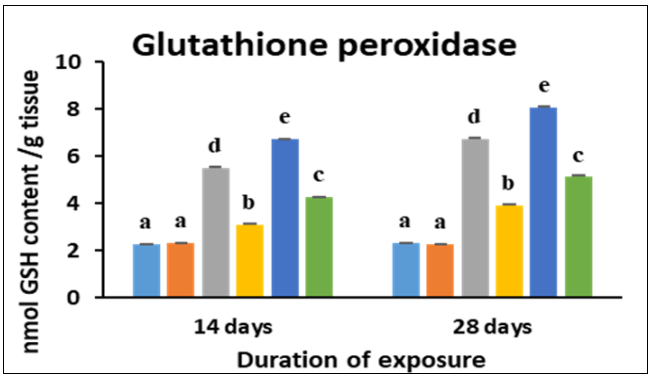
In the present study, Cr exposure resulted in a significant increase in the activity of oxidative stress enzymes, viz., superoxide dismutase, catalase, glutathione reductase, glutathione S-transferase, and glutathione peroxidase, and in lipid peroxidation (Figure 2). The increase in the activities was reported to be concentration-and duration dependent, with no significant difference among the control groups. Additionally, ameliorative effects of ascorbic acid were observed, with partial recovery of the activity of all the enzymes toward normal levels when the plants were fed ascorbic acid.











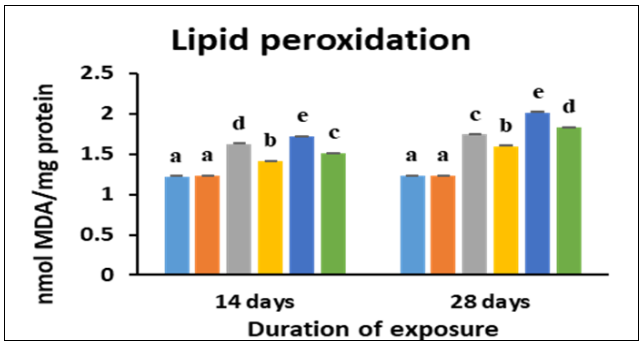


Fig 2: Effects of dietary ascorbic acid supplementation on the activity of oxidative stress enzymes in the liver of Cr-exposed *C. carpio* after 14 and 28 days of exposure

Superoxide dismutase (SOD)

The SOD enzyme is the first line of defense, as it catalyzes the reduction of superoxide radicals and is one of the reactive oxygen species produced due to oxidative stress. It acts as an active free radical scavenger. Cr exposure resulted in an increase in SOD activity during the present study, which was concentration-and duration dependent. These results are in accordance with the observations of Kumar *et al*. (2013) [20] and Rajeshkumar *et al*. (2017) [42, 28], who reported increased SOD activity in the liver of *C. carpio* following exposure to Cr and a mixture of three heavy metals, respectively. Awasthi *et al*. (2018) [8] and Chen *et al*. (2018) [11] noted similar increasing trends in SOD activity in *C. punctatus* and *Oryzias latipes* exposed to hexavalent Cr, respectively. The elevated levels of SOD activity in *C. carpio* recorded in the present study could be a result of induced antioxidant activity to counteract the oxidative stress caused by the overproduction of ROS as a result of Cr toxicity. SOD is a free radical scavenger that plays an important role in protection against oxidative stress induced by oxyradicals (ROS), which can damage cells. Furthermore, ascorbic acid, a strong antioxidant and a scavenger of ROS, reacts with the superoxide radical produced as a result of Cr toxicity, resulting in decreased SOD activity in the ascorbic acid-supplemented groups.

Catalase (CAT)

Catalase acts a step further than SOD in the first-line defense mechanism and converts hydrogen peroxide (H_2O_2) produced as a result of SOD catalytic reduction into H_2O and O_2. CAT activity also increased in a concentration-and duration-dependent manner as a result of Cr toxicity. Partial restoration of the activities toward normal was recorded in the groups supplemented with ascorbic acid. Similar to our observations in the present study, an increase in the catalase activity of *C. carpio* as a result of multiple exposures to heavy metals (Rajeshkumar *et al.* 2017 [28, 42], Vinodhini and Narayanan 2009) [36, 56] and hexavalent Cr in *C. punctatus* (Awasthi *et al.* 2018) [8] has also been reported. The increased CAT activity noted in the present study and in other studies was probably due to increased production of H_2O_2 in the liver, which is a core predecessor of the most toxic ROS, the hydroxyl (OH-) radical. The increased production of H_2O_2 might have occurred due to the increased activity of SOD, which produces H_2O_2 as an end product of its reaction (Rajeshkumar *et al.* 2017) [28, 42]. Therefore, CAT activity may increase in an attempt to protect the liver from damage caused by oxidative stress by reducing H_2O_2 into nontoxic water and oxygen molecules. In contrast, a decrease in CAT activity was recorded by Kumar *et al*. (2013) [20] and Ekeh *et al*. (2018) [13] in *C. carpio* and *C. gariepinus* due to Cr toxicity. This could be because the overproduction of SOD radicals might have an inhibitory effect on catalase activity.

Glutathione reductase (GR)

Glutathione reductase plays a critical role in the antioxidant defense mechanism of the body against oxidative stress, and a study revealed an increase in the levels of GR in the liver of *C. carpio* exposed to two sublethal concentrations of Cr (Figure 2C). Among all the treated groups, the maximum increase was recorded at T_5. In the ascorbic acid-supplemented groups (T_4 and T_6), the increase in GR activity was less pronounced than that in the T_3 and T_5 groups. A possible explanation for this increased activity of GR in the livers of Cr-exposed fish could be the increased process of reestablishing reduced glutathione levels, which might have decreased due to the production of more oxidized forms of glutathione, i.e., GSSG, in the process of Cr detoxification. The results of the present study are in agreement with the observations of Kumari *et al*. (2014) [21], who reported an increase in the activity of GR in *L. rohita* following acute exposure to Cr. In the present study, ascorbic acid supplementation partially improved the increase in the activity of GR, and similar observations of GR were made by El-Gazzar *et al*. (2014) in the case of cadmium-exposed *O. niloticus*, in which the increase in enzyme activity decreased following ascorbic acid supplementation. In contrast, Chen *et al*. (2018) [11] reported decreased GR activity in *O. latipes* exposed to hexavalent Cr.

Glutathione S-transferase (GST)

Glutathione S-transferase enzymes can catalyze the conjugation of xenobiotic substances with the reduced form of glutathione. The increase in the activity of GST was dose-and duration dependent. A less pronounced increase in activity was observed in the ascorbic acid-supplemented groups. Similarly, an increase in the GST activity of *O. latipes* (Chen *et al*. (2018) [11] and *S. schleglii* (Kim and Kang 2016) [18] has also been reported. In the case of *O. niloticus*, mixed exposure to five heavy metals (Cd, Cu, Cr, Pb and Zn) resulted in increased GST activity. Vinodhini and Narayanan (2009) [36, 56] also reported increased GST activity in *C. carpio* exposed to a mixture of four heavy metals. Similar to our results with Cr exposure, cadmium-exposed *O. niloticus* presented a decrease in GST activity following ascorbic acid supplementation. The increased activity of GST indicates the active detoxification process of Cr. The GST activity might have increased to increase the amount of ROS produced due to Cr-induced oxidative stress. Ascorbic acid itself is an antioxidant that binds with ROS, resulting in a decrease in GST activity.

Glutathione peroxidase (GPx)

Glutathione peroxidase is involved in the conversion of H_2O_2 and other peroxides into aLCohol and H_2O and the oxidation of GSH. Like other enzymes, GPx activity increased due to Cr toxicity, and ascorbic acid ameliorated GPx activity. Kumar *et al*. (2013) reported increased GPx activity in the liver of *C. carpio* exposed to hexavalent Cr. Similarly, mixed exposure to four heavy metals resulted in an increase in glutathione peroxidase activity in common carp (Vinodhini and Narayanan 2009) [36, 56], and in *C. gariepinus* and *C. auratus*, it increased GPx activity following exposure to Cr (Ekeh *et al.* 2018 [13] and Velma and Tchounwou 2010) [35, 55]. In the present study, the increased activity of glutathione peroxidase was probably caused by increased amounts of H_2O_2 and other peroxides generated due to Cr-induced oxidative stress.

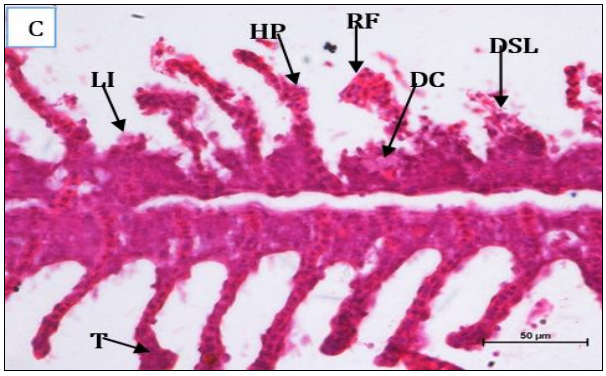
Lipid peroxidation (LPO)

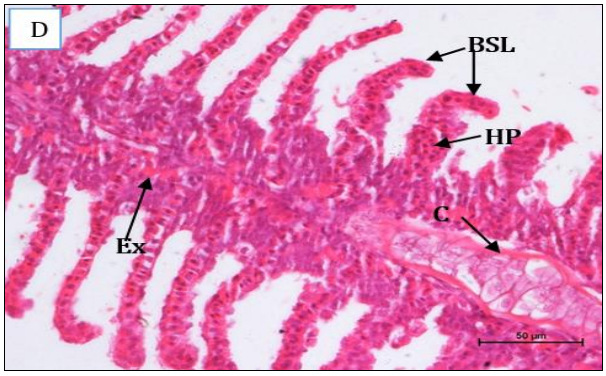
Cr toxicity in common carp resulted in lipid peroxidation in the present investigation, and ascorbic acid attenuated the effects of toxicity, as less increased levels of lipid peroxidation were observed in the supplemented groups (T_4 and T_6). Ekeh *et al*. (2018) [13], Rajeshkumar *et al*. (2017) [28, 42] and Velma and Tchounwou (2010) [35, 55] reported increased lipid peroxidation in *C. gariepinus, C. carpio* and *C. auratus*, respectively, following exposure to heavy metals. A rise in lipid peroxidation is indicative of increased oxidative stress in fish. However, the increase in lipid peroxidation observed in

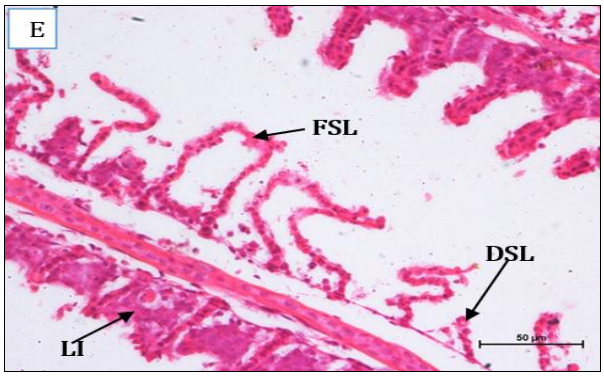
the ascorbic acid-supplemented groups might be due to the scavenging of oxidative radicals by ascorbic acid, which is an antioxidant.

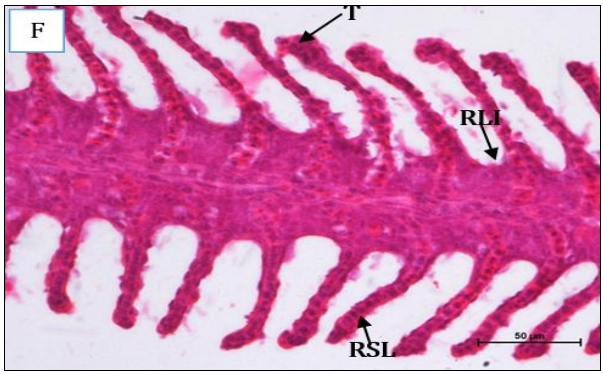
3.4 Histomorphological studies

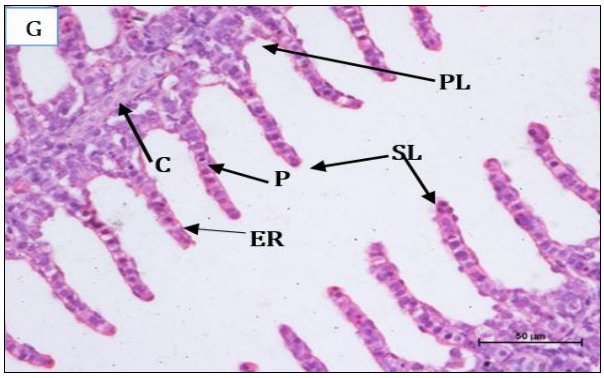
Histomorphological studies have proven to be a definite indicator of damage in fish due to toxicant exposure (Jiraungkoorskul *et al*. (2006)). Therefore, in the present investigation, histological examination of the gills, liver and kidney was performed, along with biochemical and hematological examinations, to confirm the attenuating effect of ascorbic acid in Cr-affected *C. carpio*.

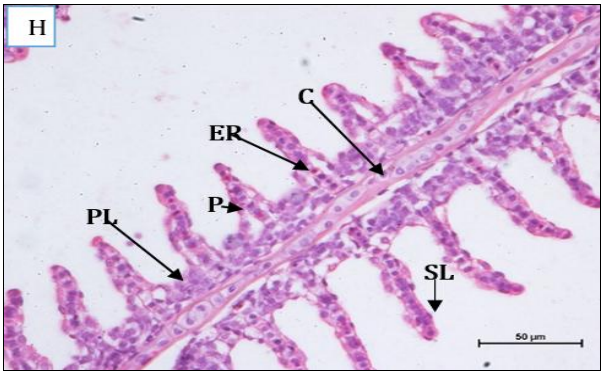


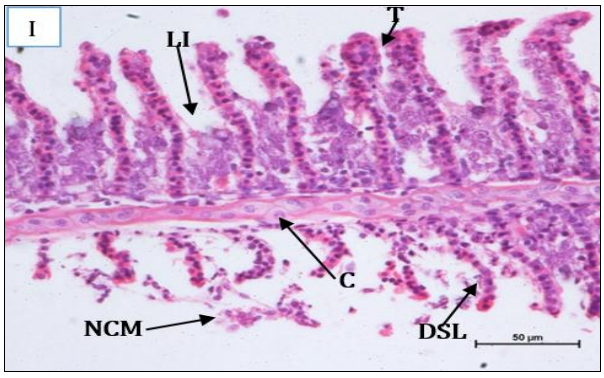


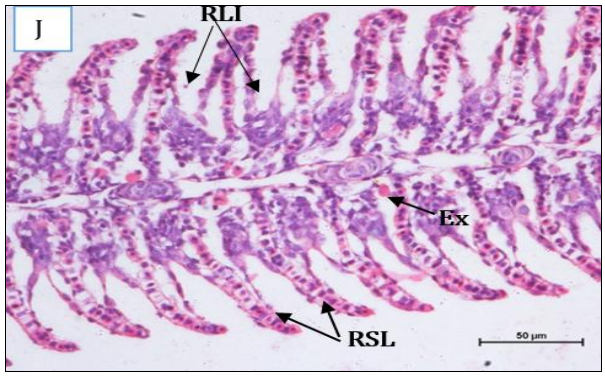


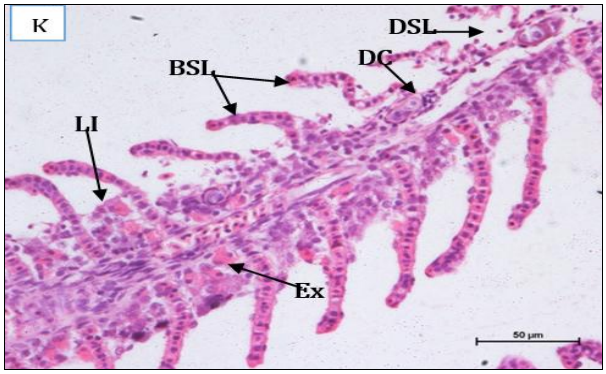


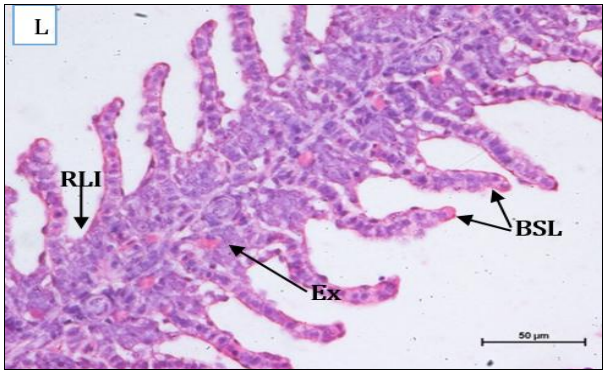


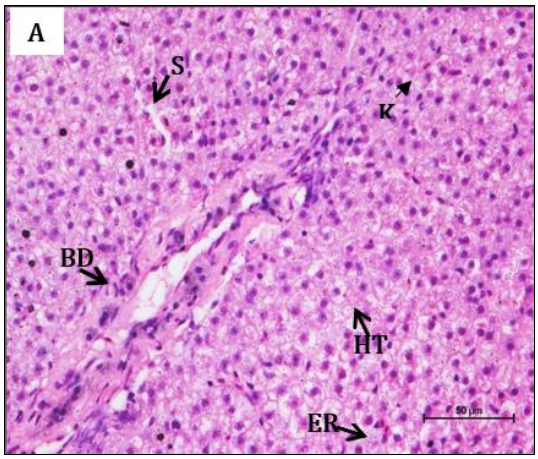


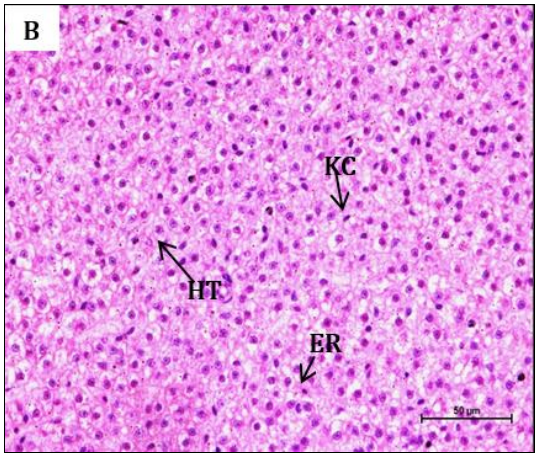


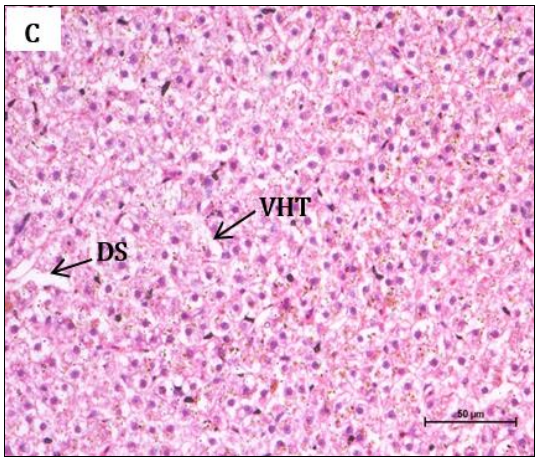


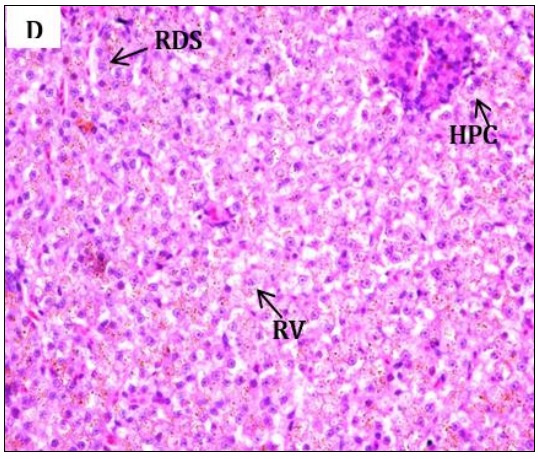








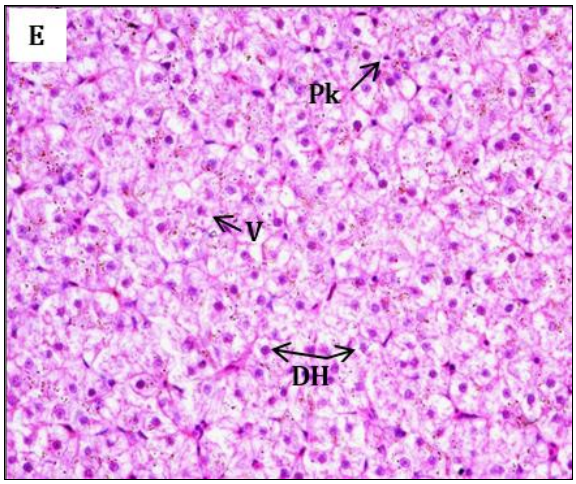


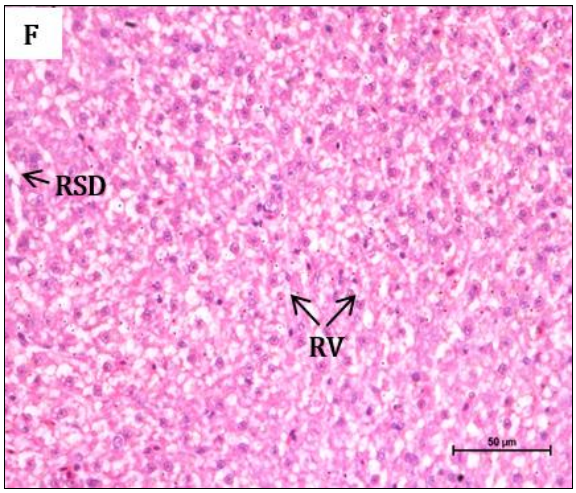


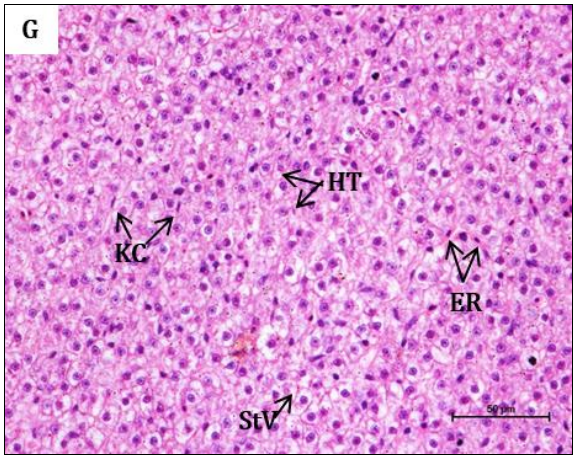
A: PL-Primary lamellae, SL- Secondary Lamellae, ER- Erythrocytes, P- Pillar cells; **B:** C- Chondrocytes, **C:** LI- Lymphocytic infiltration, HP- Hyperplasia, T- Telangiectasia, RF- Ruptured Filament, DSL- Degenerated Secondary lamellae, DC- Disorganized chondrocytes; **D:** BSL- Blunt Secondary Lamellae, Exudate; **E:** FSL- Fused secondary lamellae, DSL- Degenerated secondary lamellae, **F:** RSL- Restoring secondary lamellae, RLI- Reduced lymphocytic infiltration.

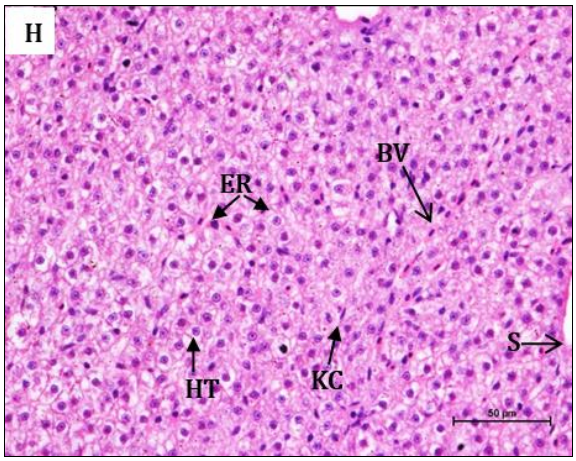
G: PL-Primary Lamellae, SL- Secondary Lamellae, P- Pillar cells, C- Chondrocytes, ER- Erythrocytes; **H:** PL-Primary Lamellae, SL- Secondary Lamellae, P- Pillar cells, C- Chondrocytes, ER- Erythrocytes; **I** LI- Lymphocytic infiltration, T-Telangiectasia, NCM- Necrosed cellular material, DSL- Degenerated secondary lamellae, Exudate, C- Chondrocytes; **J:** RLI- Reduced lymphocytic infiltration, RSL- Restoring secondary lamellae; **K;** LI- Lymphocytic infiltration, BSL- Blunt secondary lamellae, DSL- Degenerated secondary lamellae, DC- Disorganized chondrocytes, Ex- Exudate; **L:** RLI- Reduced lymphocytic infiltration, BSL- Blunt secondary lamellae, Ex- Exudate

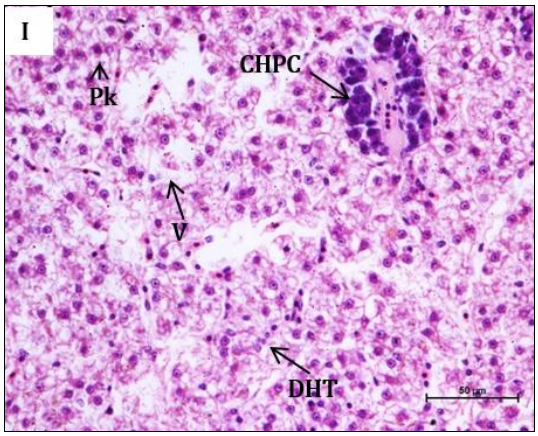
Fig 3: Ameliorative effects of dietary ascorbic acid supplementation on Cr toxicity to the gills of *C. carpio* after 14 days (A-F) and 28 days (G-L) of exposure

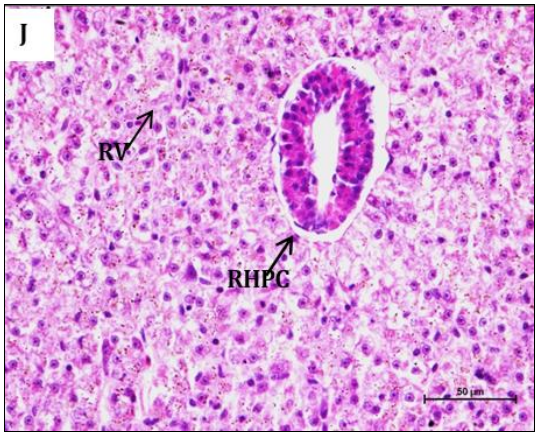


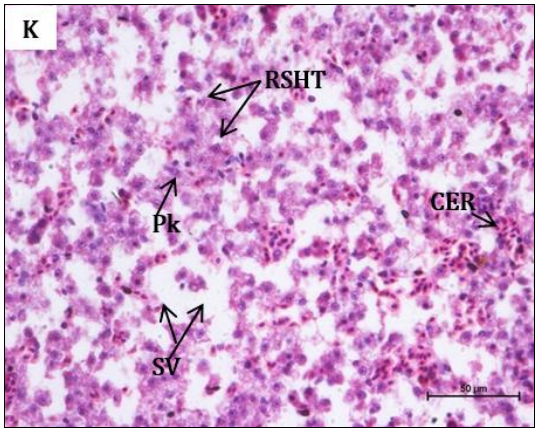


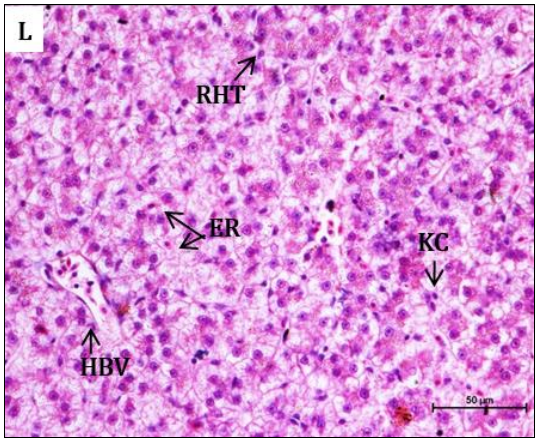










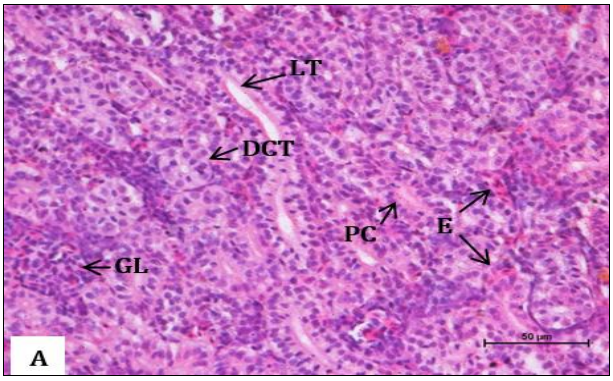


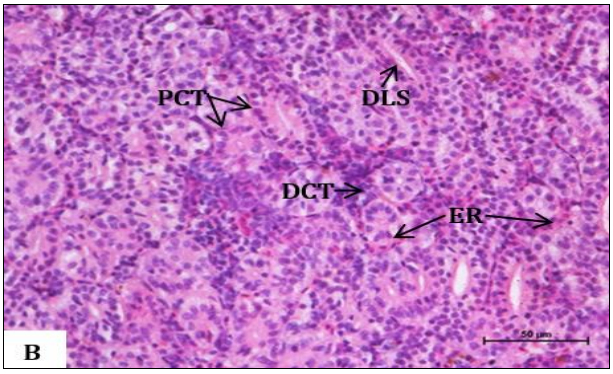
A: S- Sinusoid, KC-Kupffer cells, BD- Bile duct, H-Hepatocytes, ER- erythrocytes; **B:** KC-Kupffer cells, HT- Hepatocytes, ER- Erythrocytes; **C:** VHT-Vacuolated hepatocytes, DS-Dilated sinusoid; **D:** RDS-Reduced dilation of sinusoid, RV- Reduced vacuolization, HPC- Hepatopancreas; **E:** Pk- Pyknosis, DHT- Degenerating hepatocytes, **V:** vacuolization; **F:**

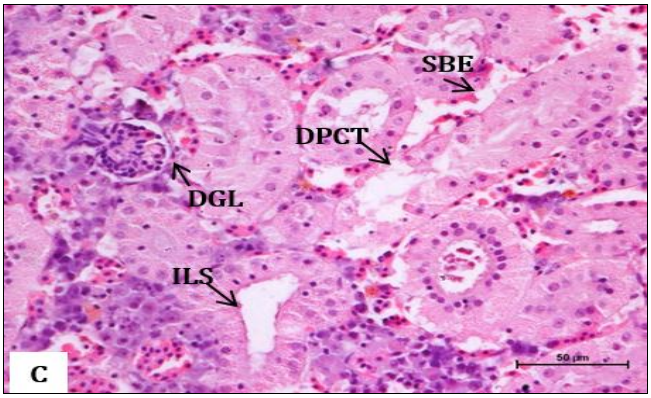
RSD- Reduced dilation of sinusoids, RVS- Reduced vacuolization

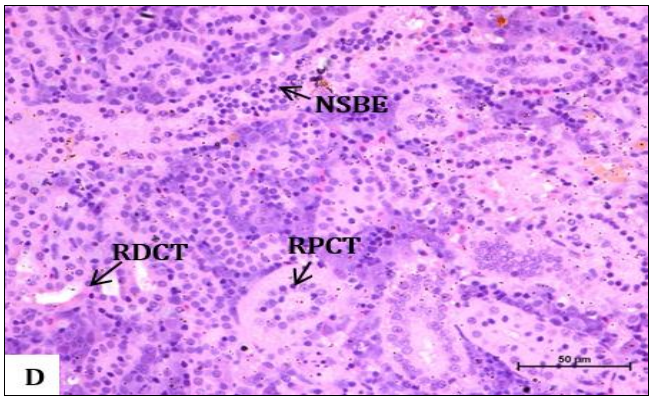
G: HT- hepatocytes, KC-Kupffer cells, ER-Erythrocytes, StV-Slight Vacuolization; **H:** BV-blood vessel, S-sinusoid, HT-Hepatocyte, KC-Kupffer cells, ER- erythrocytes; **I:** Pk-Pyknosis, CHPC-Congestion of hepatopancreas, V-vacuolization, DHT-Degenerated hepatocytes; **J:** RV-Reduced vacuolization, RHPC-Restoring Hepatopancreas; **K**: Pk-Pyknosis, RSHT-Reduced size of hepatocytes, SV-Severe vacuolization, CER-Clustered Erythrocytes; **L:** ER-Erythrocytes, HBV- Hepatic blood vessel, KC-Kupffer cells, RHT- restoring hepatocytes

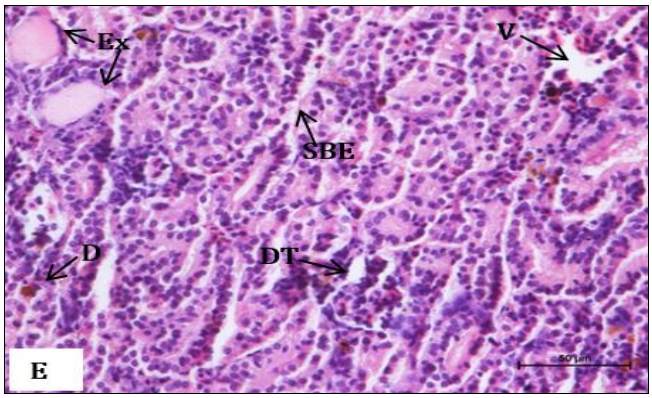
Fig 4: Ameliorative effects of dietary ascorbic acid supplementation on Cr toxicity to the liver of *C. carpio* after 14 days (A-F) and 28 days (G-L) of exposure

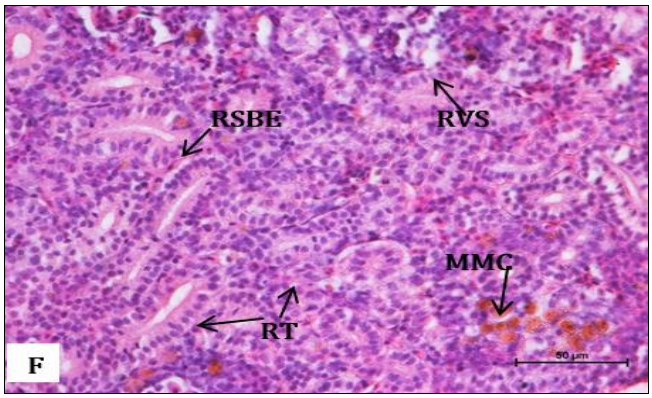


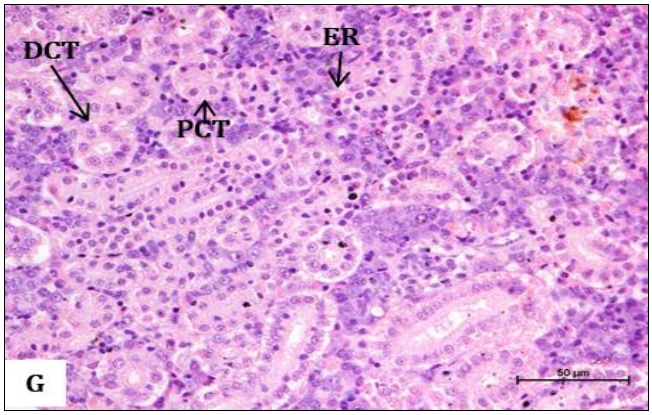


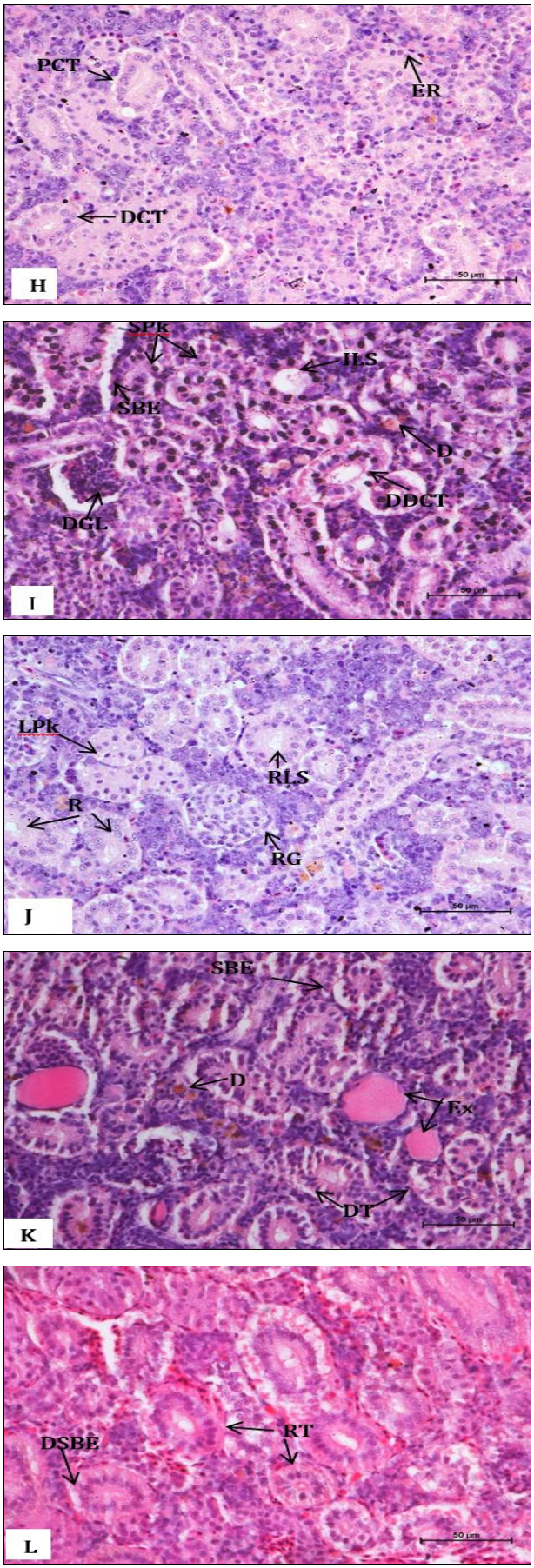












A: LT- Lumen of tubules, DCT- distal convoluted tubule, ER-erythrocytes, PCT-proximal convoluted tubule, GL-glomeruli; **B:** PCT-proximal distal convoluted tubule, DCT-distal convoluted tubule, ER-erythrocytes; **C:** DPCT-degenerative proximal convoluted tubules, DGL-degenerative glomeruli, ILS- increased lumen size, SBE-Separation between basement membrane and epithelial cells; **D:** NBSE-No separation between basement membrane and epithelial cells, RDCT-Restoring distal convoluted tubule; **E:** Ex-Exudate, DT-degenerative tubules, Dedepositions, SBE-separation between basement membrane and epithelial cells, V-vacuolization; **F:** RSBE- Reduced separation between basement membrane and epithelial cells, RT-restoring tubules, RVS- reduced vacuole size, MMC-melanomacrophage cells

G: DCT-distal convoluted tubule, ER-erythrocyte, PCT-proximal convoluted tubule; **H:** DCT-distal convoluted tubule, ER-erythrocyte, PCT-proximal convoluted tubule; **I:** DDCT-degenerative distal convoluted tubule, Dedeposition, DGL-degenerating glomeruli, SBE-Separation between basement membrane and epithelial cells, SPk-Severe pyknosis, ILS-increased lumen size; **J:** LPk-less pyknosis, RT-restoring tubules, RLS-reduced lumen size, RG-restoring glomeruli; **K:** SBE-separation between basement membrane and epithelial cells, De depositions, Ex- exudate, DT-degenerating tubules; **L:** DSBE- decreased separation between basement membrane and epithelial cells, RT- restoring tubules

Fig 5: Ameliorative effects of dietary ascorbic acid supplementation on Cr toxicity to the kidneys of *C. carpio* after 14 days (A-F) and 28 days (G-L) of exposure

3.4.1 Gills

Gills are the main organs of respiration in fishes and are involved in osmoregulation, acid base balance and the excretion of nitrogenous compounds (Alazemi *et al.* 1996) [7]. They are more prone to damage because any toxicant is present in water because of their direct contact with the toxicant. In the present investigation, microscopic examination of the gills of *C. carpio* revealed a normal histoarchitecture in both control groups, with normal sizes and structures of primary and secondary lamellae, pillar cells and erythrocytes (Figure 3 A and B). After 14 days of exposure, degeneration of secondary lamellae, hyperplasia, ruptured filaments, lymphocytic infiltration, disorganized chondrocytes and telangiectasia, i.e., swelling at the tips of secondary lamellae due to the accumulation of blood cells, were noted in the gills of the T_3 group (Figure 3 C). Similar aberrations were recorded in the T_4 group ($1/10^{th}$ LC_{50} + AsA), but the severity of damage was less than that in the T_3 group, as chondrocytes were organized and secondary lamellae were less deformed. However, exudates and hyperplasia still persisted in the gills of the T_4 group (Figure 3 D). The deleterious effects of Cr on gill histomorphology were more pronounced in the T_5 group ($1/5^{th}$ LC_{50}) than in the T_3 group ($1/10^{th}$ LC_{50}). In the T_5 group, severe damage to secondary lamellae along with fusion of lamellae was noted (Figure 3 E). Lymphocytic infiltration between the secondary lamellae was also greater than that in the T_3 group. The severity of alterations recorded in the T_6 group ($1/5^{th}$ LC_{50} +AsA) was lower than that in the T_5 group, as secondary lamellae were found to be restored to their original shape and size.

After 28 days of exposure, both control groups presented a normal histological structure of the gills characterized by normal sizes and shapes of primary and secondary lamellae, chondrocytes, erythrocytes and pillar cells (Figure 3G & H). However, the T_3 group ($1/10^{th}$ LC_{50}) presented degeneration of secondary lamellae, lymphocytic infiltration, necrosis and telangiectasia in secondary lamellae (Figure 3I). However, the degenerative changes recorded in the T_4 group were less severe than those recorded in the T_3 group. There was less lymphocytic infiltration between the secondary lamellae, while their structure was restored (Figure 3J). Severe damage to secondary lamellae and more pronounced lymphocytic

infiltration and disorganized chondrocytes, along with scattered exudates, were observed in the T_5 group (Figure 3 K). Similar observations were also recorded in *Gambusia affinis* (Begum *et al*. (2006) [9], *Oncorhynchus tshawytscha* (Farag *et al*. (2006) [14] and *C. punctatus* (Mishra and Mohanty, 2008) [24] exposed to Cr. These alterations recorded in the gills could be due to the accumulation of Cr in the gills, resulting in gill damage. Compared with those in the T_5 group, the gills in the T_6 group ($1/5^{th}$ LC_{50} +AsA) presented less severe histomorphological alterations, including reduced lymphocytic infiltration and restored secondary lamellar structure, after 28 days of exposure, possibly due to the attenuating effect of ascorbic acid supplementation (Figure 3 L). The present study results are supported by the observations of Jiraungkoorskul *et al*. (2006), who reported reduced alterations in the gill histoarchitecture of *Puntius altus* when exposed to cadmium and feed supplemented with ascorbic acid.

3.4.2 Liver

The liver plays an important role in detoxification, excretion, metabolism, digestion and storage of various substances that can cause toxicity in fish (Rana *et al.* 2015) [29, 43]. Microscopic examination of the livers of the T_1 and T_2 groups revealed a normal histoarchitecture characterized by normal hepatocytes, erythrocytes, Kupffer cells, sinusoids and bile ducts (Figure 4A & B). After 14 days of exposure, vacuolization of hepatocytes and dilation of sinusoids were observed at T_3 (Figure 4C). However, compared with T_3, T_4 ($1/10^{th}$ LC_{50}+ AsA) resulted in a reduction in the dilation of sinusoids and less vacuolization of hepatocytes (Figure 4 D). Hence, there was less tissue damage in the T_4 group than in the T_3 group, which might be due to the ameliorative effects of ascorbic acid. Furthermore, at higher concentrations (T_5:$1/5^{th}$ LC_{50}), liver damage was more pronounced than that at T_3, as revealed by pyknosis, increased vacuolization and degeneration of hepatocytes (Figure 4 E). In contrast, less vacuolization and reduced sinusoid dilation were observed in the T_6 group ($1/5^{th}$ LC_{50} + AsA) than in the T_5 group (Figure 4 F). After 28 days of exposure, both control groups (T_1 and T_2) presented normal hepatocytes, erythrocytes, Kupffer cells and sinusoids (Figure 4 G and H). However, pyknosis and degenerating hepatocytes were noted in group T_3 ($1/10^{th}$ LC_{50}), along with increased vacuolization and congestion in the hepatopancreas (Figure 4 I). However, the T_4 group ($1/10^{th}$ LC_{50}+ AsA) presented fewer alterations from the normal histoarchitecture than did the T_3 group. Compared with that of T_3, the structure of the hepatopancreas also revealed partial restoration and vacuolization (Figure 4J). Compared with those of the controls, the histomorphology of the livers of the fishes in the T_5 group ($1/5^{th}$ LC_{50}) exhibited maximum alterations, with severe vacuolization and pyknosis, and clustered erythrocytes and a reduced shape and size of hepatocytes (Figure 4 K). These results indicate that the effect increases with increasing Cr concentration. In contrast, T_6 ($1/5^{th}$ LC_{50} + AsA) resulted in the restoration of hepatocytes and normal erythrocytes (Figure 4L) and the reappearance of Kupffer cells at T_6, which was not observed at T_5. Therefore, in the present investigation, alterations observed in the histoarchitecture of the liver following Cr exposure were dependent on the concentration and exposure period. These observations are in accordance with the findings of Rana *et al*. (2015) [29, 43], who reported similar degenerative changes in the cytoarchitecture of the liver in *C. carpio* following exposure to Cr. As the liver is the main detoxifying organ, hepatic alterations indicate a self-protective mechanism in an attempt to mitigate toxicity (Mishra and Mohanty 2008) [24]. Rajeshkumar *et al*. (2017) [28, 42] also noted alterations, such as hepatocellular vacuolization and pyknotic nuclei, along with congestion in sinusoids in *C. carpio* following exposure to a mixture of heavy metals. Increased vacuolization could be due to increased fat accumulation in the cytoplasm, and an increased rate of metabolism during detoxification might have resulted in the degeneration of hepatocytes. The present study revealed less severe alterations in liver histomorphology in fish supplemented with dietary ascorbic acid, and the results are well supported by the findings of Jiraungkoorskul *et al*. (2006), who reported reduced damage to the liver of *P. altus* following exposure to cadmium upon supplementation with ascorbic acid.

3.4.3 Kidney

The kidney is a vital organ involved in the excretion of metabolites and is strongly affected by the presence of any toxicant in water. The toxic components present in the filtrate affect the structure and function of the organ involved in filtering. In the present study, the histomorphological alterations in the kidneys of fishes in both control groups (T_1 and T_2) revealed normal renal histoarchitecture in the proximal and distal convoluted tubules and glomeruli (Figure 5A & B). After 14 days of exposure, the T_3 group presented microscopic changes in the morphology of the kidney, including degeneration of convoluted tubules and glomeruli, along with increased lumen size and separation between the basement membrane and epithelial cells of tubules (Figure 5C). In T_4, ascorbic acid supplementation might have resulted in some recovery of tissue from damage since no separation between the basement membrane and epithelial cells was observed in T_4 compared with T_3 (Figure 5 D). The structure of convoluted tubules was also restored compared with that at T_3. The damage was found to increase with increasing Cr concentration, as the kidneys of the fish in group T_5 were affected more than those in the T_3 group were. Alterations in the histoarchitecture of the kidney were less severe at T_6 ($1/10^{th}$ LC_{50} + AsA) than at T_5 since reduced separation between the basement membrane and epithelial cells, regenerating tubules and reduced vacuolization were observed (Figure 5 F). The histomorphological alterations observed in the kidney increased with increasing exposure duration, as the changes observed after 28 days of exposure were more severe than those observed after 14 days of exposure. The kidneys of both the control groups (T_1 and T_2) presented normal histoarchitecture, with a normal structure of convoluted tubules and glomeruli (Figure 5 G and H) throughout the experiment. However, at T_3 ($1/10^{th}$ LC_{50}), Cr exposure caused severe pyknosis, degeneration of tubules and glomeruli, and deposition and separation between the basement membrane and epithelial cells (Figure 5 I). However, dietary ascorbic acid supplementation had an attenuating effect, as the damage caused by toxicity was lower in T_4 than in T_3. Histological examinations of the kidneys at T_4 ($1/10^{th}$ LC_{50}) revealed less pyknosis and that tubules and glomeruli restored their structure and sizes and reduced lumen size (Figure 5 J). Marked alterations, such as degenerating tubules and increased space between the basement membrane and epithelial cells along with deposition and exudates (Figure 5K), were observed at T_5 ($1/5^{th}$ LC_{50}), which were more pronounced than those at T_3. However, these alterations were

reduced at T_6 (1/5th LC_{50} +AsA). Additionally, separation between the basement membrane and epithelial cells was reduced to a great extent in T_6, and the shape and size of the tubules were restored (Figure 5 L). Our observations in the T_4 and T_6 groups are supported by the findings of Kumari and Ahirrao (2018) [22], who reported reduced degeneration of tubules, hypertrophy of glomeruli, vacuolization and necrosis in *H. fossilis* following acute exposure to cadmium and ascorbic acid supplementation.

4. Conclusion

The results of the present study revealed that Cr induced degenerative changes in *C. carpio* following acute and chronic exposure. Chronic exposure caused alterations at the hematological, biochemical and histological levels. The presence of more Cr in water bodies than permissible limits is deleterious for fish health. It could be concluded that ascorbic acid acts as an ameliorative agent because of the attenuating response against Cr toxicity observed in the present study. It can be suggested that fish farmers use ascorbic acid in fish feed to improve the health of *C. carpio* or that it be used for any other fish species.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

There is no fish specific information in the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forest and Climate Change, Government of India, and it is not mandatory to obtain ethical clearance for fish in India. However, authors followed all the applicable international, national, and/ or institutional guidelines for the care and use of animals.

6. References

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