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Enzymatic Activities in Common Carp; *Cyprinus carpio* Influenced by Sublethal Concentrations of Cadmium, Lead, Chromium

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Abstract: The aim of this study was to assess the effects of heavy metal agents on two biochemical enzyme activities, alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST), as well as alkaline phosphatase (AP) and amylase. For these studies, triplicate groups of adult fish and a control group (50 fish/group) were exposed to sublethal concentrations (10% of LC_{50}-96 hr values) of cadmium chloride, chromium (III) chloride, or lead (II) nitrate for a period of 33 days. The results indicated that AST levels were significantly ($p<0.05$) increased by exposure to each of the metals. The AST activities among the metals did not significantly differ from one another. Apart from Cr, no significant differences were detected in the activities of ALT relative to control levels; among the metals themselves, the only significant differences were seen between Cr and Cd. With regard to AP, Cd treatment resulted in significant increases in activities of the enzyme. Again, among the metals there were also differences in effect, with the outcomes significantly differing between Cr and Cd. Finally, in the case of amylase, all three metals led to significant increases in enzyme activity; the effect-trend was Pb>Cd>Cr. The present investigation illustrates that Cd, Pb and Cr are differentially toxic to the common carp following sub-chronic exposure.

Key words: *Cyprinus carpio* · Heavy Metal · Enzyme Activity

INTRODUCTION

Based on their known toxicologic profiles in many animal models, it is certainly plausible that waterborne metals could alter physiological and biochemical parameters in fish. The survival of many aquatic species (even beyond fish) depends not only on the health status (at time of exposure) of the hosts, but also on the type/length of exposure to and inherent toxicities of the metal toxicants in agreement with Brungs *et al.* [1]. Cadmium is considered one of the most toxic contaminants present in polluted waterways, causing toxicity at each level of the ecologic stratum by Rashed [2]. Even at sub-lethal concentrations, cadmium has a cumulative effect and causes serious physiologic disturbances in fish, such as induction of abnormal behavior, locomotor anomalies, or anorexia by Woo *et al.* and Brya *et al*. and Cicik *et al.* [3-5]. Lead is known to alter the hematologic system of hosts by inhibiting the activities of several enzymes involved in heme biosynthesis. Once absorbed, it is distributed particularly to the liver, kidney, heart and gonads, as well as to the immune system by ATSDR [6]. Chromium, primarily in the Cr (VI) oxidation state, is considered extremely toxic in the environment due to its high solubility and oxidative potential (the latter being an attribute associated with its ability to induce DNA lesions in exposed cells) in agreement with Reynolds [7].

Enzymes are necessary for normal cellular metabolism. The liver is a critical organ that is replete with many key enzymes used for homeostasis; degenerative changes due to metal toxicity in the liver are often reflected in alterations in the level/activity of a number of its enzymes. Enzyme activities are considered as sensitive biochemical indicators of imminent hazardous effects in fish and have been used as important parameters in the testing of water for the presence of toxicants according to El-Demerdash and Elagamy and Oruc and Uner [8, 9].

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Animals exposed to metallic contaminants in both field and laboratory experiments displayed increased or decreased enzyme activity depending on the dose, species and route of exposure in agreement with Wong and Wong; Jiraungkoorskul *et al.* and Jiraungkoorskul *et al.* [10-12]. Aspartate aminotransaminase (AST, *aka* glutamate oxaloacetate transaminase/ GOT), alanine transaminase (ALT, *aka* glutamate pyruvate transaminase/ GPT) and alkaline phosphatase (AP) are released during acute\chronic liver disorders by Huang *et al*. [13]. Alkaline phosphatase is an intestinal enzyme involved in the assimilation of nutrients and in other bodily processes, such as bone mineralization, formation and resorption by Piattelli *et al.* [14]. These enzymes are commonly used as biomarkers of acute hepatic damage; thus, bioassays of all three can serve as a diagnostic tool for assessing necrosis of hepatocytes [15, 16].

Changes in the levels of ALT and AST activities in fish serum have frequently been used as indicators of toxicant and contamination of marine ecosystems according to Hilmy and Domaity; Rashatwar and Hyas and Kim *et al.* [17-19] and as indices of liver function in these types of hosts according to Shakoori and Alam [20]. Despite this, comparisons of biochemical parameters in toxicant-stressed fish have rarely been compared to outcomes in mammalian models in agreement with Hedayati *et al*. [21]. For this reason, studies were conducted here to analyze AP, AST, ALT, as well as amylase in different tissues to determine their utility as stress biomarkers in *Cyprinus carpio* (common carp). Specifically, the measurements were performed following 33 days of exposure of these hosts to sublethal concentrations of Pb, Cd and Cr, three of the most common pollutants in waterways of many areas in the world.

MATERIALS AND METHODS

Treatment Procedures: Cyprinus carpio were obtained from the Nasr Fish Culture Pond in Sari, Iran. Prior to toxicity testing, the fish were acclimatized for 1 week under laboratory conditions (25±1°C, 12-hr light/dark cycle). Water quality parameters (TDS=600 ppm, pH=6.75, EC=1 ds/m, DO=5-8 mg/l) were measured prior to and throughout the experiment. No feeding was submitted to the studied fish during the acclimation period. At the beginning of the exposure period, fish were randomized into various tanks (at n = 50 fish/tank); average total lengths (11.68 1.92cm) and weights (25.92±6.30 g) of the fish did not significantly differ among the treated and control groups. All procedures were done in accordance with and the approval of the Institutional Animal Ethics Committee of the Sari Agricultural Sciences and Natural Resources University.

The heavy metals Cd, Cr, Pb in the chemical forms of cadmium chloride ($CdCl_2.H_2O$, BDH), chromium chloride ($CrCl_3.6H_2O$, APLICAM) 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 hr value for each metal was determined. Thereafter, triplicate treatment groups (excluding the control) were exposed to sublethal concentrations (i.e., 10% of the newly-determined LC_{50}-96 hr value for each metal according to Sobha *et al.* and Radhakrishnan [22, 23] of Cd (test value used = 8.4 mg/l), Cr (test value used = 2.0 mg/l) and Pb (test value used = 6.26 mg/l) for a period of 33 days.

Harvesting and Preparation of Fish Blood/Tissue Samples: The day after the final exposure (On day 34 of experiment), blood samples were collected (using a 23-gauge needle) from behind the anal fins of each fish. The collected blood samples were processed for subsequent use in AST and ALT analyses. The fish were then euthanized by tapping on the head and then necropsied (to isolate hepatopancreas and intestines) on a glass plate located on a box containing ice-cold water in order to maintain enzyme activity. All isolated tissues were immediately stored at -80°C until assayed for the enzyme activities. The hepatopancreas of each fish was homogenized (at 0-4ºC) using an IKA T25 digital homogenizer (Wolflabs, York, UK). For the homogenization, a 100 mM Tris–HCl buffer (pH 7.8) containing 0.1 mM EDTA and 0.1% Triton X-100 was used; samples were prepared at 1 g tissue in 9 ml buffer. The homogenates were then centrifuged at 30,000 x g for 30 min at 4°C in a (Andreas Hettich GmbH, Tuttlingen, Germany). The resultant supernatant was collected and then frozen at -80°C for later use in enzyme analyses and estimation of protein content in agreement with Furne *et al*. [24].

The preparation of harvested intestinal tissues for measures of alkaline phosphatase activity was based on the method of in agreement with Cahu *et al.* [25]. In brief, the isolated tissues were homogenized in Tris (2 mM)-mannitol (50 mM) buffer (pH 7.0). Brush border extracts were then prepared as described by Crane *et al.* [26] i.e., tissue homogenates were centrifuged at 9,000 x g for 10 min after the addition of 0.1 M $CaCl_2$. The resultant supernatants were then collected and stored at -80°C until analysis of enzyme activity or protein content. In all

cases, total protein in the test samples was measured by the Bradford method [27] using bovine serum albumin as a standard. All enzyme activities were expressed as specific activity (U/mg protein).

Enzyme Assays: Amylase (E.C.3.2.1.1) activity was determined using the 3,5-dinitrosalicylic acid (DNS) method [27, 28]. Starch substrate (1% w/v) was diluted in a buffer (pH 6.9) containing 20 mM Na_2HPO_4 and 6 mM NaCl. The substrate (250 µl) was then incubated with crude extract (50 µl) and buffer solution (250 µl) for 3-4 min at 25°C. Thereafter, 0.5 ml of 1% DNS solution was added and the samples boiled for 5 min. Distilled water (5 ml) was then added to the mixture and the absorbance of the solution was recorded at 540 nm in Unico Model 2802UV spectrophotometer (Dayton, NJ). Blanks were similarly treated, but lacked the crude enzyme extracts. Maltose (0.3-5.0 µM) was used for the preparation of a standard curve from which activities in the samples were calculated based on the OD_{540} value and extrapolation. Ultimately, the α-amylase specific activity was defined as the µmole maltose produced per min per mg protein under these specified condition.

Alkaline phosphatase (AP, E.C.3.1.3.1) activity was quantified at 37°C using 4-nitrophenyl phosphate (PNPP) as substrate in 30 mM $Na_{42}CO_3$ buffer (pH 9.8). One unit (U) was defined as 1 µg BTEE released per min per ml of brush border homogenate at 407 nm in agreement with Bessey *et al*. [29].

Both ALT and AST enzymes were determined using the Pars-Azmoon Diagnostics Infinity AST reagent Kit (Pars-Azmoon Co., Teheran, Iran) and a Sigma Diagnostics Infinity ALT Reagent Kit (Sigma, St. Louis, MO), respectively.

The obtained data were checked for normality and homogeneity of variances prior to their comparison. All data were expressed as the mean ± SD. All enzymatic assays were run in triplicate. Enzyme activities were compared using a one-way analysis of variance (ANOVA) and means compare-sons were performed using a Duncan's test at a reliability level of 5%. Data were analyzed using SPSS statistical software (Chicago, IL).

RESULTS

The sublethal levels of Pb, Cr and Cd (6.2, 2.0 and 8.3 mg/l, respectively) used in these studies differently affected the enzyme activities in the *Cyprinus carpio* hosts. Figure 1A shows that AST activities were significantly increased compared with the control values

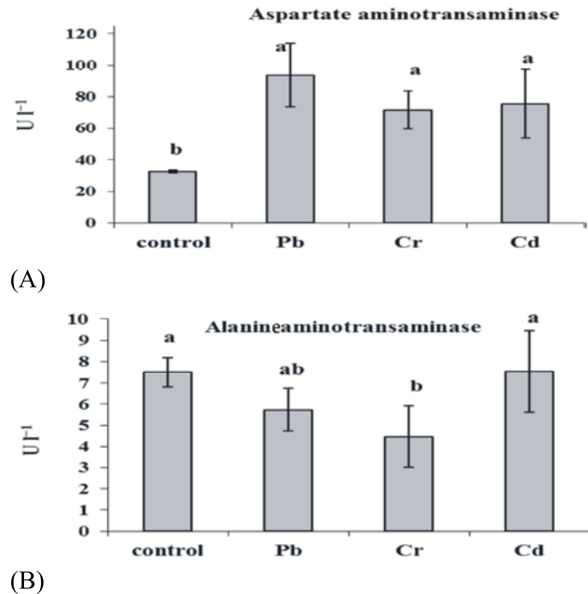

Fig. 1: Activities of (A) aspartate (AST) and (B) alanine (ALT) aminotransaminase in the serum of the common carp following exposure to sublethal concentrations of cadmium, lead and chromium for 33 days. Values of enzyme activity (shown as U/L; [mean ± SD], n = 50 fish/group) with dissimilar superscript letters are significantly different from one another (p<0.05). All assays were performed in triplicate for each fish

(32.7±0.9 U/L) due to each of the three metals. Levels of AST activities were 93.6±20.0, 93.6±20.0 and 93.6±20.0 U/L, respectively, following the Pb, Cr and Cr exposures. None of these values statistically differed from one another. Conversely, the fish subjected to Cr exhibited a significantly lower ALT activity than the control hosts (Figure 1B). In this case, while the ALT values did not differ between the fish exposed to Pb and Cd treatments, those between the Cd and Cr treatments did.

Intestinal AP activity was not significantly affected by the sublethal doses of Cr and Pb; in contrast, sublethal Cd levels significantly increased this compared to control values (P>0.05). Further-more, the differences in AP activity among the fish treated by the heavy metals were significant and ranked in effect as Cd > Pb > Cr (Figure 2A).

The sublethal levels of Pb, Cr and Cd resulted in significant increase in amylase activity in all heavy metal treatments as opposed to the control. In addition, the three metal treatments showed significant fluctuations in amylase activity on the order of Pb > Cd > Cr (Figure 2B).

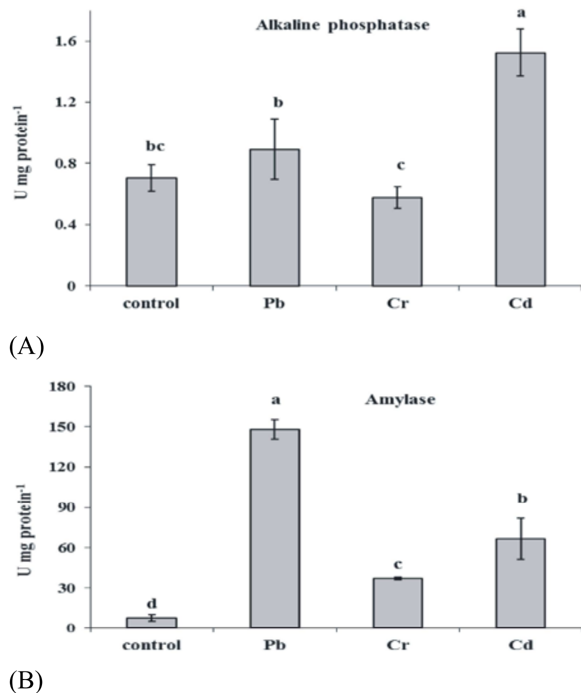

Fig. 2: Activities of (A) alkaline phosphatase in intestinal brush border and (B) amylase in hepato-pancreatic tissues. Specific activities of the common carp were following the exposure to sublethal concentrations of cadmium, lead and chromium for 33 days. Values of specific enzyme activity (shown as U/mg protein; [mean ± SD], n = 50 fish/group) with dissimilar superscript letters are significantly different from one another ($p < 0.05$)

DISCUSSION

Both field and laboratory experiments have revealed various enzymatic responses in animals including freshwater fish exposed to various pollutants and, in particular, heavy metals depending on the dose, species and means of exposure [10, 12, 30, 31, 32, 33]. For instance, the non-essential heavy metal cadmium (at 50 mg/l) reduced the activities of proteolytic enzymes in some teleosts [34, 35]. In the crab *Scylla serata*, cadmium (at 0.3-1.5 mg/l) diminished alkaline phosphatase (AP) activity in the hepatopancreas according to Dnavale and Malsurkar [36]. This study, however, recorded increased AP activity in carp as 2.5-times the control level following exposure to a sublethal concentration (8.3 mg/l) of cadmium, while chromium (at 2 mg/l) caused declines in AP activity. In contrast, Pb (at 6.2 mg/l) led to an insignificant change in AP activity in these carp. The studies by Atli and Canli [36] also showed that AP activity in the liver, intestine and serum generally increased following metal (e.g., Pb, Cd, Zn and Cu) exposures. In the catfish *Clarias batrachus*, experimental lead contamination significantly increased AP levels with increasing time in agreement with Nehar *et al*. [36]. Although lead nitrate is generally considered an activator of AP [37], such an effect was not evident in the present study. This may be either because of the very low concentration of Pb used here, the species-specificity of the metal toxicity, or due to differences in water quality. With respect to the latter item, as described in agreement with Demayo *et al.* [38], lead toxicity is often a function of water hardness; species tested and fish age.

It is known that heavy metals inhibit the activity of a wide range of enzymes according to Dixon and Webb [39]. Boge *et al.* [40] suggested that the inhibition of AP activity in the intestine of *Onchorhynchus mykiss* might be due to the oxidation of specific molecules. Inhibitory effects on the activities of all studied enzymes by Cd and Hg (*in vitro*) in the liver of *Tilapia nilotica* were linked to the breakdown of the membrane stability according to El-Demerdash and Elagamy [8]. Further-more, changes in serum enzyme activities observed in some studies were generally related to the physiological and functional alterations in metal-exposed fish resulting from the leakage of some enzymes from injured cells and modified activities of serum enzymes (11, 41), which directly reflect cell damage in specific organs [42, 43].

The concentrations of AST (or GOT) and ALT (or GPT) estimated in the experimental fish of this investigation (32.7-93.6 U/L for AST and from 4.46-7.05 U/L for ALT) corroborate those ranges mentioned for the normal concentrations in the blood (5-40 U/L for AST and 5-35 U/L for ALT: in agreement with Haung *et al.* [13]. In a similar study, fluctuations in serum AST and ALT levels in *C. carpio* were concluded to have been a result of altered biochemical metabolism induced by chromium [44]. Additionally, according to Karan *et al.* [45] and Reynders *et al.* [46] exposed *C. carpio* to cadmium and observed increases in the activities of AST and ALT. In the Indian major carp *Labeo rohita*, chromium at LC_{50} levels (61 mg/l) had no significant effects on ALT or AST activities in agreement with Vutukuru *et al.* [47]. Findings in the present study indicated that AST levels were elevated by Pb, Cr and Cd, whereas ALT activity was reduced with Cr and unaffected by Pb and Cd. The Cd-induced rise in AST activities is in agreement with the results of Shalaby [48, 49], who reported that sublethal concentration (10 ppm) of Cd caused significant increases

in AST of common carp after 7 and 15 days and in AST and ALT of Nile tilapia (*O. niloticus*) following 7 and 15 days. Several reports also stated that these plasma/serum enzymes of a number of fish species were highly increased in the fish treated with heavy metals including cadmium [17, 45, 50, 51, 52]. These correspond with the rationale that at elevated cadmium concentration, the activity of aminotransferases may increase in order to counter the energy crisis during stress, but decrease when accumulated concentrations of cadmium become too high in agreement with Macinnes *et al.* [53].

The Cr-stimulated fall in ALT activity detected in here disagrees with Vutukuru *et al.* [47] whereas Karatas and Kalay [54] and Roy [55] similarly reported decreased activities of ALT activities in *Tilapia zilli* and *Boleopthalmus dussumier*, respectively, contaminated with lead and toxicants. They also suggested disturbance in the structure and integrity of cell organelles, like endoplasmic reticulum and membrane transport system. Conversely, Cr exposure (0.05 µg/ml) in comparison to other examined heavy metals (e.g., Cu, Cd, Zn, Ag) did not alter AST and ALT activities in *Oreochromis niloticus* according to Öner *et al.* [56]. In other investigations, common carp and the catfish *Clarias gariepinus* exposed to deltamethrine (96 hr) and lead acetate (six weeks), respectively, displayed elevated ALT and AST activities [57, 58], while the same catfish exposed to different concentrations of diazinon showed no clear trends in the plasma ALT activities by Inyang *et al*. [59] as did *C. carpio* in here. In another study on *C. carpio*, De Smet and Blust [32] observed no significant changes in the levels of AST and ALT following 29 days of Cd (20 µM) contamination. These more or less contrasting consequences appear to be indicative of differences in the examined toxicants concentrations and durations, species and milieu.

The level of amylase was significantly affected in the Pb-treated carp as 15 times the control level. Amylase fluctuations were significantly different among the heavy metal treatments and also in comparison with the control having the least concentration. The amylase concentration in the carp subjected to the heavy metals was minimal at Cr treatment (Figure 2B). The elevated amylase activity has been reasoned to result from pancreatitis or from damaged amylase secretary cells according to Yousafzai and Shakoori [60]. Comparable to this study, both short and long term contamination of the Indian carp *Cirrhina mrigala* by lead acetate led to increased hepatic amylase activity by Mujeeb [61]. Increased hepatic and muscle amylase activities were also reported in a freshwater fish, *Tor putitora*, stemming from a polluted river caused by heavy metals load including chromium [60, 62].

Overall, the changes in concentrations and enzymes activities following heavy metal exposure occur either due to (i) leakage of these enzymes from hepatic cells and thus raising levels in blood, (ii) increased synthesis and (iii) enzyme induction of these enzymes according to Shakoori [63] or because of liberation of these enzymes to the blood stream when the hepatic parenchyma cells are damaged [64, 65]. Such changes in biochemical levels under the effect of heavy metal toxicity might result in impairment of energy requiring vital processes and hence give an idea about the health status of the fish population by Parvathi [44]. Our results revealed stimulation or interruption in enzyme activities showing dysenzymia in response to the heavy metals, which may reflect pancreatitis, injured intestine and cellular damage in agreement with Yousafzai and Shakoori [62].

CONCLUSION

This study indicated that several key enzymatic systems in fish could be used as sensitive bioindicators of metal contamination in aquatic systems. The changes induced by the metals examined demonstrate that modifications of several different carp enzymes, at rather low concentrations might be practical endpoints for use in biomonitoring of fish health in waterways that are routinely polluted in many countries around the world.

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