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Original article

HAEMATOLOGICAL, BLOOD BIOCHEMICAL AND HISTOPATHOLOGICAL EFFECTS OF SUBLETHAL CADMIUM AND LEAD CONCENTRATIONS IN COMMON CARP

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Summary

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The present research aimed at examining the effects of common carp (*Cyprinus carpio)* exposure to sublethal concentrations of two non-essential heavy metals: cadmium (Cd: 8.4 mg/L) and lead (Pb: 6.2 mg/L) for 15 days to evaluate occurring biochemical and haematological effects. The examined parameters included haematocrit (Hct), haemoglobin (Hb), lymphocytes (Lym), neutrophils (Neu), total protein (TP), albumin (Alb), immunoglobulin M (IgM), glucose, red and white blood cells counts (RBC & WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). Exposure to both metals significantly ($P<0.05$) reduced the amounts of WBC and MCHC. MCV values decreased ($P<0.05$) after the Pb treatment but MCV estimates with Cd exposure showed no differences. MCH levels increased in both treatments ($P<0.05$) whereas Hct, Hb, RBC, Lym, and Neu following both metal exposures were almost similar to those in the control. IgM values were elevated in fish contaminated with both Pb and Cd ($P<0.05$). The exposed fish showed fusion of gill lamellae, vessel dilatation, hyperaemia, and hyperplasia of gill epithelial cells whereas muscle histology remained unchanged. The observed responses can be secondary to low heavy metals concentrations reflecting the trigger of stress reactions in affected fish.

Key words: cadmium, common carp, haematology, histopathology, lead

INTRODUCTION

Cadmium (Cd) is considered as one of the most toxic contaminants in polluted waterways, causing toxicity at any level of the ecologic stratum (Rashed, 2001). Even at sub-lethal concentrations, cadmium has a cumulative effect and causes serious physiologic disturbances in fish, such as anorexia as well as effects on the blood cells (Witeska, 1998; Cicik & Engin, 2005). Particular attention, therefore, must be focused on fish because of their high affinity for cadmium (De Conto Cinier *et al*., 1999). Lead (Pb) is a non-essential and non-beneficial element known to alter

the haematologic system of hosts by inhibiting the activities of some enzymes involved in heme biosynthesis (ATSDR, 2005). While data exist on a variety of lead effects on fish, little attention has been paid to biochemical changes that develop more quickly in response to lead toxicity in various fish species, particularly in common carp with less known physiological effects of lead (Nehar *et al*., 2010; Ergönül *et al*., 2012). In general, lead and cadmium are classified as potentially toxic heavy metals because they are very harmful, even at low concentrations, when ingested over a long time period (Guardiola *et al*., 2013).

Physiological and biochemical profiles in fish and other aquatic organisms under heavy metal stress serve as important bioindicators in aquatic environment monitoring (Shalaby *et al*., 2005; Abbas, 2006; Abbas *et al*., 2007). Haematological values and alterations have been widely used as indices for determining the physiological and health status of fish, therefore, allowing a relatively rapid evaluation of the chronic toxicities of a compound (Blaxhall, 1972). They also represent tools to monitor stress and pathological changes (Kori-Siakpere *et al*., 2005). Therefore, measurement of serum biochemical parameters can be a useful diagnostic tool in toxicology to find their general health status and target organs affected by toxicants (Zikic *et al*., 2001; Mc Donald & Grosell, 2006).

Nonetheless, the slow progress in the establishment of baseline blood parameters for many fish species necessitates considering some biological data to which haematologic tissues respond (Ranzani-Paiva *et al*., 2003). Their changes depend on fish species, age, the cycle of the sexual maturity of spawners, and diseases (Golovina, 1996; Luskova, 1997). It should be noted that although sufficient investigations have addressed the mechanisms of fish physiological and biochemical reactions to xenobiotics, it is evident that species differences of these mechanisms exist (Folmar, 1993). Furthermore, relatively little is known about Cd and Pb effects on the immune system and histopathology of fish (Witeska *et al*., 2010); immunotoxicological aspects are also important for aquaculture in certain regions that have been slightly evaluated and resulted in controversial results (Guardiola *et al*., 2013) justifying further studies.

Due to accumulation of heavy metals in fish gills as well as its role in respiration and osmotic balance, gill tissue is considered a good indicator of long-term exposure to heavy metals and also of resultant water pollution (Wepener *et al*., 2001; Filazi *et al.,* 2003). Concerning public health issues, cadmium contamination of food is of critical significance as cadmium is involved in enduring health problems. Moreover, *C. carpio* have the ability to accumulate and concentrate cadmium to levels several orders of magnitude above those found in their environment (De Conto Cinier *et al*., 1999). Accordingly, because data are scarce and, in some cases, conflicting, this study aimed at determining changes in the histology of common carp's gill tissue following exposure to sub-lethal amounts of cadmium and lead. The present study also sought to examine haematological and biochemical changes in this species influenced by sublethal levels of Pb and Cd, the most common pollutants in waterways of many areas in the world.

MATERIALS AND METHODS

The experimental common carp *Cyprinus carpio* (11.68 ±1.92 cm and 25.92±6.3 g)

were sampled from Nasr Fish Culture pond (Sari, Iran). Prior to toxicity testing, the fish were acclimatised for one week under laboratory conditions (25±1° C, 12 h light: 12 h dark). Water quality parameters (TDS=600 ppm, pH=6.75, EC=1 ds/m) were measured during the experiment. During acclimation and toxicity test, the fish were not fed. The heavy metals Cd and Pb in the chemical forms of cadmium (II) chloride ($CdCl_2.H_2O$, BDH) and lead (II) nitrate [$Pb(NO_3)_2$, Merck, Whitehouse Station, NJ] were used in the present study. For the experiments, first the LC_{50}-96 h value for each metal was determined. There were three triplicate treatments in nine aquaria (incl. control) with nine fish per replication. The fish (excluding control) were exposed to sublethal concentrations (i.e., 10% of the determined LC_{50}-96 h value for each metal: Abedi *et al*., 2012) of Cd (test value used = 8.4 mg/L) and Pb (test value used = 6.26 mg/L) for a period of 15 days.

After the exposure period, no mortalities were observed in the experimental fish. Following the completion of toxicity test, blood samples (1 mL) were immediately taken from the caudal vein of 10 fish from each aquarium using both heparinated and non-heparinated syringes (for haematological and biochemical analyses, respectively). Red and white blood cells (RBC & WBC) were counted by Neubar haemocytometer using a special kit (Pars Azmoon Co., Iran) by diluting fluid (3 g sodium citrate, 99 mL distilled water and 1 mL formalin) counting the four corners and the center fields. Haemoglobin (Hb) was determined spectrophotometrically at 540 nm absorbance (cyanmethemoglobin method) by the use of Pars Azmoon kit. Haematocrit (Hct) was determined by standard microhaematocrit method and expressed in percentages. Erythrocyte indices – mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) – were calculated according to Dacie & Lewis (1984). Differential leukocyte counting was performed with blood smears stained with May-Grunwald⁄Giemsa. The smears (two slides per fish) were examined by light microscopy under oil immersion at 100× magnification.

In order to separate serum, blood samples (1 mL) simultaneously collected from the caudal vein s were left to coagulate at 4 °C for 15–20 min and then centrifuged (3000 rpm, 20 min). The fresh serum was then subjected to biochemical analyses. Serum biochemical analyses: glucose, total protein (TP), albumin, and IgM were determined by the use of Pars-Azmoon Kit with a EURO LYSER plus autoanalyzer.

Comparison of the control and experimental groups was statistically analysed by Student's t-test, and the results were validated when $P<0.05$.

Gill tissue and muscle samples from the experimental *C. carpio* were taken and fixed in 10% buffered neutral formalin. The tissues were processed to obtain paraffin sections (5.0 μm thick), stained with haematoxylin and eosin (H&E) according to Bancroft *et al*. (1996), and then examined under light microscope (Dybern, 1983). Histological photos were taken by a camera microscope (Olympus BX41, Japan).

RESULTS

Survival rate of control fish was 100% in the present examination. The results indicated some differences in the blood values of control and contaminated *C. carpio* (Table 1). In general, WBC, MCH, and

IgM contents differed significantly compared with the control values (Tables 1 & 2). The lead treatment caused a significant reduction in MCV (198.45±18.16) compared to the control ($P<0.05$) but cadmium-treated carps presented almost unchanged MCV values (232.93±35.77). Significant differences were observed between the lead and cadmium treatments *vs* control in MCH levels, and both treatments showed a significant increase compared to the control group. In addition, MCHC decreased in both Cd and Pb treatments as opposed to the control ($P<0.05$).

Table 2 reveals that IgM values were elevated in the fish contaminated with both lead (161.3±70.12) and cadmium (134.9±24.08) as opposed to the control (66.3±2.82; $P<0.05$). Albumin levels were almost similar in fish exposed to both heavy metals with marked differences to those recorded in control ($P<0.05$). Concentrations of TP and Glu were not significantly different ($P>0.05$) between the metal-exposed fish and the control.

The contaminated gills of common carp revealed some fusion of gill lamellae, vessel dilatation, hyperaemia, and hyper-

Table 1. Haematological parameters in the blood of *C. carpio* exposed to sub-acute concentrations of cadmium and lead for 15 days (mean±SEM, n=10)

Haematological characteristics	Cadmium	Lead	Control
Hct (%)	31.44±2.5^a	29.57 ±3.91^a	32.8±2.16^a
Hb (g/L)	73.20 ± 10.90^a	69.00 ± 13.60^a	87.60 ± 6.80^a
RBC ($\times10^{12}$/L)	1.38 ± 0.25^a	1.51 ± 0.33^a	1.42 ± 0.05^a
WBC ($\times10^{9}$/L)	4.03 ± 0.62^b	4.63 ± 1.00^b	6.70 ± 1.06^a
Lym (%)	98.56 ± 1.33^a	99.00±1.15^a	99.20 ± 0.83^a
Neu (%)	1.44 ± 1.333^a	1.00±1.155^a	0.80 ± 0.837^a
MCV (fL)	232.93 ± 35.77^a	198.45±18.16^b	230.86 ± 8.77^a
MCH (pg/cell)	53.38 ± 4.88^b	45.88 ± 3.70^c	61.70 ± 4.63^a
MCHC (%)	23.16 ± 2.27^b	23.186 ± 1.67^b	26.78 ± 2.33^a

Hct: haematocrit; Hb: haemoglobin; RBC: red blood cells; WBC: white blood cells; Lym: lymphocytes; Neu: neutrophils; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration. Different superscripts in a row indicate statistically significant differences ($P<0.05$).

Table 2. Biochemical parameters in the blood of *C. carpio* exposed to sub-acute concentrations of cadmium and lead for 15 days. (mean±SEM, n=10)

Biochemical characteristics	Cadmium	Lead	Control
IgM (mg/L)	1349.00 ± 240.80^a	1613.00 ± 701.20^a	663.00 ± 28.20^b
Alb (g/L)	10.00 ± 5.29^a	10.30 ± 3.20^a	2.50 ± 0.70^b
TP (g/L)	31.00 ± 13.00^a	21.00 ± 4.00^a	20.00 ± 0.70^a
Glu (mmol/L)	10.56 ± 2.75^a	8.72 ± 0.76^a	6.69 ± 0.92^a

IgM: immunoglubin M; Alb: albumin; TP: total protein; Glu: glucose. Different superscripts in a row indicate statistically significant differences ($P<0.05$).

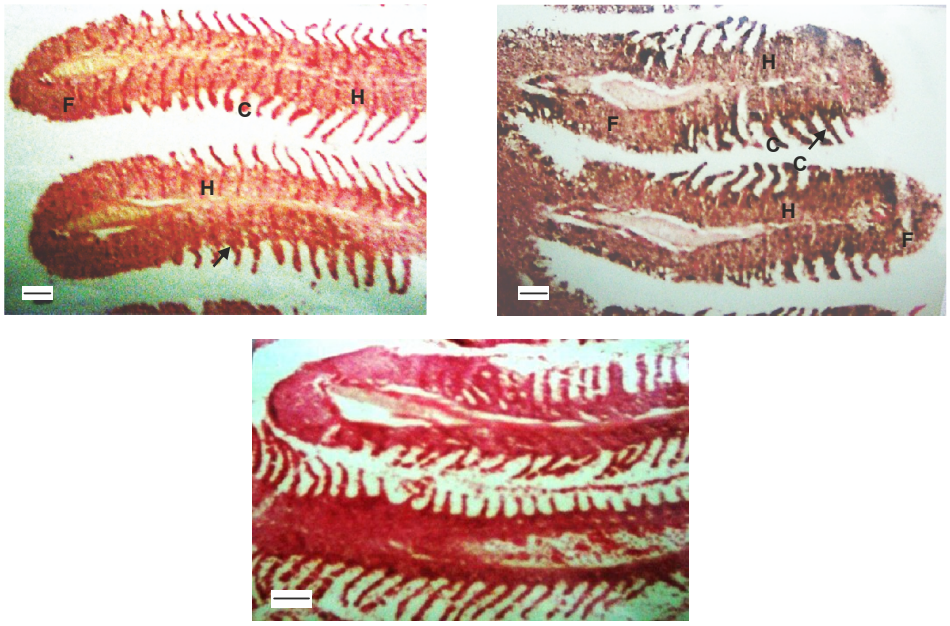

Fig. 1. Microscopic images of common carp's gill exposed to sub-acute levels of Cd (top, left) and Pb (top, right); Club cells (C), hyperplasia (H), fusion of the gill lamellae (F), and blood (arrow) in gill filaments. Control image (below) shows no histological lesion. H & E, bar=1 mm.

plasia of gill epithelial cells (Fig. 1). Compared to the normal pink gills (control), the contaminated gills turned to grayish black particularly due to the heavy metal deposits leading to swollen gills as well. The primary lamellae in the control fish appeared normal with well-defined, branched secondary lamellae. The wrinkled and curled secondary lamellae in the exposed fish seemed to reveal necrosis, dissociation of epithelial cells, haemorrhage and damage of epithelial cells of both primary and secondary lamellae. In addition, lead exposure led to more pronounced histological effects noted above than those from cadmium (Fig. 1).

DISCUSSION

Our experimental *C. carpio* displayed no significant elevations in the levels of blood glucose when exposed to sublethal concentrations of cadmium and lead, which disagrees with those found by Hontela *et al*. (1996) in rainbow trout and by Ergönül *et al*. (2012) in the same species treated, respectively, with cadmium and lead. The glucose fluctuations as a result of metal contaminations are apparently dose-dependent as was also detected in *C. carpio* exposed to gallium, in which levels of blood glucose markedly increased at high (4.0 mg L^{-1}) compared to low (2.0 mg L^{-1}) metal concentrations (Yang & Chen, 2003).

IgM is the only important immunoglobulin class described in fish, and the major antibody of primary response in higher vertebrates. According to our results, mean IgM values were significantly higher in fish contacted with both Cd and Pb sub-acute levels. In *Tilipia nilotica* on

the other hand, cadmium sulphate exposure (0.5 p.p.m) led to significant decrease in IgM level from the first week until the end (Zaki *et al*., 2010), which the authors attributed to a high cortisol secretion indicated by hyperglycaemia in the exposed fish as well as suppression of immune system of exposed fish rendering them susceptible to any infective agent. Finally, intoxication of gilthead seabream, *Sparus aurata*, with waterborne Cd (5 μM $CdCl_2$ or 1 $mg.L^{-1}$) resulted in insignificant elevation of serum IgM levels after 10 and 30 days of exposure (Guardiola *et al*., 2013).

In *Rutilus rutilus caspicus* exposed to sublethal concentrations of Cd and Pb for a period of 4 days, lead reduced lymphocyte but increased monocytes and neutrophils; cadmium, on the other hand, elevated the number of lymphocytes while decreased the number of monocytes and neutrophils (Akbari *et al*., 2010). In the present study, the number of lymphocytes remained unchanged with both lead and cadmium exposure, and both treatments showed significant decreases in WBC compared to the control. Likewise, Witeska *et al*. (2009) detected that 5 and 10 mg/L of Cd caused pronounced and prolonged reductions in leukocyte counts of common carp exposed to Cd whereas WBC and leukocyte counts showed no marked fluctuations in the same species exposed to sub-acute lead levels (Ergönül *et al*., 2012). The concentrations of Hct, RBC, Hb, and Neu showed no considerable fluctuations in the blood of common carp exposed to both cadmium and lead as opposed to those measured in the control. This may indicate that the fish did not show a distinct anaemia as was also reported in the same species by Witeska (2001). In the same way, Hct levels remained almost intact after 4–67 days of *C. carpio* exposure to sub-lethal concentrations (0.02 mg/L) of cadmium (Ergönül *et al*., 2012). Furthermore, no significant changes in the erythrocyte count, concentration of haemoglobin, and percentage of haematocrit were determined in carp fingerlings contacted with cadmium (Palaèkova *et al*., 1992). Contrarily, contamination of *C. carpio* with sub-lethal concentrations of lead led to marked rises in both RBC and haemoglobin levels (Ergönül *et al*., 2012). The above more or less contrasting findings might demonstrate, besides dose dependency of Cd impacts, the role of other environmental and experimental conditions in the results obtained.

Shalaby (2007) found significant decrease in MCH and MCHC values of *Oreochromis niloticus* after exposure to cadmium. Similar findings were recorded by Köprücü *et al*. (2006), and Adeyemo (2007) under the influence of heavy metals and pesticide stress in different fish species. In the present work, significant decreases were noticed in MCHC caused by both lead and cadmium treatments compared to control. Common carp intoxicated with sublethal doses (10 $\mu g\ kg^{-1}$ body weight) of cadmium for 60 days (Brucka-Jastrzębska & Protasowicki, 2005) displayed significant differences in the haematocrit, MCHC, MCV, erythrocyte and leukocyte counts, which roughly corroborate our findings.

Proteins are responsible for the transport, accumulation and detoxification of cadmium in plasma. Albumin as a nonenzymatic antioxidant in vertebrates' serum may have an important role in protection against metal damage. De Smet *et al*. (1998) reported that an albumin-like protein was not recognised in plasma of common carp and that cadmium is not bound to albumin due to the absence or at least of very low concentrations in carp

plasma; they suggested that carp transferrin to be a major metal-binding protein (De Smet *et al*., 2001). Significant alterations were not detected in total protein levels of our *C. carpio* exposed to each of Pb, Cd and control, whereas amounts of albumin showed a statistical difference between the metal exposed and control fish with almost similar values recorded for both Cd and Pb treatments. A similar finding on total protein has been reported in common carp contaminated with subacute levels of lead (Ergönül *et al*., 2012). Correspondingly, albumin densities significantly increased following fish exposure to sublethal trivalent chromium in our previous study on *C. carpio* (Abedi *et al*., 2013). On the other hand, a decreasing trend of common carp's serum albumin was observed in all heavy metals tested followed by steady increasing phase when the fish were exposed to sub-lethal concentrations of the metals including Pb (Gopal *et al*., 1997), which more or less corresponds our results.

Due to direct contact of fish gills with the pollutants and also extensive epithelial surface, structural and physiological changes in this tissue can be used as indicators of environmental pollution and also as a tool for investigating the relationship between exposure to pollutants and biological responses (Oliveira Ribeiro *et al*., 2006). Hans *et al*. (2006) reported that accumulation of cadmium in some organs of common carp was dependent on time of exposure and doses of cadmium. The intoxicated gills of common carp in the present study revealed some pathological symptoms. The fusion of gill lamellae seems to be resulted from changing and/or coagulation of mucus through altering the composition of glycoprotein on the surface of gill cells caused by heavy metals (Wedemeyer *et al*., 1990). This creates hypoxia conditions leading to oxygen uptake from water surface by the fish; consequently, the contact of gill filaments with the air causes a coalition of the filaments and ultimately, hinders sufficient water flow through the filaments rendering reduced respiration and breathing disorders.

Cadmium ions are taken in by fishes both through the alimentary tracts and through gills, while lead mainly through the respiratory system (Spry & Wiener, 1991). Thence, a comparison of Cd and Pb effects on the carp's gill (Fig. 1, right) reveals that lead exerted more obvious injuries than those from cadmium (Fig. 1, left). Also, vessel dilatation and hyperemia were seen in the gills, which are considered to be acute responses. Epithelial cells hyperplasia and fusion of secondary gill lamellae, along with their clubbed deformation, are brought about as chronic reactions against heavy metals (Poosti & Marvasti, 1999). Our microscopic observations mostly indicate chronic abnormality of gills affected by both Cd and Pb; altogether, it appears that such histological lesions in fishes are mainly nonspecific and the type and classification of toxicants can rarely be identified through observations (Mallat, 1985).

In conclusion, the results of this study indicate undesirable situation of common carp's gill. Our findings also reveal that the fish growth and living are influenced by constant exposure to toxic substances. From the viewpoint of public health, fish that are permanently exposed to such pollutants can be dangerous and cause diseases if the amounts of these substances in their bodies exceed the standard ranges for human consumption. Obviously, such changes in the fish body as a bioindicator warn human health because the food chain is affected by the environment.

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