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Effects of Metal (Cd, Cu, Zn) Interactions on the Profiles of Metallothionein-Like Proteins in the Nile Fish *Oreochromis niloticus*

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Metals are present as mixtures in the aquatic environment because of their consistent release from various sources. Studies on the toxicity of metals have mostly focused on the effects of single metal exposures rather than combined exposures (Lange et al. 2002). Metal accumulation in fish tissues is determined from the differences between uptake and depuration. When metal uptake rates exceed the depuration rates then tissue accumulation of metals occur (Heath, 1987). Metal uptake rate is determined by factors such as concentration, temperature, chelating agents, pH, ions and the sex of animals (Heath 1987; Roesijadi and Robinson, 1994). Metal interaction can be seen as synergism, antagonism or additive effects (Heath 1987; Brzoska and Moniuszko-Jakoniuk, 2001). Interactions can affect the absorption, distribution, excretion and also biological functions of metals.

Metallothioneins (MTs) are ubiquitous low molecular weight (6000-7000) proteins that are heat-resistant, absent in aromatic amino acids and rich in cystein contents. MTs occur naturally in tissues and serve as potential donor for the essential metals (Cu, Zn) and also they bind non-essential metals (Cd, Hg) for sequestration (Roesijadi and Robinson, 1994). Both essential and non-essential metals situmulate MT synthesis though capacity of induction may differ among metals (Hogstrand and Haux 1990; Wu et al. 1999; Atli and Canli 2003). There is no evidence in the literature that MTs bind Fe and Pb (Reichert et al. 1979; Roesijadi and Robinson, 1994; Atli and Canli, 2003). However, Cheung et al. (2004) showed hepatic MT mRNA induction after exposure of Tilapia to Pb. Because metals are present in mixture in the aquatic environment, it is desirable to estimate the induction of MT-like proteins when metals given in single and mixture. This would enable us to estimate better the behavior of MTLP in natural condition and be useful in environmental monitoring.

Tilapia (*Oreochromis niloticus*) is a widely distributed freshwater fish that can persist in a highly polluted habitat and has the potential for the development as a biological monitor of environmental pollution (Ueng et al. 1996). Our previous study showed that Tilapia has Zn and Cu binding MT-like proteins naturally and is able to induce Cd binding MT-like proteins when exposed to this metal (Atli and Canli, 2003). The aim of this study is determine the induction of

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Metallothionein-like proteins (MTLP) in the liver of *Oreochromis niloticus* in relation to metal (Cd, Cu, Zn) interactions. Metal and sulphydryl levels in the eluates of liver tissues were determined. Total metal accumulation in the liver of *Oreochromis niloticus* was also measured.

MATERIALS AND METHODS

O. niloticus were continuously cultured in Çukurova University. The fish were obtained from culture pools and transferred to the laboratory where the experiments were held. They were acclimatized to the laboratory conditions for one month before the experiments. The laboratory was illuminated for 12 hours with fluorescent lamps (daylight 65/80 W) and kept at 20±1.0 °C. During the experiments, the tap water used in the experiments had a pH value of 7.99±0.9 and a total hardness of 320±7.24 mg $CaCO_3$/L and an alkalinity of 245.5±12.5 mg $CaCO_3$/L. The aquariums were aerated with air stones attached to an air compressor to saturate with oxygen (6.44±1.64 mg O_2/L). The experiments were conducted in glass aquariums sized 33*33*40 cm, each containing 4 fish (3 replicates) in 30 L of contaminated test solution or tap water only (for controls). Fish were exposed to 1 mg/L concentration of copper ($CuSO_4$. $5H_2O$), cadmium ($CdCl_2.H_2O$) and zinc ($ZnCl_2$) for 14 days in single and mixture conditions. Control and metal containing aquariums were renewed after every two days to minimize metal loss and for the refreshment of the medium. Water changing was done after feeding to reduce contamination of the environment with food remains.

After 14 days of exposure period, all fish from three replicate tanks were taken out and killed by a blow to the head. Total lengths were measured to the nearest mm. Mean lengths of fish (129 ± 7.25 mm) did not differ significantly ($P>0.05$) among different exposure regimes and control. Liver tissues of 12 fish (3 replicates) of the same exposure group were pooled and mixed very well. They were allocated for the metals analyses and for the gel filtration. All samples were frozen at –20 °C for two week before the analyses.

Liver tissues were homogenized in 10 mL buffer containing 40 mM Tris-HCl, 0.25 M Sucrose (pH 7.4) at 9500 g for 90 sec (Janke & Kunkel Ultra Turrax T25) and centrifuged at 2500 g for 10 min. The supernatants were decanted carefully to clean glass tubes and heated in water bath at 80 °C for 10 min. Heat treated samples were centrifuged at 2500 g for 10 min to remove the coagulated particules and supernatants were transferred to clean tubes for gel filtration processes. These supernatants (5 mL) was run on a column (2.7 x 60 cm) packed with Sephadex G-75 using an elution buffer containing 50 mM Tris (pH 8.0). Ninety tubes each containing 5 mL eluate were collected from the column for each run. All spectrophotometric analyses and metal measurement were carried out in these eluates. Protein absorbance of the eluates was measured at 254 nm and 280 nm. A series of molecular weight markers (Alcohol dehydrogenase 150.000, Albumin 66.000, Carbonic anhydrase 29.000, Cytochrom C 12.400 and Aprotinin 6500) was used to determine approximate molecular weights of proteins in the eluates. Linear regression equation was calculated between elution

volume and log molecular weight of the markers and following equation was found; y= 5.814-0.666x, r^2 = -0.999. Sulfhydryl group analysis in the eluates was carried out using the Ellman's reagent (Ellman, 1958). Five eluates were pooled to measure their metal levels in MTLP fraction. Metal concentrations in eluates of liver homogenates and dry liver tissues were measured using a flame atomic absorption spectrophotometer (Perkin Elmer AS 3100) with the method explained elsewhere (Canli, 1995). The detection limits of metals were 0.002, 0.001 and 0.002 μg/mL for Cu, Cd and Zn.

RESULTS AND DISCUSSION

Total metal accumulation in the liver of *O. niloticus* is given in Table 1. Concentrations of copper, zinc and cadmium in the liver were influenced significantly by metal exposure types. Cadmium concentrations increased significantly after 14 days of exposure to this metal. Highest increase occurred when Cd was given alone and this was followed by Cd-Zn mixture. However, lowest increase occurred in Cd-Cu mixture. Copper concentrations increased significantly in all treatments ($P<0.05$) in the liver of *O. niloticus*. However, zinc concentrations in the liver did not increase significantly ($P>0.05$) after 14 days of exposure except Cd-Zn mixture experiment, contrarily there were some decreases in Zn alone and Cu-Zn mixture experiments ($P<0.05$). This is interesting because exposure concentration used here higher than environmentally realistic levels indicating Zn is well regulated. The regulation of zinc by fish was demonstrated (Heath, 1987; Dethloff et al. 1999; Lange et al. 2002; Atli and Canli, 2003). However, regulation of cadmium and copper is not evident for fish, though copper regulation in crustacean is well documented (Heath 1987; Canli 1995).

Interactions of metals affect metal accumulation in tissues of fish and other aquatic animals. Generally, metal accumulation is higher when metals are given in single rather than combination (Mance, 1987). Cadmium-zinc interaction is well known in different stages of metal metabolism in animal cells (Brzoska and Moniuszko-Jakoniuk, 2001). The authors reviewed the interaction between cadmium and zinc and concluded that interaction between the two metals is very important in cadmium toxicity. It was pointed that dietary Zn intake increases sharply during Cd intoxication and, also Cd toxicity increases during Zn deficiency. The present data also support this because highest total Zn accumulation in the liver of *O. niloticus* occurred in Cd-Zn mixture, suggesting it was due to a reaction to prevent Cd toxicity. Although cadmium did not alter copper levels considerably, copper altered cadmium levels (Table 1). Cadmium levels fell sharply when copper was present in exposure medium. This could be as a result of interaction between the two metals. Cd-Cu interaction appears to interfere to accumulation of both metals (Mance, 1987; Heath, 1987). Pelgrom et al. (1994) showed that exposure to Cu, Cd and mixture of these metals resulted different accumulation levels of Cu and Cd in fish *Oreochromis mossambicus*. They indicated that exposure to a single metal caused an increase in whole body content of the metal exposed to, though exposure to the mixture of metals presented complex interaction mechanisms. Their data showed that whole body cadmium content significantly decreased when copper was present in the medium.

Their results also supported the present data as total cadmium concentrations in liver tissue fell sharply when cadmium and copper were present in the exposure medium. Dethloff et al. (1999) exposed *Oncorhynchus mykiss* to copper and zinc mixture and found that Cu levels increased both in single and mixture exposures, whereas zinc levels were highly variable in various exposures. The present results also demonstrated similar patterns of Cu and Zn accumulation.

The elution profiles of liver homogenates from controls and metal exposure experiments were presented in Figures 1-8. Generally, the figures show that there were three protein peaks at 254 nm and 280 nm. The first peak obtained between 13-20 fractions and contained high molecular weight proteins greater than 70000 Da. However, this peak was not observed in Cd alone and Cd-Zn mixture experiments. These proteins contained sulfhydryl groups and also bound trace amount of Zn. Because of their high molecular weight they are not metallothioneins or MTLP. The absorbance at 254 nm of this fraction increased considerably in exposures in which copper was present. The second peak obtained between 21-35 fractions and contained medium molecular weight proteins approximately 43000 Da. Although these peaks were detected at 254 and 280 nm there was no detection at 412 indicating the absence of sulfhydryl groups. Both their molecular weight and absence of sulfhydryl groups indicate that they are also not MT or MTLP. Absorbance at 254 nm of this fraction increased considerably in exposure in which Cd alone and Cd-Zn mixture were present. The third peak obtained between 35-60 fractions and contained MTLP because these proteins contained sulfhydryl groups, metals and have low molecular weight. All proteins detected here are heat-resistant at 80 °C. This is an important characterization of MTs because there are many metal containing proteins or enzymes that could be measured in eluates if heat treatment was not applied here. Our previous studies (Atli and Canli, 2003) showed that *O. niloticus* has metal containing proteins that were removed after heat-treatment. Thus, the heat-treatments of tissue homogenates are essential to purify proteins for gel filtration studies because other metal and sulfhydryl groups containing proteins interfere with MTLP profiles. Heat-treatments were successfully applied to purify MTLP in gel filtration studies (De Conto Cinier et al. 1998; Wu et al. 1999; Atli and Canli, 2003; Cheung et al. 2004).

The liver of *Oreochromis mossambicus* had similar elution profiles as this fish also produced three protein peaks each containing thiol groups and metals (Wu et al. 1999). Studying subcellular distribution of cadmium in *Salmo gairdneri* showed that there were three protein peaks in the liver, among these only MT fraction contained cadmium (Olsson and Hogstrand 1987). Olsson and Haux (1986) showed that copper and zinc levels in hepatic MTs did not increase significantly with increases in exposure levels in the liver of perch *Perca fluviatilis*. Similarly, Cu and Zn levels in MT fractions of *O. niloticus* exposed to Cu and Zn did not increase considerably (Atli and Canli, 2003). This may be high basal levels of Cu and Zn MTs in fish tissues. However, high levels of copper and zinc bound on MT or MT-like proteins can also increase in animals that lived for a long period in metal contaminated environments (Hogstrand and Haux 1990; Cheung et al. 2004). Copper levels in MTLP fraction of *O. niloticus* also

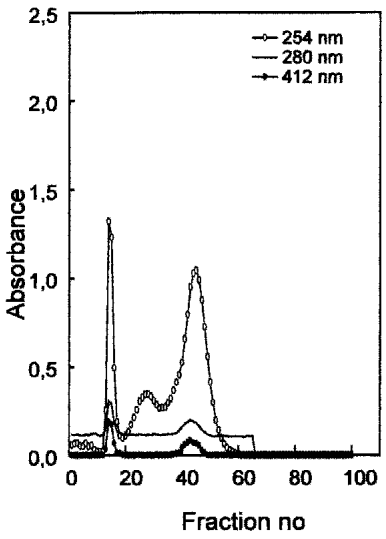

Figure 1. Sephadex G-75 elution profile of liver from control O. *niloticus.*

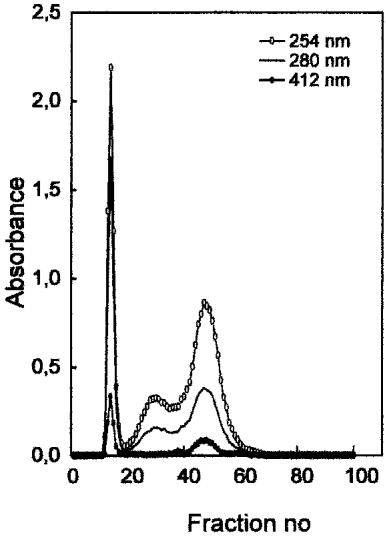

Figure 2. Sephadex G-75 elution profile of liver from Cu-exposed *O. niloticus.*

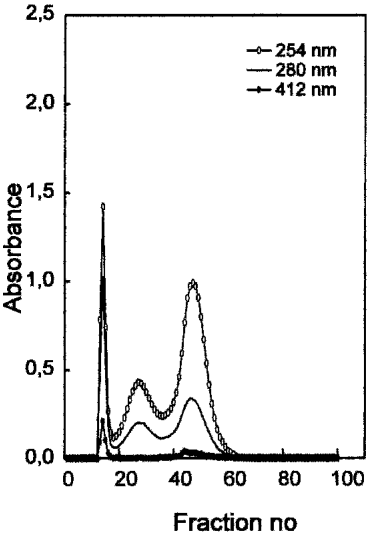

Figure 3. Sephadex G-75 elution profile of liver from Zn-exposed *O. niloticus.*

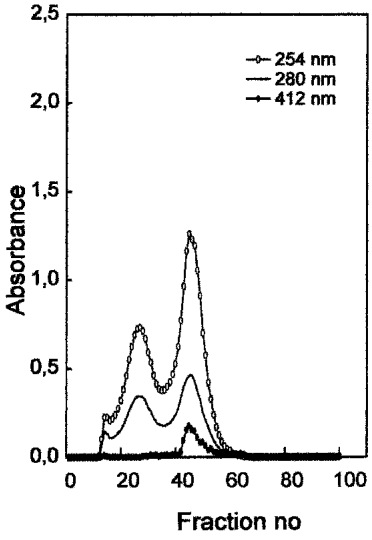

Figure 4. Sephadex G-75 elution profile of liver from Cd-exposed *O. niloticus.*

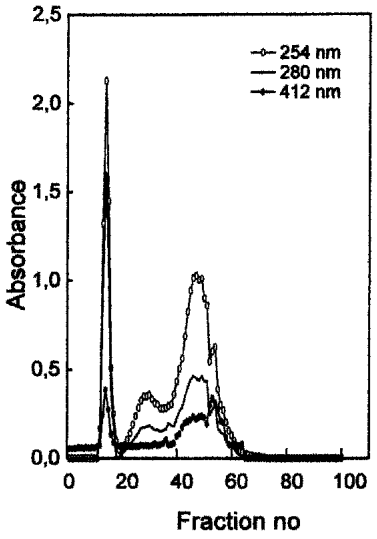

Figure 5. Sephadex G-75 elution profile of liver from *O. niloticus* exposed to Cu-Zn mixture.

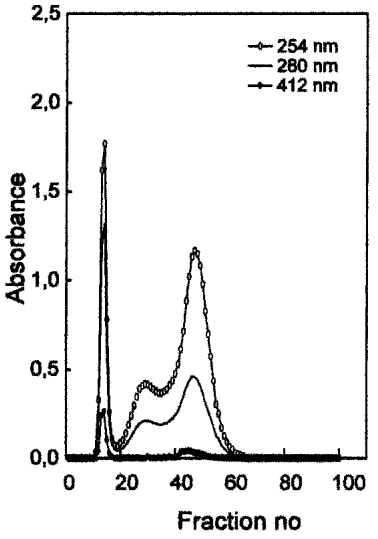

Figure 6. Sephadex G-75 elution profile of liver from *O. niloticus* exposed to Cd-Cu mixture.

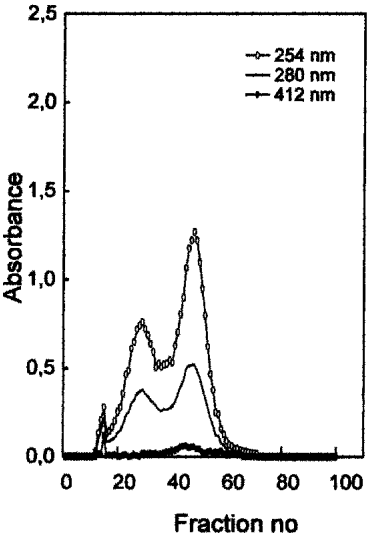

Figure 7. Sephadex G-75 elution profile of liver from *O. niloticus* exposed to Cd-Zn mixture.

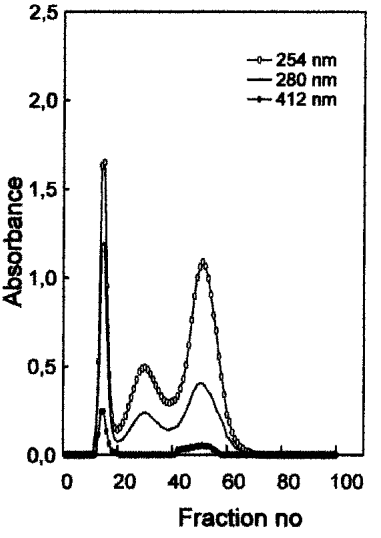

Figure 8. Sephadex G-75 elution profile of liver from *O. niloticus* exposed to Cd-Cu-Zn mixture.

Table 1. Metal concentrations (μg metal/mg d.w.) and associated standard errors in the liver of *Oreochromis niloticus* after 14 days of exposures. * indicates the significant differences between control and individual exposure type (P<0.05).

Exposure Type	Cadmium	Zinc	Copper
Control	0.64 ± 0.04	82.18 ± 6.00	128.53 ± 7.26
Cd	82.58 ± 10.04*		
Zn		52.90 ± 12.20	
Cu			234.53 ± 32.19*
Cd-Zn	61.91 ± 7.93*	202.04 ± 36.56*	
Cd-Cu	16.10 ± 3.02*		269.51 ± 43.81*
Zn-Cu		57.56 ± 2.95*	191.03 ± 27.21*
Cd-Cu-Zn	19.62 ± 3.49*	88.92 ± 0.74	256.58 ± 38.82*

Table 2. Metal concentrations (μg metal/mL) in eluates of liver homogenates of *Oreochromis niloticus* exposed to 14 days to metals. Fractions pools given below are obtained from eluates in which MTLP were present. ND: not detected (<0.002 μg/mL).

Exposure Type		Fraction Pools				
		35-40	41-45	46-50	51-55	56-60
Control	Cd	ND	ND	ND	ND	ND
	Cu	0.252	0.376	0.435	0.286	0.156
	Zn	2.823	2.90	0.756	0.174	0.073
Cd		ND	0.161	0.077	ND	ND
Cu		0.438	0.688	0.595	0.332	0.213
Zn		0.317	0.519	0.297	0.123	0.059
Cd-Cu	Cd	ND	0.029	0.013	ND	ND
	Cu	0.457	0.748	0.522	0.243	0.160
Cd-Zn	Cd	ND	0.059	0.068	ND	ND
	Zn	0.407	0.674	0.405	0.122	0.043
Cu-Zn	Cu	0.393	0.648	0.522	0.286	0.203
	Zn	0.648	0.535	0.365	0.200	0.139
Cd-Cu-Zn	Cd	ND	0.016	0.048	ND	ND
	Cu	0.355	0.282	0.090	0.043	0.047
	Zn	0.200	0.524	0.538	0.305	0.160
Mol. Weight		18197-10889	9840-6540	5900-3930	3540-2350	2120-1410

increased in the present study indicating excess levels of copper uptake from the medium. MT concentrations tend to increase in fish tissues when they are exposed to excess levels of metals via various routes (De Conto Cinier et al. 1998; Wu et al. 1999; Dang et al. 2001; Cheung et al. 2004; Lecoeur et al. 2004).

The profiles of MTLP fraction were not changed considerably at 254, 280 and 412 nm except some increase in Cd alone experiment (Fig 1-8). However, metal contents in MTLP fraction increased sharply especially those of cadmium and copper (Table 2). This table shows that cadmium levels in 41-45 fraction pool in where MTLP proteins present increased in the following order; control< Cd-Cu-Zn <Cd-Cu <Cd-Zn <Cd. This order for copper in the same fraction pool was; Cd-Cu-Zn < control< Cu-Zn< Cu< Cu-Cd. The order for zinc was opposite as the controls showed highest Zn concentration. Exposure of animals to any stress factor may increase metabolic activity which means it also increases respiration. Increased metabolic activity may mean increased demands for Zn containing proteins and/or enzymes such as carbonic anhydrase which is very important for CO_2 transport in the blood. Mason et al. (2004) indicated that Zn donation from MTs to apo-carbonic anhydrase was evident, which may also help to understand the increase of total Zn in the present study. Sharp increase of cadmium levels in MTLP fraction was shown in aquatic animals after exposure to this metal. However, copper levels in MTLP fractions do not always follow the same trend. (Canli et al. 1997; Ueng et al. 1996; De Conto Cinier et al. 1998; Wu et al. 1999; Atli and Canli, 2003; Lecoeur et al. 2004). Although copper levels in MTLP fraction increased in the present study, some studies indicate that copper increase was not evident (Olsson and Haux, 1986; Olsson and Hogstrand, 1987; Atli and Canli, 2003; Lecoeur et al. 2004). Exposure concentration, durations and also species differences could play very important roles in threshold of copper increase in MTLP fraction. It seems that the threshold exposure conditions exceeded in the present study as copper levels in MTLP fraction increased two folds.

Metallothioneins are naturally present in all animal cells as storage form of the essential metal and have conspicuous roles in the extracellular and intracellular control of these metals. Therefore, the basal levels of MTs are considered to be involved in the essential metal regulation. The induction of MTs was shown after exposure to metals, most important inducers being Cd, Hg, Zn and Cu (Roesijadi and Robinson, 1994; Cheung et al. 2004). Natural occurrence of copper and zinc MTs was shown in tissues of fish, though cadmium MTs were absent naturally in control animals or animals from the field (Olsson and Hogstrand 1987; Canli 1995; Ueng et al. 1996; De Conto Cinier et al. 1998; Wu et al. 1999) unless animals were caught from a contaminated site (Hogstrand and Haux 1990; Dallinger et al. 1997; Olsvik et al. 2001). In this study, heat-resistant, low molecular weight, metal-binding proteins that contain sulfhydryl groups were demonstrated in the liver of *O. niloticus*. In controls, only Cu and Zn containing MTLP were present though all metals were detected in MTLP fractions after exposure to metals both in single and mixture exposures.

It is evident that MTs could be a sensitive indicator of heavy metal contamination in the aquatic environment (Hogstrand and Haux 1990; Dallinger et al. 1997;

Canli et al. 1997; De Conto Cinier et al. 1998; Lange et al. 2002; Atli and Canli, 2003). The use of MTs or MTLP as a detection tool for essential metal exposures could be limited when metal uptake rate could not be reached to a threshold level, though this limit could be achieved much lower concentrations in non-essential metal exposures. Therefore, it is generally suggested that MTs could be a sensitive indicator of non-essential metals such as cadmium rather than essential metals (Lange et al. 2002; Atli and Canli, 2003; Lecoeur et al. 2004). As the present study showed, exposure type of metals could also alter the induction of MT (Lange, 2002) indicating the fact that metal interactions should also be taken into account when natural monitoring studies are carried out. Special attention should be given to the threshold levels of both essential and non-essential metal levels necessary to trigger MT induction in the aquatic environment.

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