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Long-Term Toxicity Effects of Cadmium and Lead on *Bufo raddei* Tadpoles

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Cadmium (Cd) and lead (Pb), entering aquatic systems from industrial and consumer waste can cause contamination of the aquatic environment. As a result of bioaccumulation when they enter the food chain, Cd and Pb can cause serious damage to physiological processes or tissues in aquatic organisms even at concentrations far below the lethal level (He et al., 2005).

Erythrocytic micronuclei (EMN) and erythrocytic nuclear abnormalities (ENA) have been routinely applied to assess water quality and the genotoxicity of contaminants on organisms (Chandra and Khuda-Bukhsh, 2004; Zhu et al., 2004). Superoxide dismutase (SOD) is an enzyme that can scavenge superoxide anions and protect organisms from adverse effects. Adenosine triphosphatase (ATPase) is an enzyme that catalyzes adenosinetriphosphate hydrolysis and provides chemical energy for organisms. The activities of SOD and ATPase have been used as biomarkers for assessing water quality (Achuba, 2002; Brauner et al., 2003).

Like fish, tadpoles are inevitably affected by heavy metals in aquatic systems. Perez-Coll and Herkovits (1990; 1996) and Herkovits and Perez-Coll (1993) have documented the accumulation of Cd or Pb in the tissues and organs of embryos and tadpoles. Lefcort et al. (1998) reported that heavy metals could alter the survival, growth, metamorphosis and antipredatory behavior of *Rana lutei-ventris* tadpoles. Rosenberg et al. (1998) studied the sublethal effect of lead on erythrocyte osmotic fragility in *Bufo arenarum*. Naab et al. (2001) reported that Zn could inhibit the pentose phosphate pathway in the ovaries of adult *B. arenarum* females, resulting in a decrease of the embryo survival ratio. Carattino et al. (2004) showed that both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities could be used as biomarkers for Cu but not for Cd toxicity. Fonovich de Schroeder (2005) proposed a possible role for glucose 6-phosphate dehydrogenase as a biomarker for Zn in *B. arenarum* ovarian tissue. *Bufo raddei* is the most common species in North China and is highly adaptable to polluted environments. Our previous work (Huang et al., 2004; Long et al., 2004) showed that Cd and Pb increased the mortality and malformation ratio and prolonged the development and metamorphosis period in tadpoles, but had little effect on the hatching of *B. raddei*. Although various reports have shown that heavy metals can affect tadpoles' growth and development, little is known about the chronic toxicity effects of Cd and Pb on the physiological processes of *B. raddei.* tadpoles. The purpose of this research is to explore the effects of Cd and Pb on EMN and ENA frequencies, SOD and ATPase activities in target organs of *B. raddei* tadpoles with increasing concentrations of heavy metal and exposure times.

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Materials and Methods

Bovine serum albumin (BSA) and Giemsa were obtained from Sigma (USA). All other chemicals and reagents were of analytical grade and were procured from local chemical companies.

Table 1 Relative EMN and ENA frequencies in tadpoles exposed to various Cd and Pb concentrations for 45, 60 and 75 days

Biomarkers	Heavy metal	Concentration	Exposure time (days)		
			45	60	75
EMN	Cd	Control	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04
		0.0015 ppm	2.83 ± 0.15	6.00 ± 0.20A★	1.80 ± 0.44•
		0.03 ppm	3.67 ± 0.24a	9.00 ± 0.23Ab★	2.20 ± 0.21•
		0.15 ppm	4.00 ± 0.29ab	9.50 ± 0.29Ab★	2.60 ± 0.19a•
	Pb	Control	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04
		0.7 ppm	1.67 ± 0.07	4.00 ± 0.18A☆	1.10 ± 0.04•
		1.4 ppm	2.67 ± 0.13a	7.50 ± 0.24Ab★	1.70 ± 0.07•
		14 ppm	5.50 ± 0.24ABC	11.00 ± 0.33ABC★	4.20 ± 0.11Abc•
		70 ppm	12.00 ± 0.45ABCD	18.50 ± 0.48ABCD★	5.40 ± 0.17ABC★•
ENA	Cd	Control	1.00 ± 0.06	1.00 ± 0.03	1.00 ± 0.05
		0.0015 ppm	12.33 ± 0.45	1.07 ± 0.05★	1.41 ± 0.37★
		0.03 ppm	14.08 ± 0.36A	1.93 ± 0.03a★	2.12 ± 0.13a★
		0.15 ppm	14.42 ± 0.47Ab	2.14 ± 0.04a★	2.88 ± 0.20ab★
	Pb	Control	1.00 ± 0.06	1.00 ± 0.03	1.00 ± 0.05
		0.7 ppm	1.92 ± 0.06	1.04 ± 0.03	1.76 ± 0.10
		1.4 ppm	13.75 ± 0.40AB	8.00 ± 0.19AB★	2.24 ± 0.18a★•
		14 ppm	14.17 ± 0.37AB	8.43 ± 0.40AB★	2.76 ± 0.15a★•
		7 0ppm	13.33 ± 0.32AB	8.21 ± 0.33AB★	3.41 ± 0.16ab★•

Results were analyzed by one-way ANOVA and expressed as mean ± SE (n = 10). Statistically significant differences are indicated by: a ($P < 0.05$) or A ($P < 0.01$) when compared with control; b ($P < 0.05$) or B ($P < 0.01$) when compared with 0.0015 ppm Cd or 0.7 ppm Pb; c ($P < 0.05$) or C ($P < 0.01$) when compared with 1.4 ppm Pb; and D ($P < 0.01$) when compared with the 14 ppm Pb group at each time. ☆ ($P < 0.05$) or ★ ($P < 0.01$) indicate statistical differences of 60 d and 75 d compared with 45 d; and • ($P < 0.01$) indicates statistical difference between 75 d and 60 d at each metal concentration

Adult *B. raddei* were collected from Tian-qing Park in Lanzhou, China, an unpolluted place (the concentrations of Cd and Pb were 1.56×10^{-5} μg/L and 6.50 μg/L respectively in water, and 2.25×10^{-4} μg/L and 4.00 μg/L in sediment). Experiments were conducted in three series to determine the frequencies of EMN and ENA, and activities of SOD and ATPase in tadpoles exposed to Cd and Pb for 45, 60 and 75 days. In each series, we set 16 aquaria (a total of 48 aquaria were used) of the same size for eight groups each, in two replicates: control (no metals); 0.0015 ppm Cd, 0.03 ppm Cd and 0.15 ppm Cd (using $CdCl_2 \cdot 2.5H_2O$); 0.7 ppm Pb, 1.4 ppm Pb, 14 ppm Pb and 70 ppm Pb [using $Pb(NO_3)_2$]. The concentrations of Cd and Pb were set referring to the 96 hr lethal concentrations (Cd 0.3317 ppm, Pb 140 ppm). One hundred embryos were hatched, reared in each aquarium with 20 liters of dechlorinated tap water in the laboratory and fed sufficient food of boiled spinach, at room temperature (20 ± 1°C) on a 14-h light:10-h dark cycle; during our experiments from April to May, this rhythm was similar to the ambient light cycle. The water in each aquarium was changed every two days to replenish the Cd and Pb. At the end of 45, 60 and 75 days of exposure over 80% of the tadpoles survived, 10 tadpoles in each aquarium were sacrificed and their blood, liver, skin and intestine were sampled for further testing.

Blood samples of tadpoles were taken from the heart and two slides per tadpole were prepared. Slides were fixed for 15 min in methyl alcohol before staining with Giemsa (19:1 diluted by pH 6.98 phosphate buffer) for 15 min, washed several times with distilled water, imaged with a Nikon microscope under an oil immersion lens (1,000x magnification) and counted at permillage (polychromatocytes to 5,000 blood cells).

Liver, skin and intestine samples were homogenized in 1.0 ml of SOD homogenization buffer (0.1 M Tris-HCl buffer, pH 8.2). The homogenates were centrifuged at 12,000 rpm for 20 min. SOD activity (unit/mg protein) was assayed by a modified pyrogallol auto-oxidation method (Marklund and Marklund, 1974). One unit of SOD activity was defined as the amount of protein that caused a 50% inhibition of the pyrogallol auto-oxidation rate. The protein contents in various samples were estimated by the method of Bradford (1976) using BSA as standard.

Liver, skin and intestine were homogenized in 1.0ml ATPase homogenization buffer (0.35 M Mannitol, 1.0ppm BSA, 0.25 M Sucrose, 0.001 M EDTA, 0.01 M Tris-HCl

Table 2 Relative SOD activities in the liver, skin and intestine of tadpoles exposed to various Cd and Pb concentrations for 45, 60 and 75 days

Tissue	Heavy metal	Concentration	Exposure time (days)		
			45	60	75
Liver	Cd	Control	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.06
		0.001 5ppm	0.91 ± 0.06	0.57 ± 0.04	1.60 ± 0.08a☆•
		0.03 ppm	0.94 ± 0.04	0.34 ± 0.03a	1.95 ± 0.07a☆•
		0.15 ppm	0.71 ± 0.04	0.30 ± 0.04a	1.98 ± 0.10a★•
	Pb	Control	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.06
		0.7 ppm	0.89 ± 0.12	1.50 ± 0.17	1.58 ± 0.14
		1.4 ppm	1.08 ± 0.08	1.34 ± 0.11	1.83 ± 0.22
		14 ppm	2.37 ± 0.09	1.40 ± 0.17	4.07 ± 0.54abc☆○
		70 ppm	4.09 ± 0.25abcd	1.35 ± 0.18☆	3.86 ± 0.55abc○
Skin	Cd	Control	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.04
		0.0015 ppm	1.72 ± 0.24a	1.32 ± 0.29	2.39 ± 0.41a○
		0.03 ppm	1.90 ± 0.26a	1.11 ± 0.24☆	2.58 ± 0.45a○
		0.15 ppm	2.52 ± 0.45Ab	1.18 ± 0.27★	2.93 ± 0.54A•
	Pb	Control	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.04
		0.7 ppm	1.97 ± 0.25	1.38 ± 0.15	3.63 ± 0.38a
		1.4 ppm	1.49 ± 0.15	1.93 ± 0.17	4.35 ± 0.34a☆
		14 ppm	2.45 ± 0.21	1.30 ± 0.12	11.75 ± 2.16ABC★•
		70 ppm	2.76 ± 0.24a	1.31 ± 0.14☆	12.07 ± 1.92ABC★•
Intestine	Cd	Control	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.04
		0.0015 ppm	0.63 ± 0.06	0.97 ± 0.17	1.61 ± 0.30a☆○
		0.03 ppm	0.72 ± 0.05	0.95 ± 0.25	1.67 ± 0.20a☆○
		0.15 ppm	0.77 ± 0.05	0.72 ± 0.20	2.14 ± 0.34Ab★•
	Pb	Control	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.04
		0.7 ppm	2.19 ± 0.23	1.63 ± 0.20	4.26 ± 0.50a
		1.4 ppm	2.01 ± 0.30	2.22 ± 0.30	5.50 ± 0.54a
		14 ppm	2.62 ± 0.19	1.47 ± 0.20	12.44 ± 1.71ABC★•
		70 ppm	2.97 ± 0.35a	1.49 ± 0.25☆	12.81 ± 2.59ABC★•

Results were analyzed using one-way ANOVA and were expressed as mean ± SE (n = 10). Statistically significant differences are shown by: a ($P < 0.05$) or A ($P < 0.01$) when compared with the control; b ($P < 0.05$) or B ($P < 0.01$) when compared with 0.0015 ppm Cd or 0.7 ppm Pb; c ($P < 0.05$) or C ($P < 0.01$) when compared with 1.4 ppm Pb; and d ($P < 0.05$) when compared with the 14 ppm Pb group at each time. ☆ ($P < 0.05$) and ★ ($P < 0.01$) indicate statistical differences of 60 d and 75d compared with 45 d; and ○ ($P < 0.05$) or • ($P < 0.01$) indicate statistical difference between 75 d and 60 d at each metal concentration

buffer, pH 7.2). The homogenates were centrifuged at 4,000 rpm for 20 min. ATPase activity was assayed by the malachite green method of Hess and Derr (1975), and expressed as μg of inorganic phosphate liberated per hour per mg of protein.

To conveniently compare with different exposure time at each concentration and organ, the frequencies of EMN and ENA and the activities of SOD and ATPase were expressed as relative values (the values of treatment groups compared to those of control groups). Differences were detected by one-way analysis of variation (ANOVA) followed by Bonferroni's test for multiple comparisons. Two-way ANOVA was performed to analyze the interaction of treatment concentration and time, and post hoc comparisons were made with Bonferroni's test if needed. The relations between metal concentration and EMN/ENA frequency or SOD/ATPase activity were conducted by partial correlation analysis. Statistically significant levels at $P < 0.05$, $P < 0.01$ were adopted.

Results and Discussion

Our results showed that the EMN and ENA frequencies, and ATPase activity in tadpoles were significantly increased with increasing Cd and Pb concentrations (EMN-Cd: $r = 0.5285$, $P = 0.006$; EMN-Pb: $r = 0.9004$, $P < 0.001$; ENA-Cd: $r = 0.3366$, $P = 0.048$; ENA-Pb: $r = 0.3485$, $P = 0.040$; ATPase-Cd: $r = 0.4173$, $P = 0.034$; ATPase-Pb: $r = 0.5061$, $P < 0.001$). The differences in the

Table 3 Relative ATPase activities in liver, skin and intestine of tadpoles exposed to different Cd and Pb concentrations for 45, 60 and 75 days

Tissue	Heavy metal	Concentration	Exposure time (days)		
			45	60	75
Liver	Cd	Control	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.03
		0.0015 ppm	0.57 ± 0.05	1.46 ± 0.19☆	1.36 ± 0.29☆
		0.03 ppm	0.74 ± 0.06	1.58 ± 0.27☆	1.46 ± 0.28☆
		0.15 ppm	0.65 ± 0.06	1.63 ± 0.31☆	1.61 ± 0.39☆
	Pb	Control	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.03
		0.7 ppm	0.52 ± 0.12	1.79 ± 0.27☆	4.79 ± 0.48A★•
		1.4 ppm	0.64 ± 0.17	2.80 ± 0.33ab★	5.36 ± 0.58A★•
		14 ppm	0.50 ± 0.12	4.60 ± 0.52ABc★	5.38 ± 0.70A★
		70 ppm	0.58 ± 0.13	5.16 ± 0.56ABC★	7.56 ± 1.18ABCD★•
Skin	Cd	Control	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.04
		0.0015 ppm	0.28 ± 0.03	1.64 ± 0.33☆	2.13 ± 0.40☆
		0.03 ppm	0.52 ± 0.05	2.10 ± 0.32☆	2.07 ± 0.42☆
		0.15 ppm	0.54 ± 0.06	2.55 ± 0.50ab☆	2.57 ± 0.38a☆
	Pb	Control	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.04
		0.7 ppm	0.31 ± 0.06	2.46 ± 0.29a★	4.22 ± 0.60 A★○
		1.4 ppm	0.27 ± 0.05	2.33 ± 0.31a★	3.99 ± 0.54 A★•
		14 ppm	0.34 ± 0.10	3.17 ± 0.54a★	4.62 ± 0.57 A★○
		70 ppm	0.38 ± 0.10	3.43 ± 0.60ac★	4.92 ± 0.71 A★○
Intestine	Cd	Control	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.05
		0.0015 ppm	0.58 ± 0.14	2.60 ± 0.45a★	3.97 ± 1.12A★
		0.03 ppm	0.38 ± 0.07	2.86 ± 0.60a★	6.34 ± 1.21AB★•
		0.15 ppm	0.50 ± 0.10	3.55 ± 0.62A★	6.78 ± 1.20AB★•
	Pb	Control	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.05
		0.7 ppm	0.52 ± 0.14	1.32 ± 0.30	2.72 ± 0.37A★○
		1.4 ppm	0.63 ± 0.18	1.36 ± 0.34	2.34 ± 0.44a☆
		14 ppm	0.72 ± 0.21	1.31 ± 0.25	3.26 ± 0.47A★○
		70 ppm	0.85 ± 0.20	1.67 ± 0.36	3.48 ± 0.56Ab★○

Results were analyzed by one-way ANOVA and are expressed as mean ± SE (n = 10). Statistically significant differences are shown by: a ($P < 0.05$) or A ($P < 0.01$) when compared with control; b ($P < 0.05$) or B ($P < 0.01$) when compared with 0.0015 ppm Cd or 0.7 ppm Pb; c ($P < 0.05$) or C ($P < 0.01$) when compared with 1.4 ppm Pb; and D ($P < 0.01$) when compared with the 14 ppm Pb group at each time. ☆ ($P < 0.05$) or ★ ($P < 0.01$) indicate statistical differences of 60 d and 75 d compared with 45 d; and ○ ($P < 0.05$) or • ($P < 0.01$) indicate statistical difference between 75 d and 60 d at each metal concentration

SOD and ATPase activities between the three organs in each group were also found in this study: the SOD activity increased significantly ($P < 0.01$–0.05) in the skin of tadpoles exposed to Cd for 45 days; at 60 days, SOD activity decreased significantly ($P < 0.05$) in the liver in the 0.03 ppm and 0.15 ppm Cd groups, while ATPase activity increased significantly ($P < 0.01$–0.05) in the intestine due to Cd and in the liver and skin due to Pb exposure. For exposure times lasting from 45 to 75 days, the EMN and ENA frequencies generally decreased (Table 1), whereas SOD and ATPase activities increased in each treatment group (Tables 2 and 3). Two-way ANOVA indicated that the EMN and ENA frequencies, and the SOD and ATPase activities in tadpoles were significantly ($P < 0.01$–0.05) affected by exposure time, concentration and their interaction, although EMN and ENA frequencies were primarily influenced by treatment concentration whereas SOD and ATPase activities were mainly influenced by exposure time. Partial correlation analyses showed that there were no significant ($P > 0.05$) correlation between EMN/ENA frequency and SOD/ATPase activity.

The micronucleus test has been widely employed in fish to study the genotoxicity of heavy metals, and the changes of EMN and ENA frequencies were dependent upon fish species (Ayllon and Garcia-Vazquez, 2000), heavy-metal dose or concentration (Chandra and Khuda-Bukhsh, 2004) and exposure time (Zhu et al., 2004). A moderate dose-dependent increase in micronuclei of fish exposed to $CdCl_2$ in acute experiments (6, 24, 48, 72 and 96 h) was reported by Chandra and Khuda-Bukhsh (2004), and our results

showed a similar tendency, indicating that Cd and Pb could induce genotoxic effects on tadpoles even in a chronic experiment. Moreover, although the total number and the average turnover time of erythrocytes were unknown, and although their development and metamorphosis time were prolonged from about 65 to 100 days (Huang et al., 2004; Long et al., 2004), the EMN and ENA frequencies decreased with increasing exposure time, which suggested that tadpoles could protect themselves from a lightly polluted environment by partly regulating the factors that could cause genetic damage.

Heavy metals can cause toxic effects on organisms by generating oxygen free radicals (OFRs), while SOD can scavenge superoxide anions (one species of OFRs) and protect the organism from these adverse effects. Previous studies have documented that SOD activities in fish both under field and laboratory conditions are organ-specific (Achuba, 2002) and exposure-time dependent (Zikic et al., 2001), and our results confirm that SOD activity in tadpoles is also related to these two factors. Although there were no significant correlations between EMN/ENA frequency and SOD activity, the latter increased significantly with prolonging treatment time, which suggested that tadpoles could moderately adjust SOD activity to protect themselves against a polluted environment.

ATPase activity could be considered as an early warning of pollutant-induced damage to the ionic and osmoregulatory system (Stagg et al., 1992), and its activity in fish gill may be a sensitive indicator to metal exposure (Ay et al., 1999). ATPase activity in fish in vitro is generally inhibited by heavy metals, whereas in vivo effects are not so clear. Brauner et al. (2003) observed that ATPase activity decreased in chronic experiments, which was similar to the results of Morgan et al. (1997) in acute experiments. In contrast, our results documented that ATPase activity in tadpoles in vivo was positively correlated with exposure time and concentration, which might be because some compensatory mechanisms were involved in the homeostatic mechanisms (Stagg et al., 1992). If the exposure concentration was not high enough to cause irreversible change to the enzyme structure, tadpoles would increase ATPase activity in correlative organs by increasing the number and/or the turnover rates of the enzyme. Although no significant correlations between EMN/ENA frequency and ATPase activity were obtained, the increase of ATPase activity with increasing exposure time indicated that tadpoles can provide more chemical energy by catalyzing adenosinetriphosphate hydrolysis for their self-protection from the polluted environment. In addition, Cd and Pb influenced the EMN and ENA frequencies primarily depending on concentration, whereas SOD and ATPase activities depended more strongly on exposure time.

In conclusion, our results show that changes of the EMN and ENA frequencies and the SOD and ATPase activities in the target organs of *B. raddei* tadpoles are related to exposure time and the concentration of Cd and Pb. The present study indicates that, with increasing exposure time, tadpoles can moderately increase their enzyme activities for self-protection in the polluted environments. However, further studies are needed to discover the metal tolerance mechanism in aquatic organisms.

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