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Effects of Rare Earth Metal Ions and Their EDTA Complexes on Antioxidant Enzymes of Fish Liver

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In China, rare earth elements (REEs) were widely used in agriculture, forestry, animal husbandry and aquaculture as microelement fertilizers or animal food, which incurred high environmental concern. Many studies on REEs chemical behavior in the environment and their effects on ecosystem and human health have been carried out (Ran and Liu 1993; Liu 1993; Ji and Cui 1997). Generally, the fraction of the water soluble REEs is about 10% or lower of the total REEs in soil samples. In aquatic environment, the concentrations of dissolvable REEs in sediment are very important to evaluate the bioaccumulation of REEs in algae. In a way, rare earth is a inhibitor for tumor, which is not only due to its directly action on cellar, but due to its improvement abilities in immunity system and biochemical metabolism of the organism. Recently, the bioconcentration and elimination of REEs in Carp (Sun et al. 1996) and in algae (Sun et al. 1997) have been reported. The bioaccumulation processes of REEs by algae were highly dependent on chemical species. Adding organic ligands, which can form RE-organic complex species, led to a great reduction of the REEs bioaccumulation in algae. The distribution and bioavailability of REEs in different parts of aquatic microcosm have been investigated (Yin et al. 1997). REEs were adsorbed rapidly to sediments, resulting in high residue levels and comparatively higher bioaccumulation factors of REEs in shellfish. However, few reports have been published concerning the toxicological effects of different species of rare earth on the laboratory aquatic organisms.

Large quantities of reactive oxygen intermediates (ROIs) resulted from metabolism of extraneous chemicals were discovered to cause various damages to organisms. Exposure to these ROIs producers could induce the antioxidant enzyme activities (superoxide dismutase, catalase and glutathione peroxidase). Such induces could serve as an important adaptation to the conditions of oxidative stress (Mather-Mihaich and DiGiulio 1991; Babo et al. 1992; Thomas et al. 1993). Studies using bivalve molluscs showed that experimental exposure of *Rangia cuneata* and *Geukensia demissa* to paraquat provoked a significant transient induce of CAT activity (Wenning and DiGiulio 1988). Enhancement of SOD and CAT activities were observed in the laboratory to a highly PAH- and PCB-contaminated sediment (DiGiulio et al. 1989). Hence, there was the expectation

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that changes in the activities of antioxidant enzymes could be used as early biomarkers for exposure and toxicity in free-living organisms. In addition, time-effectiveness of action of rare earth metal ions on enzymes has been reported. For instance, the inhibition of La^{3+} on *rhus vernicifera* laccase was transient (Wang et al. 1994), but its inhibitory level on calcium ATPase strengthened as time went on (Taro and William 1990).

Our previous study reported that rare earth metal ions (Gd^{3+}, Y^{3+}) could accumulate in different internal organs of fish and bioaccumulation values of Gd^{3+} and Y^{3+} in the liver were relatively higher than other organs of goldfish. Gd^{3+} and Y^{3+} affected the enzyme activities in goldfish liver (Wang et al. 1999).

The objective of the present study is to reveal the toxicological effects of different species of rare earth to goldfish and validate the potential use of antioxidant enzymes as early biomarkers of REEs in aquatic ecosystem. For this purpose, the exposure tests were performed to observe the changes of SOD CAT activities of goldfish *carassius auratus* with the control at selected times. The toxicological effects on goldfish were estimated with contrast and analysis of the test data.

MATERIALS AND METHODS

Lanthanum, Gadolinium and Yttrium were selected as representatives of light, medium and heavy rare earth elements, respectively. The chemicals were of an analytical grade and purchased from Shanghai Yuelong Metal limited Company. The REEs stock solutions with a concentration of 1000mg/l were prepared by dissolving the metal salts in 10.0ml (1:1) HNO_3, then adding distilled water to 1.0L. $Na_2EDTA \cdot 2H_2O$ (Ethyleneediamine tertraacetic, A.R.) furnished by Shanghai Chemical Reagent First Plant was used in preparation for RE-EDTA complexes. EDTA complex species of RE in test solutions were calculated by using MINTEQA2 (Brown and Allison 1990) with RE-EDTA making up over 99% of the total RE species.

Goldfish *Carassius auratus* was purchased from a local Aquatic Research Institute, whose average body length and weight were about 9cm and 12g, respectively. All goldfish was acclimated to the water dechlorinated by active carbon for 10 days before exposure and their mortality was below 2%. A commercial assorted feed was in crumb and given once a day.

Goldfish was randomly selected into the aquariums with the rate of fish/water 5.88g/l and 32 goldfish as a group. Three groups were exposed to RE metal ion and its EDTA complex solutions at the concentration of 0.015mM (according to Wang et al. 1999 and real concentration of aquatic environment) with one group

used for the control. During the experiment, the pH values and the hardness of all the solutions were respective 6.5±0.2 and about 100mg/l, and the test solutions were always aerated and renewed every 24 hours. Exposure experiment was repeated for different rare earth elements (La, Gd and Y).

Three to five goldfish was taken from each group for parallel samples at the different time intervals. Goldfish samples were weighed, dissected and their livers were separated for weight after rinsing with distilled water. About 0.15g of goldfish liver was homogenized after addition of 2.0ml of 6.7mM phosphate buffer pH 7.0 for assessment of CAT and SOD activities. The extraction were centrifugalized at 1×10^4 rpm for 10 minutes and preserved at -38℃for analysis. All above operations were under the temperature of below zero.

Determination of the protein in goldfish livers was carried out by the method of Bradford (1976). In brief, protein reagent was prepared by dissolving 100mg Coomassie Brilliant Blue G-250 in 50ml 95% ethanol and adding 100ml 85% (W/V) phosphoric acid to this solution. The absorbance of 0.1ml protein solution mixed with 5.0ml protein reagent was measured by a 722 spectrophotometer at 595nm after 2min. The weight of protein was calculated by the corresponding absorbance according to a standard curve.

Liver CAT activity was assayed by ultraviolet spectrophotometer according to Xu et al. (1997). 10μl sample was added to 3.0ml of H_2O_2 phosphate buffer pH 7.0 (0.16ml of 30% H_2O_2 to 100ml of 0.067M phosphate buffer) and the variation of H_2O_2 absorbance in 60 seconds was measured with a 7520 spectrophotometer at 250nm. One unit of enzyme activity was defined as the amount of the enzyme which resolved half of the concentration of H_2O_2 in 100 seconds at 25℃.

SOD activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol using a modification of method of Marklund et al. (1974). Samples were assayed in a solution of 8.7ml of 50mM phosphate buffer pH 8.24 and 0.3ml of 3mM pyrogallol (dissolved in 10mN HCl). The rate of pyrogallol auto-oxidation was measured with a 7520 spectrophotometer at 325nm. One unit of enzyme activity was defined as the amount of the enzyme which gave 50% inhibition of the auto-oxidation rate of 0.1mM pyrogallol in one milliliter solution at 25℃.

RESULTS AND DISCUSSION

Activities of CAT and SOD in goldfish livers during exposure to La^{3+}, Gd^{3+}, Y^{3+} and their EDTA complexes were shown in table 1, table 2, and table 3, respectively. It was observed that there were different effects on these enzyme

Table 1. Activities (Units/mg Protein) of CAT and SOD (Mean±SD) in liver of *Carassius auratus* exposed to La^{3+} and La-EDTA solutions from 0 to 24 day

Day	CAT			SOD		
	Control	La^{3+} (2.0mg/l)	La-EDTA (2.0mg La/l)	Control	La^{3+} (2.0mg/l)	La-EDTA (2.0mg La/l)
0	11.42±0.57	11.40±0.44	11.41±0.47	0.984±0.035	1.002±0.087	0.995±0.022
2	12.43±0.93	12.32±0.95	$8.32±0.66^{b}$	1.484±0.051	$1.233±0.090^{c}$	$1.331±0.092^{a}$
4	13.64±1.15	14.36±0.21	12.52±1.20	1.167±0.080	$0.780±0.055^{d}$	$0.884±0.022^{d}$
6	11.81±0.46	$13.17±0.85^{a}$	11.80±0.92	1.075±0.034	$0.643±0.033^{d}$	$0.699±0.050^{d}$
9	14.83±0.54	$18.44±0.80^{b}$	15.10±0.21	1.089±0.074	$0.796±0.071^{d}$	$0.881±0.061^{c}$
13	10.40±0.83	9.37±0.91	$8.43±0.27^{b}$	0.610±0.020	0.589±0.036	0.596±0.004
18	9.35±0.37	$7.46±0.72^{c}$	$7.45±0.70^{c}$	0.751±0.070	$0.868±0.033^{a}$	0.874±0.078
24	—	—	—	—	—	—

Note. Results are expressed as means±SD of four duplicates. Values with a superscript are significant different from the control. a, P=0.1; b, P=0.05; c, P=0.02; d, P=0.01

Table 2. Activities (Units/mg Protein) of CAT and SOD (Mean±SD) in liver of *Carassius auratus* exposed to Gd^{3+} and Gd-EDTA solutions from 0 to 24 day

Day	CAT			SOD		
	Control	Gd^{3+} (2.0mg/l)	Gd-EDTA (2.0mgGd/l)	Control	Gd^{3+} (2.0mg/l)	Gd-EDTA (2.0mg Gd/l)
0	9.64±0.22	9.61±0.13	9.65±0.12	0.512±0.022	0.510±0.013	0.513±0.023
2	9.09±0.52	8.69±0.32	$7.22±0.25^{d}$	0.521±0.025	0.472±0.032	0.480±0.024
4	9.09±0.90	9.66±0.76	8.84±0.56	0.637±0.018	$0.563±0.020^{d}$	$0.580±0.022^{b}$
6	8.69±0.54	$10.87±0.83^{c}$	8.56±0.76	0.637±0.031	$0.490±0.015^{d}$	$0.553±0.008^{c}$
9	6.86±0.21	$8.17±0.52^{c}$	$7.81±0.62^{a}$	0.649±0.042	$0.508±0.026^{d}$	$0.583±0.026^{a}$
13	8.25±0.56	$9.08±0.26^{a}$	$9.44±0.58^{a}$	0.389±0.012	$0.362±0.008^{b}$	$0.423±0.010^{c}$
18	12.66±1.12	11.53±0.92	13.29±1.20	0.345±0.011	$0.403±0.009^{d}$	$0.422±0.009^{d}$
24	10.53±0.55	9.46±0.50	10.02±0.54	0.389±0.016	$0.461±0.022^{c}$	$0.506±0.023^{d}$

Note. Results are expressed as means±SD of four duplicates. Values with a superscript are significant different from the control. a, P=0.1; b, P=0.05; c, P=0.02; d, P=0.01

Table 3. Activities (Units/mg Protein) of CAT and SOD (Mean±SD) in liver of *Carassius auratus* exposed to Y^{3+} and Y-EDTA solutions from 0 to 24 day

Day	CAT			SOD		
	Control	Y^{3+} (2.0mg/l)	Y-EDTA (2.0mg Y/l)	Control	Y^{3+} (2.0mg/l)	Y-EDTA (2.0mg Y/l)
0	5.87±0.13	5.86±0.10	5.88±0.14	0.320±0.005	0.322±0.003	0.324±0.007
2	6.78±0.51	$4.55±0.36^{d}$	$5.88±0.28^{a}$	0.327±0.016	0.302±0.025	$0.419±0.037^{c}$
4	6.05±0.48	5.62±0.50	6.51±0.55	0.265±0.018	$0.234±0.006^{b}$	$0.218±0.005^{c}$
6	4.62±0.11	4.43±0.33	5.16±0.49	0.400±0.025	$0.320±0.011^{d}$	$0.275±0.002^{d}$
9	5.69±0.41	5.88±0.02	$7.06±0.36^{c}$	0.373±0.020	$0.438±0.008^{d}$	0.395±0.015
13	6.50±0.22	6.40±0.25	7.11±0.25	0.313±0.010	$0.374±0.010^{d}$	$0.375±0.027^{b}$
18	7.51±0.46	7.05±0.05	7.61±0.52	0.310±0.009	$0.387±0.031^{c}$	$0.419±0.040^{d}$
24	6.76±0.56	6.29±0.37	6.74±0.47	0.307±0.002	$0.412±0.006^{d}$	$0.420±0.003^{d}$

Note. Results are expressed as means±SD of four duplicates. Values with a superscript are significant different from the control. a, P=0.1; b, P=0.05; c, P=0.02; d, P=0.01

activities between RE metal ions and their EDTA complexes. CAT activities of

goldfish exposed to La-EDTA (Table 1) and Gd-EDTA (Table 2) for two days significantly decreased (tests for statistical significance, P=0.05 and P=0.01, respectively), while those exposed to La^{3+} and Gd^{3+} were not different from the control. It was also the same that SOD activities of goldfish exposed to Y-EDTA for four days were lower than those exposed to Y^{3+} (P=0.02 and P=0.05 respectively). Such observations may result from the contribution of organic complexes in enhancing bioaccumulation and toxicity of metals (George and Coombs 1977). In the present study adding EDTA may enhance the decrease of CAT activities of goldfish at exposure to La^{3+} and Gd^{3+}, and the decrease of SOD activities of goldfish at exposure to Y^{3+}. Similarly, Winner and Gauss (1986) noted that in the presence of humic acid, the bioavailability of trace metals was poorly correlated to the bioaccumulation and toxicity in Daphnia. Borgmann and Charlton (1984) reported that Cu complexed with organic matters existing in natural water tended to be more toxic than that of Cu^{2+}.

On the contrary, Several reports have also indicated the ability of organic ligands in reducing the toxicity of trace metal through complex, even that the metal-induced inhibition of yeast hexokinase activity could reverse by EDTA (Neet et al. 1982). In the present study it was shown that the decrease of CAT activities in the second day were alleviated when exposing to Y-EDTA (Table 3, P=0.1). SOD activities of goldfish exposed to La-EDTA and Gd-EDTA for nine days (P=0.02) were higher than La^{3+} and Gd^{3+} (P=0.01), respectively (Table 1, Table 2). These results provided an evidence for the role of EDTA in the detoxification of rare earth metal ions. The present study showed that EDTA complexes may either enhanced or reduced toxicity of different REEs on CAT and SOD activities, which may be due to the different complexation ability of RE metal ions and await further study. Hence, synthetic chemicals like EDTA, DTPA, NTA, various amino acids (Poldoski 1979; Blust et al. 1986), naturally occurring organic matters like humic acids (HA) (Paulaauskis and Winner 1988) have been used in toxicological assessments of metals.

The activation rates of CAT and SOD in goldfish livers calculated by dividing the increase of enzyme activities by the control at exposure to RE metal ions and RE-EDTA were shown in table 5 and table 6, respectively. It was observed that activation rates of each antioxidant enzyme presented a similar time course of changes neither dependent on the species of RE nor on different REEs. CAT activities of goldfish with intoxication were decreased against those of the control following two-day exposure to all RE solutions, such as the activation rates of CAT of goldfish exposed to Gd^{3+} and Gd-EDTA were –4.40% and -20.6% (P=0.01), respectively. Then CAT activities increased and reached the highest values in the ninth day, for example, the activation rates of CAT of goldfish exposed to Gd^{3+} and Gd-EDTA arrived at 19.1% (P=0.02) and 13.9% (P=0.1), respectively. After nine-day exposure, CAT activities in goldfish livers decreased again and finally were inhibited. Such transient induce of CAT was similarly observed in bivalves exposed to paraquat (Wenning and DiGiulio 1988), menadione and bezo(α)pyrene (Livingstone et al. 1990), and the WAF of crude

Table 5. Activation rates of CAT and SOD (%) in liver of *Carassius auratus* exposed to rare earth metal ions from 0 to 24 day

	CAT			SOD		
Day	La^{3+} (2.0mg/l)	Gd^{3+} (2.0mg/l)	Y^{3+} (2.0mg/l)	La^{3+} (2.0mg/l)	Gd^{3+} (2.0mg/l)	Y^{3+} (2.0mg/l)
0	0	0	0	0	0	0
2	-0.860	-4.40	-32.9	-16.9	-9.40	-7.65
4	5.28	6.27	-7.11	-33.2	-11.6	-11.7
6	11.5	25.1	-4.11	-40.2	-23.1	-20.0
9	24.3	19.1	3.34	-26.9	-21.7	17.4
13	-9.90	10.1	-1.54	-3.44	-6.94	19.5
18	-20.1	-8.93	-6.13	15.58	16.8	24.8
24	—	-10.2	-6.95	—	18.5	34.2

Table 6. Activation rates of CAT and SOD (%) in liver of *Carassius auratus* exposed to RE-EDTA complexes from 0 to 24 day

	CAT			SOD		
Day	La-EDTA (2.0mg La/l)	Gd-EDTA (2.0mg Gd/l)	Y-EDTA (2.0mg Y/l)	La-EDTA (2.0mg La/l)	Gd-EDTA (2.0mg Gd/l)	Y-EDTA (2.0mg Y/l)
0	0	0	0	0	0	0
2	-33.1	-20.6	-13.3	-10.3	-7.87	-9.79
4	-8.21	-2.75	7.60	-24.3	-8.95	-17.7
6	0.0800	-1.50	11.7	-34.9	-13.2	-31.3
9	1.82	13.9	24.1	-19.1	-10.2	5.90
13	-18.9	14.4	9.38	-2.30	8.74	19.8
18	-20.3	4.98	1.33	16.4	22.6	35.2
24	—	-4.88	-0.300	—	30.1	36.8

and lubricant oils (Cajaraville et al. 1992). CAT activity was also increased transiently in livers of eels *Anguilla anguilla* exposed to the pesticide dinitro-*o*-cresol (Braunbeck and Völkl 1991). In addition, it was reported that CAT activity finally decreased in mussels exposed to dioctyl phthalate, clofibrate and bezo(α)pyrene (Cancio et al. 1998). The role of CAT in antioxidant defense was to resolve and eliminate H_2O_2 produced by organelles (DiGiulio et al. 1989).

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

After entering the fish liver, two species of rare earth underwent metabolism in cells to release ROIs, which contributed to CAT activation. The possible mechanism referred to transient induce of CAT was that, at long exposure times, the direct toxic action of RE on certain protein synthesis would impede a prolonged induction of CAT activity.

SOD activities in goldfish livers at exposure to RE solutions were decreased gradually from the beginning day and reached the lowest values in the sixth day, such as the activation rates of SOD of goldfish exposed to Y^{3+} and Y-EDTA reached –20.0% and –31.3% (P=0.01), respectively. Following the inhibition, SOD activities increased gradually and eventually were remarkably activated, for

instance, the activation rates of SOD of goldfish exposed to Y^{3+} and Y-EDTA were 34.2% and 36.8% (P=0.01), respectively. SOD in fish liver was one of metal enzymes possessing Cu/Zn or Mn, whose important function in antioxidant defense was to catalyze the disproportionation of $O_2^{\bullet -}$ (DiGiulio et al. 1989).

$$2\,O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Because La^{3+}, Gd^{3+} and Y^{3+} were all cations and not the similarities of substrate (as the form of anion), it was not likely for them to lie in the site of Cu/Zn which should be occupied by substrate (Wang et al. 1998). In addition, the effects of both RE metal ions and RE-EDTA on SOD were analogous, therefore, the inhibition of RE on this enzyme may not due to the replacement of metal ions in enzyme molecular, but due to the alteration of distribution or shape of electric-charges in enzyme molecular. After a long-term exposure, ROIs were released through the metabolism of RE in liver cells and thereby induced the activity of SOD. The utility of oxidative-stress associated phenomena still remains speculative, but available information motivates continued research (DiGiulio et al. 1993).

As shown in table 5, it was observed that the activation rates of CAT of goldfish exposed to Y^{3+} were near the zero, however, CAT activities of goldfish exposed to La^{3+} and Gd^{3+} were apparently activated during the experiments. In addition, both the inhibition level and time of Y^{3+} on SOD activities were less than La^{3+} and Gd^{3+}. It may be concluded that the toxicological effects of light REEs (La as representative) on antioxidant enzymes of goldfish liver were close to those of medium REEs (Gd as representative), but different from those of heavy REEs (Y as representative), which may be related to their different ion radius. George et al. (1971) indicated that the inhibition effects of RE ions on trypsase were characterized by $Lu^{3+} >> Er^{3+} \geq Sm^{3+} > La^{3+}$, i.e. inhibition abilities increased with the reduction of the radius of RE ions.

In general, the difference of effects between RE metal ions and RE-EDTA on antioxidant enzymes was significant and the effects on CAT and SOD were also different from each other. Therefore, the effects of REEs on antioxidant enzymes of fish liver depended not only on the different REEs and the species of RE, but also on the types of enzymes. In addition, a transient induce of antioxidant enzyme CAT and an eventually significant induce of SOD were observed in goldfish liver at exposure to RE solutions. It appeared that the assessment of CAT and SOD activities was suitable for the detection of pollutant-induced alterations. Therefore, we have demonstrated the potential use of antioxidant enzymes as early biomarker of REEs in aquatic environment; further study was necessary to assess ecological risk in agricultural application of REEs.

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