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Effects of Metal (Ag, Cd, Cr, Cu, Zn) Exposures on Some Enzymatic and Non-Enzymatic Indicators in the Liver of *Oreochromis niloticus*

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Abstract Freshwater fish *Oreochromis niloticus* exposed to 0.05 μg/mL of Cu, Cd, Zn, Ag and Cr for up to 30 days. Only Ag, which exceeded environmentally realistic concentrations by a factor of >100 caused fish mortality within this period. Metals increased aspartate transaminase (AST) activity, while they decreased alanine transaminase (ALT) activity, except Cr exposure. Concentrations of free –SH group decreased whereas protein concentrations did not alter following metal exposures. Detectable metal accumulation occurred in the liver of Cd, Cu and Zn exposed fish. This study emphasized that both enzymatic and non-enzymatic mechanisms may be useful in understanding the degree of metal toxicity in fish liver.

Keywords Heavy metals · Liver · *Oreochromis niloticus* · –SH group · Transaminases

The contamination of aquatic environment by metals due to natural and anthropogenic sources is a worldwide environmental concern. Exposure to metals may lead to several toxic effects in aquatic animals, including tissue damage, respiratory changes, alterations of biochemical and physiological mechanisms, and ultimately mortality (Heath 1995). Therefore, the enzymatic and non-enzymatic parameters gain importance as sensitive tools to estimate the effects of metal exposures before the occurrences of hazardous effects in organisms. AST and ALT are the most important enzymes acting as transaminases involved in amino acid metabolism and they are known to be sensitive to metal exposures (Almeida et al. 2001; Levesque et al. 2002; Gravato et al. 2006). On the other hand, determinations of sulfhydryl group levels which metals have high affinity towards, and total protein levels could be beneficial in estimating the toxicity of metals (Gravato et al. 2006).

Liver is the major site of metal storage and excretion in fish and as a result of its major role in metabolism and its sensitivity to metals in the environment, particular attention has been given to liver in toxicological investigations (Parvez et al. 2006). Biochemical parameters assessed in fish may be an useful tool by providing quantitative measurement of metals impact as well as valuable information of ecological relevance on the effects of metals. The objective of this study was to investigate the response of AST and ALT activities and to determine levels of free sulfhydryl groups and total protein in the liver of *Oreochromis niloticus* exposed to 0.05 μg/mL concentration of Cu, Zn, Cd, Cr and Ag for 0, 5, 10, 20 and 30 days.

Materials and Methods

Freshwater fish *O. niloticus* have been cultured in Çukurova University (Turkey) for more than 20 years. Fish were taken from the culture pools and transferred to the laboratory where they were acclimatized in experimental aquaria for one month before the experiments. Experimental room was air conditioned (20 ± 1°C) and illuminated for 12 h with fluorescent lamps (daylight 65/80 W). The experiments were carried out in glass aquariums sized 40 × 40 × 100 cm that contained 130 L contaminated test solution or only test water (dechlorinated) for controls. During the experiments, pH and oxygen levels were estimated as 8.32 ± 0.08 and 5.96 ± 0.44 mg O_2/L, respectively (Orion 5 Star multimeter). Total

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hardness (with EDTA titration method) and alkalinity (acidimetry method) were measured as 340 ± 29 mg $CaCO_3$/L, 248.6 ± 13.1 mg $CaCO_3$/L, respectively.

Fish were exposed to 0.05 μg/mL concentration of Cu ($CuCl_2 \cdot 2H_2O$), Zn ($ZnCl_2$), Cd ($CdCl_2 \cdot H_2O$), Cr (K_2CrO_4) and Ag ($AgNO_3$) for 0, 5, 10, 20 and 30 days. Trisodium citrate 2-hydrate was used to prevent the metal precipitation in aquaria. A total of eight fish were used for each exposure period for each metal. Thirty-two fish were exposed to each metal in the aquaria and eight fish were removed at each sampling time. Eight control fish were used for each period to eliminate effects of factors other than metal exposures. As there were no significant differences ($p > 0.05$) in the studied parameters of different controls, all controls values were pooled. Total lengths (15.7 ± 1.21 cm) and weights (61.5 ± 12.8 g) of fish did not differ significantly ($p > 0.05$; n = 200) among different exposure treatments and control. The aquariums of both control and exposed groups were cleaned every two days in the morning after 1 h feeding period to minimize metal loss and to reduce contamination with food remains. All experimental waters were completely renewed every 2 days.

After 0, 5, 10, 20 and 30 days of metal exposures, fish were killed by transaction of spinal cord and liver tissues were dissected out with clean equipments. They were immediately stored at −80°C (Revco Ultima II) until the analysis. Liver tissues were homogenized (1/10 w/v) in homogenization buffer containing 20 mM Tris (pH 7.8), 250 mM Sucrose and 1 mM Na_2EDTA at 9.500 g (Janke & Kunkel Ultra Turrax T25) for 2 min. Homogenates were centrifuged then for 20 min (+4°C) at 13,000 g (Hettich Universal 30 RF) and the supernatants were used for the analysis. The AST and ALT activity and concentration of total protein in the supernatant were measured by using an Olympus AU 400 biochemical analyzer according to the procedure of Bergmeyer et al. (1985). Reagents were obtained from Olympus Life and Material Science (Europe, Ireland) for the analysis. Sulfhydryl group concentration was determined spectrophotometrically (Ellman 1958) using a Cecil 5000 series spectrophotometer at 412 nm. Metal concentrations in the liver were measured using a flame atomic absorption spectrophotometer (Perkin Elmer AS 3100). The detection limits of metals were 0.001, 0.002, 0.002, 0.003 and 0.002 μg/mL for Cd, Zn, Cu, Cr and Ag, respectively. Accuracy of the AAS and validity of measurements were tested with a reference material (TORT 1 lobster hepatopancreas, National Research Council, Canada). Mean values and standard deviations of the reference material were 10% of the ranges. Metal levels in the tap water were below the detection limits. Calculations of parameters were done as U/mg prot for AST and ALT activities, nmol/mg prot. for sulfhydryl group concentration, mg/g wet weight for total protein concentration and μg/g dry weight for total metal concentration.

Statistical analysis of data, presented as mean and standard error, was done using SPSS statistical package program. One-way ANOVA was applied to compare variables among control and treatments at each exposure period. Post hoc comparisons were done using LSD test to determine which individual groups were significantly different from control, when significant differences were found ($p < 0.05$).

Results and Discussion

This study investigating environmentally realistic concentration of metals, except Ag which was >100-fold higher showed that metals can be hazardous even in such low levels for *O. niloticus*. Mortality occurred in Ag exposed fish between 12th and 16th days, in this study. The other metals did not cause any fish mortality during the exposure period. Therefore, there are no data for 20th and 30th days for Ag exposure. Apart from the effects on the studied parameters, metals like Ag can also be lethal for fish even in such level (0.05 μg/mL) that may not be toxic for the other metals. High toxicity of Ag to fish associated with ionoregulatory disturbances in fish (Morgan et al. 1997) was also in agreement with this study. Generally, Ag is amongst the most toxic heavy metals together with Hg, As and Cu and, these were followed by Cd, Pb and Zn (Heath 1995).

The liver transaminase activities were significantly changed following exposure to all metals. The AST activity was stimulated by all metals (Fig. 1). The highest increase was observed in fish exposed to Zn for 10 days (1.322 ± 0.2 U/mg prot.) when compared to control value (0.321 ± 0.02 U/mg prot.) On the other hand, ALT activity decreased following metal exposures, except Cr exposure (Fig. 2). The lowest activity was determined in fish exposed to Zn for 10 days (0.116 ± 0.01 U/mg prot.), whereas the highest ALT activity was determined in fish exposed to Cr for 10 days (0.364 ± 0.04 U/mg prot.) when compared with that of control value (0.241 ± 0.02 U/mg prot.). Significant increases in AST activity and decreases in ALT activity may depend upon the liver damage following metal stress and the metal effects are observed maximally at initial exposure durations. Although AST enhances at day 20 and day 30, the metal effects tend to decline and ALT activities did not differ from control values following prolonged exposure period, except for one or two metals. Various responses of AST and ALT activities were recorded depending upon the metal species, their concentrations and exposure durations (Zikic et al. 2001;

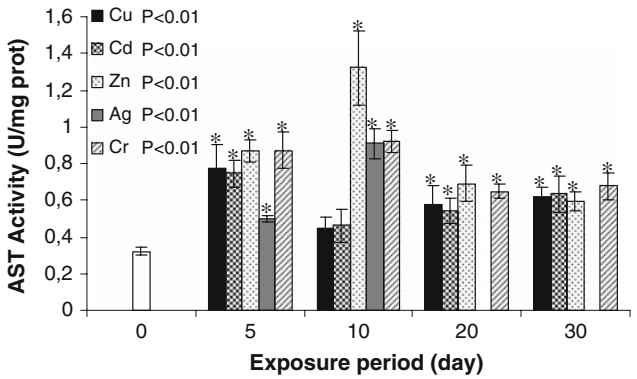

Fig. 1 AST activity in the liver of *O. niloticus* exposed to metals. Data are expressed as mean (n = 8) ± standard error. *p*-Values indicate the results of one-way ANOVA while *asterisks* indicate the results of LSD test

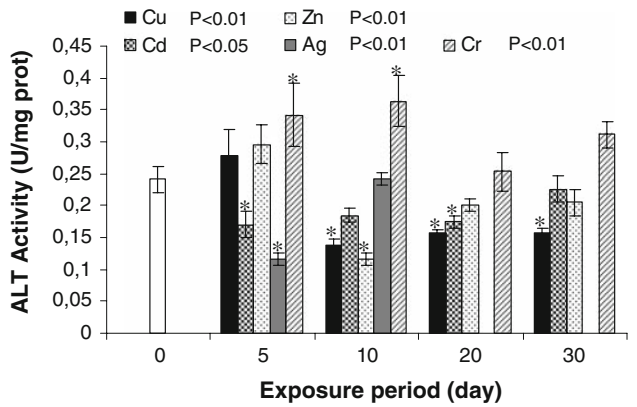

Fig. 2 ALT activity in the liver of *O. niloticus* exposed to metals. See Fig. 1 for details

Vutukuru et al. 2007). Almeida et al. (2001) demonstrated that no alteration was found in both liver AST and ALT activities in *O. niloticus* exposed to 320–2560 µg Cd/L for 7 days and they indicated that these enzymes may not limit gluconeogenesis. Nevertheless, De Smet and Blust (2001) indicated that elevated activities of AST and ALT in liver, kidney and gill tissue of *Cyprinus carpio* following 0.8, 4.0 and 20 µg/L Cd exposures for 29 days were due to increased protein breakdown to deal with the energy requirement. Vutukuru et al. (2007) indicated that Cr did not change the serum AST and ALT activity whereas as caused a significant increase in the enzyme activities due to possible leakage of enzymes across damaged plasma membranes or the increased synthesis of enzymes by the liver. Gill et al. (1991) observed inhibitions in ALT and AST activities in the liver, kidney and gills of *Barbus conchonius* following 12.6 mg Cd/L for 48 h. Data from the field also show that enzyme activities in contaminated and uncontaminated waters differ significantly. It was shown that chronic exposure of *Perca flavescens* to Cu, Cd and Zn impairs growth and alters the seasonal cycling of carbohydrate and lipid metabolisms as well as the activities of metabolic enzymes such as AST and ALT and also AST activity was found to be higher in the liver of fish from contaminated lakes (Quebec, Canada) than the reference site (Levesque et al. 2002). The enhancement of the aminotransferase activities may occur in order to counter the energy demand during metal stress, however decrease in their activities may be observed as a result of high metal accumulations in the tissues. Thus, aminotransferases can be measured sensitively to assess the levels of contamination in the environment and toxicity of metals before the occurrences of detrimental effects.

Total protein concentration did not change following metal exposures compared to that of control value (70.0 ± 2.45 mg prot/g wet weight) (Fig. 3). Similarly, De Smet and Blust (2001) observed no changes in total protein content in different tissues of *C. carpio* including liver following Cd exposures. Levesque et al. (2002) also recorded that the amount of liver protein was not different among the control and metal contaminated regions of lakes in Quebec (Canada). On the other hand, Almeida et al. (2001) reported that there was a decrease in total protein concentration in the liver of *O. niloticus* following Cd exposures and indicated that this decrease may represent a great protein reserve depletion induced by Cd treatment, liberating amino acid for gluconeogenesis. Abdel-Tawwab et al. (2007) also suggested that low liver protein levels after increasing Cu concentrations could be associated with disturbances in the liver protein synthesis and protein breakdown due to the Cu toxicity or increased export of liver proteins like Cu binding proteins such as ceruloplasmin, albumin, metallothioneins into the circulations. Proteins are a major constituent in the metabolism of fish and metals may be involved in the functioning of these molecules, therefore it is significant to analyze the changes in protein metabolism after metal exposure.

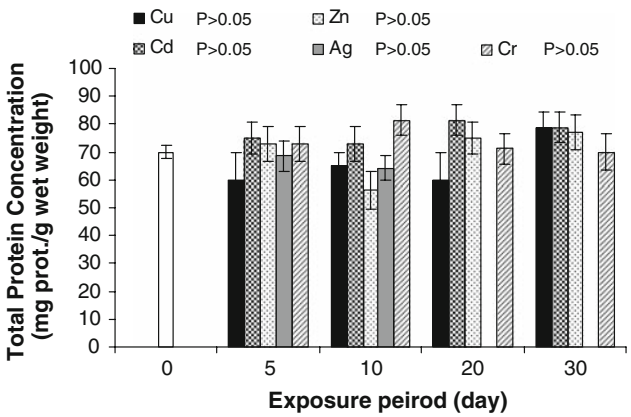

Fig. 3 Total protein concentration in the liver of *O. niloticus* exposed to metals. See Fig. 1 for details

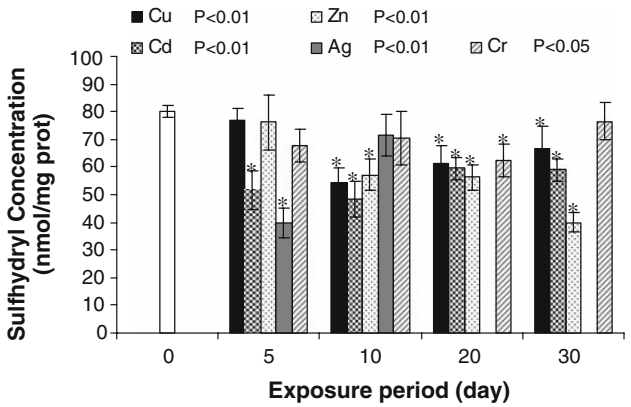

Fig. 4 Sulfhydryl concentration in the liver of *O. niloticus* exposed to metals. See Fig. 1 for details

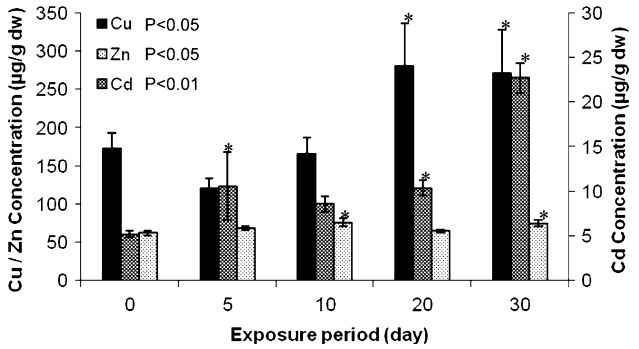

Fig. 5 Cd, Cu and Zn concentrations in the liver of *O. niloticus* exposed to metals. See Fig. 1 for details

Concentration of free sulfhydryl group significantly decreased following all metal exposures (Fig. 4). The lowest sulfhydryl group concentration was measured in fish exposed to Ag for 5 days (39.7 ± 5.56 nmol/mg prot.) when compared to that of control (80.02 ± 2.28 nmol/mg prot.). Significant decreases of sulfhydryl group levels after all metal exposures may be attributed to high affinity ofSH groups to metals. Free –SH groups are measured with the method used here. When metals bind on –SH group, it is not measurable with the method as molecular structure changes. Thus, the decrease of –SH group may be explained by binding of metal on –SH groups (Gravato et al. 2006). It was known that sulfhydryl groups are important molecules in metallothioneins (MTs) that are low molecular weight (~7000 Da), cysteine-rich (33%), heat-resistant metal binding metals. Binding of non-essential metals such as Cd to MTs prevents the reaction of the metals with other cellular molecules, thereby providing protection against metal toxicity and MTs are also involved in storage of essential metals such as Cu and Zn (Heath 1995). The other important molecule, containing –SH groups, is glutathione (GSH) that is a tripeptid basically acts as an intracellular reductant and nucleophile. It has a pivotal role in antioxidant defence system against metals causing oxidative stress and it was suggested that altered GSH levels can be associated with increased biosynthetic enzyme activities and binding of metals to –SH groups of GSH, respectively (Elia et al. 2003; Ahmad et al. 2005; Gravato et al. 2006). Therefore, using these non-enzymatic parameters in ecotoxicology researches could be beneficial together with enzymatic parameters to determine the toxicity of metals.

Total metal concentrations (µg/dry weight) in the liver of the control fish were measured as 5.22 ± 0.38 for Cd, 173.4 ± 20.2 for Cu and 62.5 ± 2.73 for Zn (Fig. 5). Following metal exposures, significant metal accumulation occurred only for Cd, Cu and Zn in the liver of *O. niloticus*. Cd accumulation rate was highest when compared to accumulation rate of other metals. Ag and Cr levels in both control and metal exposed fish were lower than detection limit of the instrument. There are many studies showing metal accumulation in fish tissues in the literature. Those studies showed that metal accumulation generally occur in tissues in relation to exposure concentrations and periods, though some other factors such as ions, pH, hardness and temperature of water also play significant roles in accumulation (Eroglu et al. 2005).

In conclusion, variable responses of liver biochemical parameters (summarized in Table 1 as percentages) to metal exposures of *O. niloticus* could be due to metals, their concentrations and durations. The alteration in aminotransferase activities indicates changes in energy metabolism in response to an enhanced energy demand to compensate the stress situation. It was shown that these

Table 1 A summary of maximum effects of metals on the enzymatic and non-enzymatic parameters in the liver of *O. niloticus*

Parameters	Cd	Cu	Ag	Zn	Cr
AST	↑	↑	↑	↑	↑
	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
	132%	141%	183%	312%	186%
ALT	↓	↓	↓	↓	↑
	$p < 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
	30%	43%	52%	52%	51%
Total protein	–	–	–	–	–
	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Sulfhydryl	↓	↓	↓	↓	↓
	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.05$
	39%	32%	50%	50%	22%
Total metal	↑	↑	ND	↑	ND
	$p < 0.01$	$p < 0.05$	$p < 0.002$	$p < 0.05$	$p < 0.003$
	335%	57%	µg/mL	20%	µg/mL

Significant increases or decreases in the levels of the parameters were indicated by up and down arrows respectively, together with % variations. ND = Not detected (below detection limit)

enzymes are influenced by metals before their accumulation in liver. The extent and duration of these responses can, at least partly, be attributed to the concentrations of free –SH containing, metal binding molecules such as MTs and GSH. Biomarkers represent metal-induced changes in biological systems that can serve as linkers between environmental contamination and its effects on the ecosystem health. In this view, this study provides a beneficial data with studying both enzymatic and non-enzymatic biochemical parameters in the detoxification organ liver to show the health status of fish before the occurrence of the detrimental effects and give valuable information about the chronic adverse effects caused by metals in the water, even in relatively low level. Nevertheless, further research is required to assess the other metabolic effects in other tissues of fish.

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