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Histological changes in juvenile common carp (*Cyprinus carpio L.*) fed on high levels of chromium chloride hexahydrate

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ABSTRACT

We designed this experiment to examine the impact of high levels of dietary Cr(III) on the histological structure of the gut, liver, kidney, and spleen of the juvenile common carp (*Cyprinus carpio L.*; average weight 15 ± 0.69 g). Fish were fed with different levels of dietary Cr as chromium chloride $CrCl_3.6H_2O$ (0.0, 4.0, 6.0, and 8.0) mg kg^{-1} diet for 10 weeks at 25 ± 1.5ºC. At the end of the experiment, no mortalities were recorded. High levels of dietary Cr impaired the normal histological structure of the tested organs. Different Cr treatments showed signs of toxicity in the liver and gut that involved the appearance of inflammatory foci cells, pyknotic nuclei, foci of melanomacrophages, vacuole formation, degeneration of hepatocytes and erythrocytes aggregation in the liver and lifting of the lining epithelium, vacuolation, and surface erosion in the intestine. Cr treatments showed remarkable changes in the kidney that included renal tubular separation, cytoplasmic vacuolation, aggregation of red blood cells, degeneration of renal tubules, necrosis of hematopoietic tissue, and oedema. Injuries and damage were observed in the spleens of fish fed with Cr-fortified diets, including necrosis, depletion of lymphoid tissues, and vacuoles formation. In conclusion, ingestion of high levels of dietary Cr(III) generated adverse health effects, and the application of Cr(III) in fish diets should be cautious.

KEYWORDS: Chromium chloride, histopathology, toxicity, common carp (*Cyprinus carpio L.*)

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1. Introduction

Chromium (Cr) has various oxidation states, but the most important are hexavalent [Cr(VI)] and trivalent [Cr(III)] chromium. Cr(VI), can easily cross cell membranes, and it represents the toxic form of chromium (Pechova and Pavlata, 2007; Mohamed et al., 2020). The toxic effects of chromium are due to an association of chromium with several macromolecules such as genetic materials within the cytosol, leading to toxic and mutagenic changes (Bakshi and Panigrahi, 2018).

Despite the fact that Cr(III) is poorly absorbed by the gastrointestinal tract, it is considered to be the most stable form and is essential to maintain and regulate normal glucose, protein, and lipid metabolism (Anderson, 1995).

The nutritional value of Cr(III) in its organic and inorganic forms in fish has gained some attention in recent years. In this context, Cr-Nicotinate and Cr-Picolinate improved glucose utilisation in tilapia (*Oreochromis niloticus* x *O. aureus*) (Pan et al., 2002) and Cr yeast modulated the immune response of rainbow trout (*Oncorhynchus mykiss*) (Gatta et al., 2001). Further, Cr-Picolinate increased weight gain in grass carp (*Ctenopharyngodon idellus*) (Li and Zhou, 2008).

Similarly, Cr chloride, Cr-yeast, and Cr-Picolinate supplementation significantly promoted growth performance, feed conversion ratio, and protein efficiency in common and mirror carp (*C. carpio*) (Ahmed et al., 2012a, b; Ahmed et al., 2013). On the other hand, some studies reported that dietary Cr had no effect on the parameters tested (Selcuk et al., 2010; Mehrim, 2014). The source of dietary chromium, diet ingredients, fish size, duration of the study, and the concentration of Cr added seem to be critical factors that altered Cr metabolism.

Based on the results of previous studies, the most positive effects were obtained when Cr was incorporated at a level not exceeding 2.0 mg Cr kg^{-1} (Ahmed, et al., 2013), whereas high Cr supplementation to the fish diet resulted in growth reduction (Tacon and Beveridge, 1982; Giri et al., 2014), negative impacts on blood parameters (Liu et al., 2010) and different histopathological changes in liver and gut tissues (Ahmed et al., 2012a). Velma and Tchounwou (2013) reported that Cr(VI) can cause DNA damage to liver and kidney cells in goldfish.

Very few studies have been published on the toxicity of dietary Cr(III) for fish, and the tolerable level of dietary Cr(III) for fish is unknown.

This experiment was conducted to assess the impact of high levels of dietary Cr(III) on the histological structure of different vital organs (liver, gut, kidney, and spleen) of common carp (*C. carpio*).

2. Material and Methods

2.1. Experimental fish and husbandry

This trial was carried out at the Vertebrate Department of the Marine Science Centre (MSC) at the University of Basrah, Iraq. Common carp (*C. carpio*) were supplied from the MSC farm (Basrah, Iraq). After 4 weeks of acclimation, 10 fish (average weight 15 ± 0.69 g) were randomly distributed into 12 fiberglass tanks (each measuring approximately 75×75×45 cm with a capacity of roughly 250 L, each tank provided with aerated freshwater at a rate of 4.5 l min^{-1}) comprising a semi-closed aquaculture system. All treatments were performed in triplicate. Fish were fed 3% biomass provided in equal rations four times a day for 10 weeks.

During the trial, the mechanical filters have been changed continuously to maintain the water quality (Sera Filter Wool) meanwhile; the water was partially changed every 24 hours.

The average water temperature was 25 ± 1.5°C, and the pH (6.2–7.8) was measured by a portable pH meter (Model 8685; AZ Instrument Corp). The average dissolved oxygen (not lower than 93.2 %) in the water was monitored daily by an oxygen meter (Lovibond®, SensoDirect). NH_3, NO_2^- and NO_3^- values were kept at the acceptable level during the feeding trial and supervised using a Freshwater Master Test Kit (Aquarium Pharmaceuticals).

2.2. Diets ingredients and preparation

Ingredients and chemical composition of the experimental diets are presented in Table (1).

Three experimental diets were fortified with 4.0, 6.0, and 8.0 mg Cr kg^{-1} as chromium chloride hexahydrate ($CrCl_3.6H_2O$). The basal diet (without Cr supplementation) was fed to the control group. Dry ingredients were weighed and mixed thoroughly, followed by the addition of liquids (oil and distilled water).

A certain amount of $CrCl_3.6H_2O$ was dissolved in 500 ml of distilled water and added gradually to the mixture. The dough was extruded to produce small pellets using a craft machine (Model No. CR-43, China). Diets were dried at 45º C and stored in plastic bags at 4º C until use.

Table 1. Ingredients and proximate composition of the experimental diets

Ingredients	Control	T1	T2	T3
Fish Meal	250	250	250	250
Corn starch	385	381	379	377
Pea Protein	171.5	171.5	171.5	171.5
Corn gluten	160	160	160	160
Sunflower Oil	33.5	33.5	33.5	33.5
a Cr added (mg kg^{-1})	0.0	4.0	6.0	8.0

[a] $CrCl_3.6H_2O$: Sigma-Aldrich Company, UK.
All other ingredients were purchased from local markets.

2.3. Histological study

At the end of the nutritional trial, 2 fish were taken from each replicate tank. Fish were anaesthetised using buffered MS222 and then sacrificed for routine histological examination. The intestines, livers, spleens, and kidneys were excised, rinsed in physiological saline, and fixed in 10% neutral buffered formalin for 48 h. The tissues were dehydrated in an ascending concentration of ethyl alcohol, embedded in paraffin, and sectioned at 7 mm. Then, the tissue sections were stained with haematoxylin and eosin (H&E), and 10 sections of each tissue from each specimen were examined using a Krüss light microscope. Olympus digital camera was used at a magnification of 400X to determine the normal and abnormal structure of organs. The thickness of each section was 7 µm, then; haematoxylin and eosin were used for tissues staining.

3. Results

3.1. Histological observations

3.1.1. Gut histological observations

No histopathological changes were found in the control group, while gut samples from different levels of Cr treatments showed signs of pathologies (Figure 1).

The occasional area of necrosis, adherence of villi, and the occasional villus tip with some erosion of the surface and hyperplasia in the epithelial cells layer were recorded in the fish fed on diets containing 4.0 and 6.0 mg Cr kg^{-1}. One or more of these pathologies were observed in four out of six fish examined in the mentioned treatments (Figure 1).

Feeding the fish on an 8 mg Cr kg^{-1} diet resulted in 5 fish out of 6 diagnosed with the same types of gut pathologies, including lifting of the epithelium from the basement membrane, the appearance of vacuoles, and aggregation of blood cells in some areas of the mucosal layer (Figure 1)

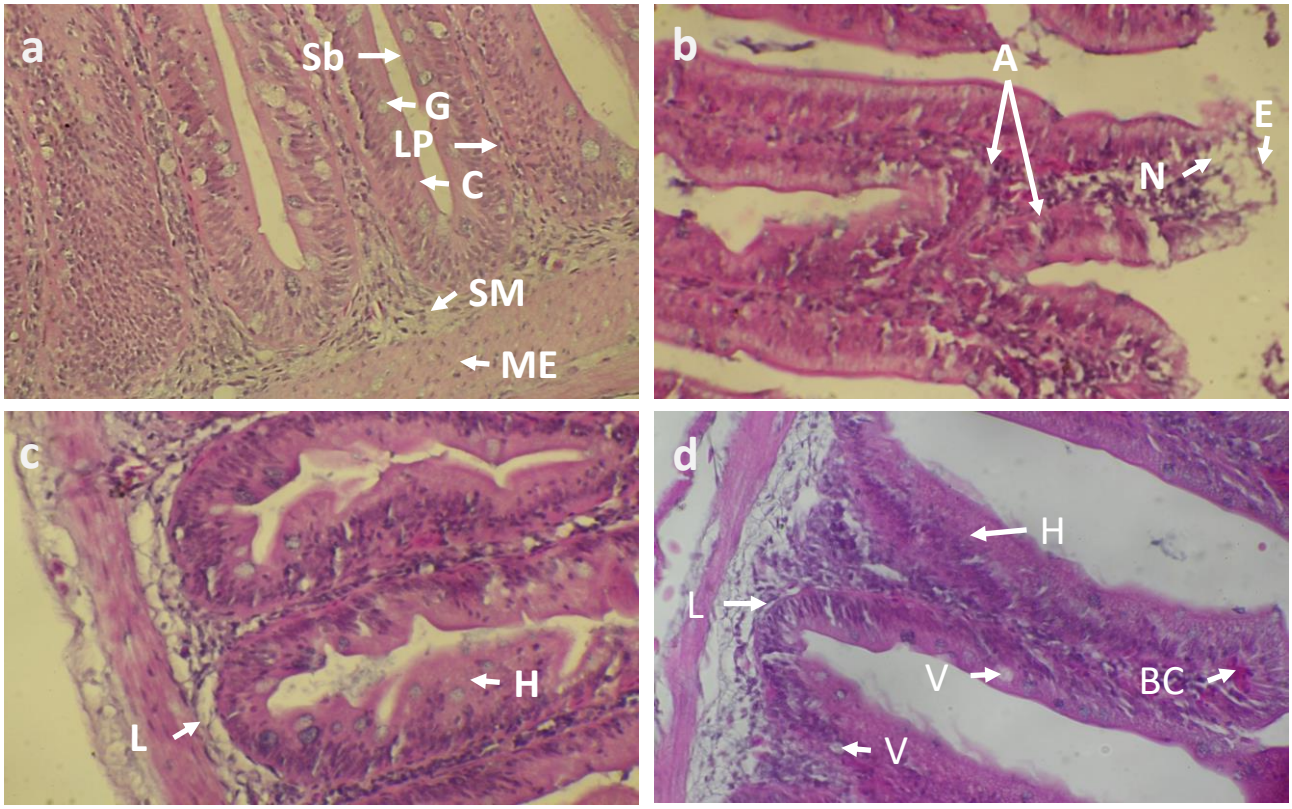

Figure 1. intestinal morphology in juvenile common carp (*C. carpio* L.) following dietary exposure to (a) control; (b) 4 mg of Cr(III); 6 mg of Cr(III); or 8 mg of Cr(III). The gut histology structure of the control groups appeared to be normal with columnar cells (C), goblet cells (G), and the striated border (Sb). The lamina propria (Lp) is located under the mucosal epithelial layer. The submucosa (SM) contains blood vessels and connective tissue cells. The inner circular smooth muscle fibres of the muscularis externa are visible in the control group. All Cr treatments showed injuries including necrosis in the layer (N); erosion of villi (E); adherence of villi (A); hyperplasia (H); lifting epithelium (L); the appearance of vacuoles (V); aggregation of blood cells close to the mucosal layer.

3.1.2. Liver histological observation

The livers tissue of the control group animals showed normal histology with a continuous mass of hepatic parenchymal cells that arranged in cords around the blood sinusoid (Figure 2). The livers of fish fed on 4.0 mg Cr kg^{-1} showed minor changes compared to the controls with some mild lipidosis, swollen and hypochromatic nuclei, and elongation of some blood vessels in four out of six fish sampled at the end of the trial. Similarly, 6.0 and 8.0 mg Cr kg^{-1} in fish diet caused similar types of lesions to those observed in low concentration of dietary Cr; in addition, the occasional foci of inflammatory cells, some cells with pyknotic nuclei, foci of melanomacrophages, vacuole formation, and degeneration of hepatocytes and aggregation of erythrocytes were recorded. One or more of these lesions were found in all six fish examined in both 6.0 and 8.0 mg Cr kg^{-1} treatments (Figure 2). Kadir Eryılmaz and Hasan Kuzuoğlu; the Kuzuoğlu group owner and to the late Prof. Dr. İbrahim Okumuş who directed the Black Sea salmon studies.

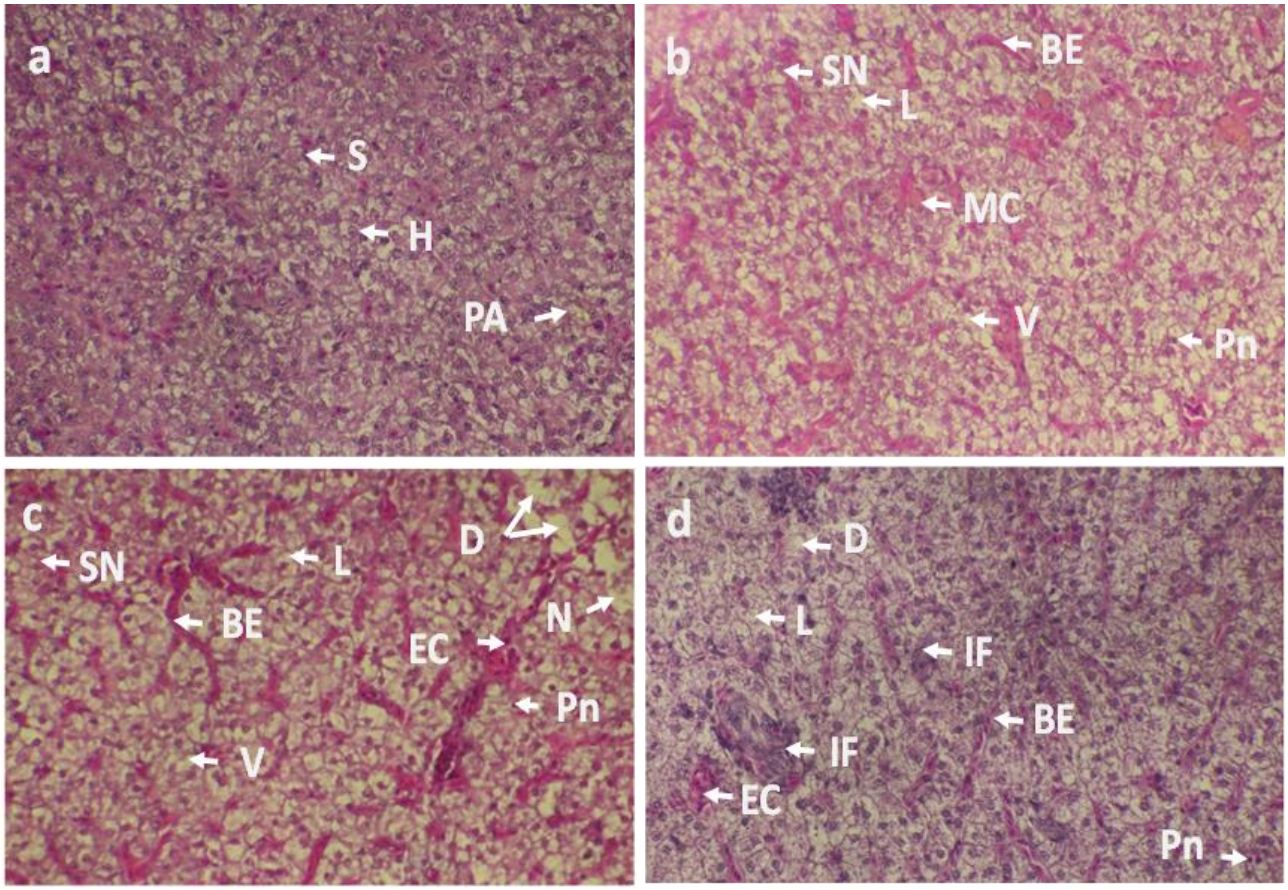

Figure 2. Liver morphology in juvenile common carp (*C. carpio*) following dietary exposure to (a) control; (b); 4.0 mg of Cr(III); 6.0 mg of Cr(III); 8.0 mg of Cr(III). The livers of control fish showed normal polygonal hepatocytes (H), and normal pancreatic acini (PA) with sinusoid space (S). All Cr treatments showed injuries, including swollen and hypochromatic nuclei (SN); blood vessel elongation (BE); lipidosis (L); vacuole formation (V); cells with pyknotic nuclei (Pn); foci of melanomacrophages (MC); foci of inflammatory cells (IF); degeneration of hepatocytes (D); aggregation of erythrocytes (EC).

3.1.3. Spleen histological observation

Spleen morphology of carp was normal in the control animals (Figure 3). Various treatments (4.0, 6.0, and 8.0) mg Cr kg^{-1} caused similar changes in the spleen. These changes involved foci of necrosis, depletion of lymphoid tissues (appearing as space containing fewer cells), foci of melanomacrophage deposits, vacuole formation, and oedema. These changes were observed in five out of six fish examined from each concentration (Figure 3).

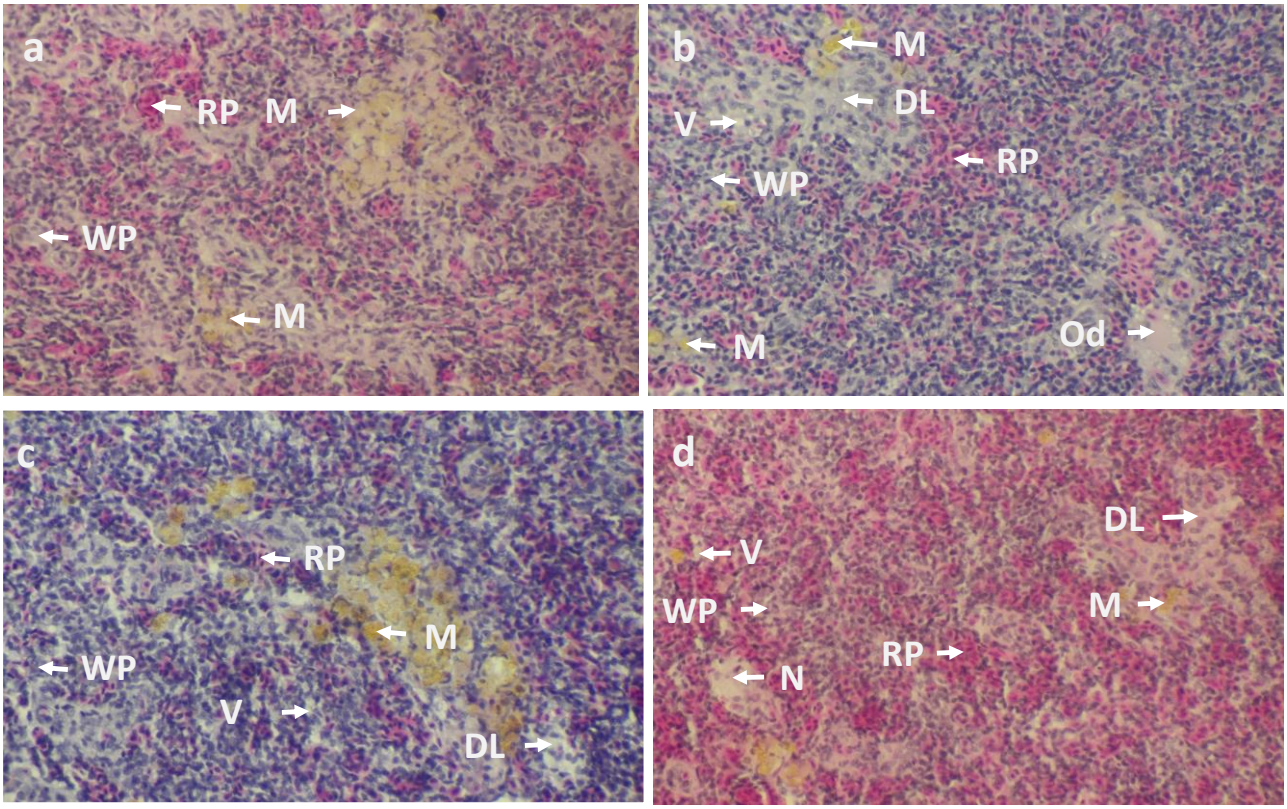

Figure 3. Spleen morphology in juvenile common carp (*Cyprinus carpio L.*) following dietary exposure to (a) control; (b); 4.0 mg of Cr(III); 6.0 mg of Cr(III); 8.0 mg of Cr(III). The spleen of the control fish showed normal histology, with defined red (RP) and white (WP) pulp as well as foci of melanomacrophage deposits (M). All Cr treatments showed similar types of injuries, including necrosis (N), depletion of lymphoid tissues (DL), increased melanomacrophage deposits (M), vacuole formation (V), and oedema (Od).

3.1.4. Kidney histological observation

The kidneys of control group animals showed the normal structure (Figure 4). The histology of kidney samples taken from fish fed on 4.0 mg Cr kg^{-1} showed occasional renal tubular separation, cytoplasmic vacuolation, degeneration of renal tubules, and minor elevation of melanomacrophage deposits. These changes were observed in three out of six fish examined. High levels of Cr (6.0 and 8.0) mg Cr kg^{-1} in fish diet resulted in similar types of lesions to those observed in the lower concentration of Cr (4.0 mg kg^{-1}), with a few necrotic cells in the hematopoietic tissue in addition to foci of oedema and aggregation of red blood cells. These changes were observed in five out of six samples taken from each concentration (6.0 and 8.0 mg Cr kg^{-1}).

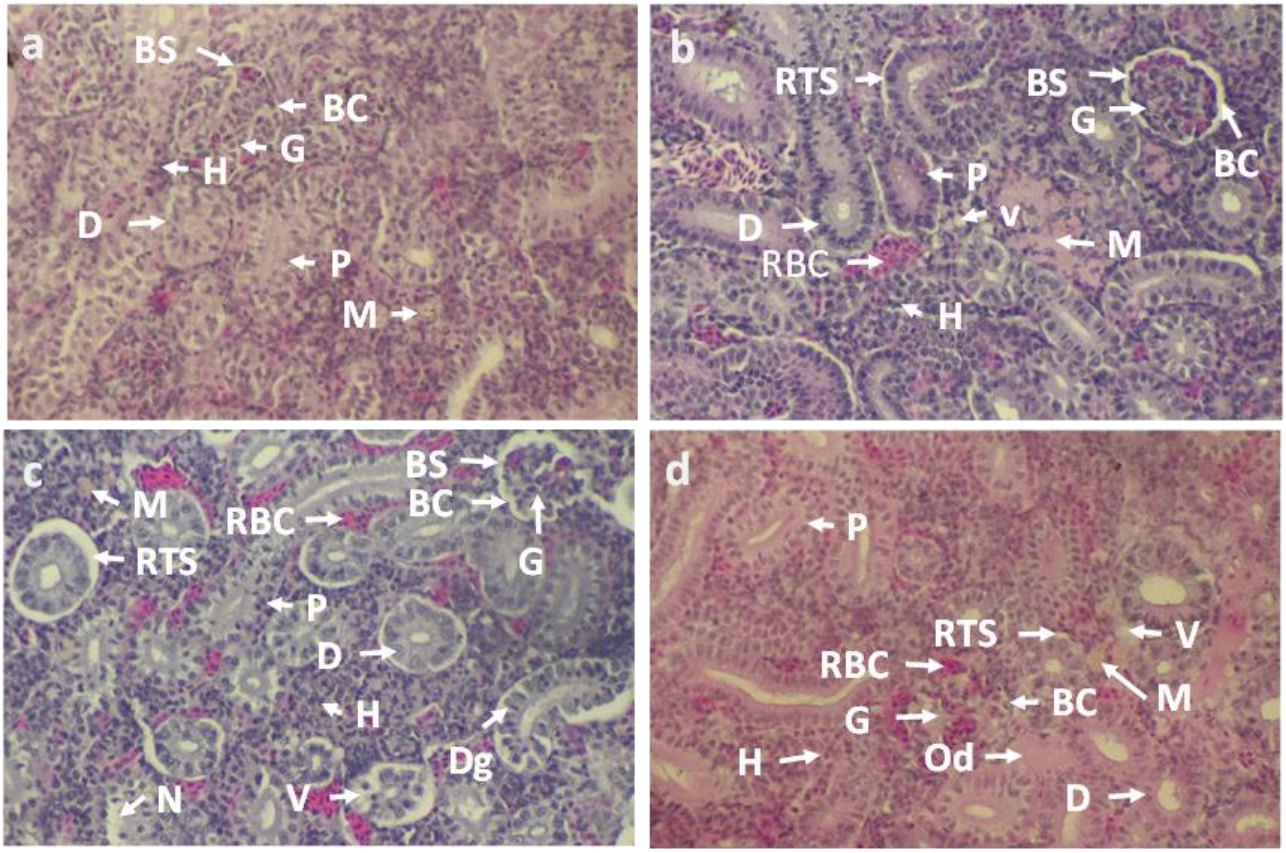

Figure 4. Kidney morphology in juvenile common carp (*C. carpio*) following dietary exposure to (a) control; (b); 4.0 mg of Cr(III); 6.0 mg of Cr(III); 8.0 mg of Cr(III). Kidneys of control fish showed normal histology with parietal epithelium of Bowman's capsule (BC); glomerulus (G); Bowman's space (BS); proximal tubules (P); distal tubules (D); hematopoietic tissue (H); and melanomacrophages (M). All Cr treatments showed injuries that included renal tubular separation (RTS); cytoplasmic vacuolation (V); aggregation of red blood cells (RBC); degeneration of renal tubules (Dg); necrosis of hematopoietic tissue (N); and oedema (Od).

4. Discussion

It is generally accepted that Cr(III) is less toxic than Cr(IV) and is an essential micronutrient for its beneficial role in glucose/insulin homeostasis; however, biological reductants, such as L-cysteine and nicotinamide adenine dinucleotide (NADH) can reduce Cr(III) to Cr(II), and the latter form can react with hydrogen peroxide to generate hydroxyl radicals (Ozawa and Hanaki, 1990). Under stress conditions, the anti-oxidative system may be damaged, and the reactive oxygen species will accumulate in the body and affect cell functions (Pizzino et al., 2017).

In animal studies, the histological examination is widely used to detect signs of disease not easily recognised by gross examination and is a vital means of supervising general fish health (Gallhoefer et al., 2013; Musumeci, 2014).

Therefore, different vital organs from the experimental fish were collected after 10 weeks of Cr treatments to assess the response of fish to dietary Cr(III) supplementation. The liver is responsible for different functions, such as uptake, metabolism, storage, redistribution of nutrients, and secretion of molecules into the blood (Genten et al., 2009).

In the present study, the livers of fish fed on high levels of dietary Cr(III) supplementation showed different signs of deterioration in the hepatic structure compared to the control groups, such as swollen and hypochromatic nuclei, blood vessel elongation, lipidosis, vacuole formation, cells with pyknotic nuclei, foci of

melanomacrophages, foci of inflammatory cells and degeneration of hepatocytes and erythrocytes.

Hinton et al. (2001) has been suggested that deterioration of hepatic structure and function not only affects the liver itself, but may also impair the function of other organs, leading to death of the organism. In our previous study, the liver of common carp (*C. carpio*) showed vacuoles formation when fish were fed on high levels of chromium chloride supplementation (2.0 mg kg^{-1} diet) (Ahmed et al., 2013).

Hyperplasia, cellular disorganization and hepatic cells necrosis were found in Labeo rohita after 60 days of exposure to 1/10th LC50 of Cr after 96 h (Muthukumaravel and Rajaraman, 2013).

In fish and mammals, the gastrointestinal tract plays an important role in the absorption of xenobiotics (Hinton et al., 2001). In the present study, high levels of dietary Cr induced different signs of abnormality that included erosion and adherence of villi, hyperplasia, and lifting epithelium, the appearance of vacuoles and aggregation of blood cells close to the mucosal layer. It seems that large doses of an element in the diet may cause tissue toxicity and local irritation of the intestine due to the direct contact with the metal (Di Giulio and Hinton, 2008).

The necrosis and fusion in the mucosal folds were observed in the intestine of mirror carp (*C. carpio*) when fed on 2.0 mg $kg^{-1}$of Cr (III) for 8 weeks (Ahmed et al., 2012 a).

Gut histology could interpret the reduction in growth performance in fish fed on diets fortified with high levels of Cr compared to the control group. It is likely that the damage to gut structure and integrity affected the process of food digestion and nutrient absorption.

In human studies, the absorbed Cr will be distributed to all parts of the body, with the highest levels being found in the kidney, liver, spleen and bone (Stearns et al., 1995).

Histological examination of the kidneys of fish fed on Cr treatments indicated significant induction of Cr toxicity; high concentrations of dietary Cr induced marked changes in kidney histology, such as renal tubular separation, cytoplasmic vacuolation, and aggregation of red blood cells in the sinusoid and degeneration of renal tubules.

The effect of dietary Cr (III) supplementation on the kidneys of fish has not been investigated compared to Cr (IV) supplementation. The kidney of *Channa punctata* (Bloch) exposed to 20 mg Cr (IV) L^{-1} displayed hypertrophied epithelial cells of renal tubules, reduction in tubular lumens, and contraction of glomeruli (Mishra and Mohanty, 2008).

It has been reported that the spleen in some fish species may have diverse functions, but in general, the spleen is involved in immune responses and is considered to be a storage organ for both red and white blood cells (Lawrence and Hemingway, 2003). The microscopic sections of the spleen from different concentrations showed an increase in melanomacrophage deposits, vacuole formation, and oedema. These alterations are most likely a result of the hyper-function of the spleen connected with the active erythropoiesis and destruction of abnormal erythrocytes (Gorgieva et al., 2010).

Despite the consensus on the poor ability of Cr(III) to cross cell membranes, it seems that carp have a significant ability to absorb Cr(III) from their diet during the juvenile stage. In human studies, it has also been reported that Cr absorption ability is higher in the early stages and decreases with advancing age (Zafra-Stone et al., 2007). Further, human studies have demonstrated that the normal adult response time to inorganic Cr treatment requires at least 1 month; whereas, in children, response time to inorganic Cr was typically 24 hrs (Milner, 1990). In conclusion and based on evidence from some recent studies, it seems that the absorption of dietary Cr is a complicated process and varies from one organism to another; therefore, more consideration should be given to the safe usage of inorganic Cr and the application of this form in fish diet should be cautious, especially in the early stages of fish life.

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