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Effect of Cadmium Ion on Peroxidase Activity of Serum in Cow: An *in vitro* Study

[1]H. Tayefi-Nasrabadi, [2]Gh. Moghaddam, [3]Z. Rajabi and [4]Gh. Dehghan
[1]Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[2]Department of Animal Sciences, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
[3]Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[4]Department of Plant Sciences, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran

Abstract: For clarifying some possible mechanism of cadmium toxicity, the effect of increasing amounts of Cd^{2+} ion on peroxidase activity was investigated *in vitro* in serum of cow. The H_2O_2-mediated oxidation of o-dianisidine was used to assess the peroxidase activity. Results show that after preincubation of serum with 0.25-100 mM Cd^{2+} concentration for 5 min, peroxidase activity was inhibited compared to the control and decreased rapidly with increasing metal concentrations. The enzyme was completely inhibited after 5 min preincubation in 100 mM Cd^{2+}. When the preincubation of serum and Cd^{2+} was prolonged to 0.5, 1 and 24 h, the enzymatic activity decreased more rapidly with increasing metal concentration and the enzyme was completely inhibited at lower metal concentrations (at 50 mM Cd^{2+} after 30 min preincubation, at 30 mM Cd^{2+} after 60 min preincubation and at 5 mM Cd^{2+} after 24 h). By considering of long biological half-life of cadmium in body of animals, it suggested that the damage caused by exposure to heavy metals is often not only dose-dependent, but also time-dependent. Even though detoxifying enzymes may not show any effect after brief exposure to low concentrations of heavy metals, prolonged incubation will affect the enzymatic activity, leading eventually to complete inactivation.

Key words: Cadmium, time dependent, peroxidase activity, serum, cow

INTRODUCTION

Cadmium is a widely distributed metallic pollutant of our environment, which can be absorbed into biological systems through direct uptake as well as by accumulation in food chains. It has toxic effects on all living systems, whether human, animal, plant or bacterium (Liu *et al.*, 2008; Martelli *et al.*, 2006; Wierzbicka *et al.*, 2007; Loka Bharathi *et al.*, 1990). Cadmium impairs the function of essential elements, such as zinc, in some key enzymatic systems. Furthermore, it can cause several pathological processes as cancer, renal dysfunction, anemia, testicular necrosis, sterility, arteriosclerosis, central nervous system damage, skeletal deformation, DNA strand breakage *in vivo* and *in vitro* and it is responsible for a decrease in the biomass production in plants and inhibition of growth in human and animals (Ramesh, 2007; Ouariti *et al.*, 1997; Saplakoglu and Iscan, 1997; Coogan *et al.*, 1992). Numerous studies have led to the conclusion that elevated Cd^{2+} levels promote and/or exacerbate oxidative cell damage by an enhanced production of highly toxic oxygen species (Lopez *et al.*, 2006; Wang *et al.*, 2004; Xie *et al.*, 2007) and it has been shown that antioxidant defense enzymes play a key role in the protection against heavy metal toxicity (Pillai and Gupta, 2005). Peroxidases are important detoxifying enzymes serving to rid cells of excess H_2O_2 under normal and stress conditions, including contamination by toxic levels of heavy metals (Richards *et al.*, 1998; Fieldes *et al.*, 1998). However, extreme stress conditions may affect the activity of the detoxification enzymes themselves. Thus, it is important to understand the behavior of those enzymes in the presence of highly toxic metals. For clarifying some possible mechanism of cadmium toxicity in animals, the effect of increasing amounts of Cd^{2+} ion on peroxidase activity was investigated *in vitro* in serum of cow at this study.

MATERIALS AND METHODS

Chemicals: O-dianisidine dihydrochloride was obtained from Sigma Chemical Co. Hydrogen peroxide (30% solution); $CdSO_4$ and all the other chemicals used in this reseach were obtained from Merck and were of reagent grade.

Corresponding Author: H. Tayefi-Nasrabadi, Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

Sampling: The blood samples were collected from cows by jugular venepuncture. After 30 min blood was centrifuged at 2000×g for 10 min at 4°C to separate serum. Serum samples of all subjects were kept at -20°C until analysis (not later than 2 weeks).

Serum peroxidase activity assay: Serum peroxidase activity was measured by following the H_2O_2-dependent oxidation of o-dianisidine at 460 nm, using an extinction coefficient of 11.3 $mM^{-1}.cm^{-1}$ (Worthington, 1988). O-dianisidine stock solutions (2.5 mg mL^{-1}) were prepared by dissolving o-dianisidine in distilled water. H_2O_2 stock solutions (3 mM) were prepared daily by appropriate dilution of 30% H_2O_2 in distilled water. Stock solutions of $CdSO_4$ (1 M) were prepared in distilled water. The assay was performed in 0.1 M citrate buffer, pH 5.0. For assay done in the presence of Cd^{2+} ions, appropriate amounts of $CdSO_4$ stock solution were mixed with 0.1 M citrate buffer; the final volume was always 3 mL and the concentration of Cd^{2+} ions varied from 0.25-100 mM. The pH was readjusted to 5.0 whenever required. Serum (50 μL) was added and incubated with the Cd^{2+} ions for 5, 30, 60 min and 24 h before addition of 100 μL o-dianisidine. The reaction was then started by adding 100 μL H_2O_2 as usual and absorbance was record after an incubation period of 15 min. In order to prepare of Horseradish Peroxidase (HRP) standard curve, the same principle as described for the serum peroxidase activity was done with one exception: Instead of serum, 50 μL HRP (110, 135, 780 and 1800 mU mL^{-1} HRP) was added to the reaction mixture. Serum peroxidase activity was calculated from absorbance readings relating to the HRP standard curve. Results were expressed as milliunits per milliliter. All assays were carried out at room temperature (~22-25°C) using an UNICO 2100 PC spectrophotometer. Results were average of at least three separate experiments.

RESULTS AND DISCUSSION

Standards peroxidase kinetic follows a linear order as shown in Fig. 1 for peroxidase activity.

The effect of Cd^{2+} on the oxidation of o-dianisidine by cow serum peroxidase in the presence of H_2O_2 was determined by following the formation of oxidized o-dianisidine at 460 nm, under end point conditions and after 5-, 30-, 60 min and 24 h preincubation of the serum with the metal ion. Incubation of serum with Cd^{2+} (0.25-100 mM) resulted in inhibition of the serum peroxidase activity. The amount of inhibition depended on the length of incubation of the serum with the metal ion and on Cd^{2+} concentration (Table 1). As shown in Table 1, incubation for 5 min at room temperature of serum with up to 0.5 mM Cd^{2+} not have any effect on the peroxidase activity. In the presence of 1-50 mM Cd^{2+}, serum peroxidase activity was inhibited compared to the control and decreased rapidly with increasing metal concentrations. For example, at 1, 25 and 50 mM Cd^{2+}, serum peroxidase activity decreased from 1713±5 mU mL^{-1} for the control to 1557±3, 654±7 and 222±2 mU mL^{-1}, respectively. The enzyme was completely inhibited after 5 min preincubation in 100 mM Cd^{2+}. When the preincubation of serum and Cd^{2+} was prolonged to 30-60 min and 24 h, serum peroxidase activity decreased more rapidly with increasing metal concentration and the enzyme was completely inhibited at lower metal concentrations (at 100 mM Cd^{2+} after 5 min preincubation, at 50 mM Cd^{2+} after 30 min preincubation, at 30 mM Cd^{2+} after 60 min preincubation and at 5 mM Cd^{2+} after 24 h preincubation). Interestingly, the same value of peroxidase activity was found for various combinations of Cd^{2+} concentration and incubation time. For example,

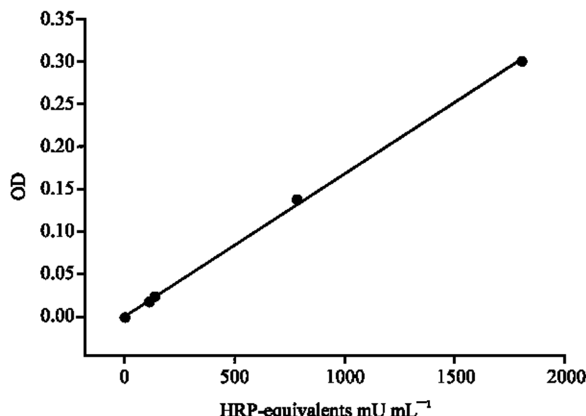

Fig. 1: Standardization of peroxidase activity assay

Table 1: Values for cow serum peroxidase activity after 5, 30, 60 min and 24 h preincubation with various Cd^{2+} concentrations

	Serum peroxidase activity (mU mL^{-1}) after			
$[Cd^{2+}]$ (mM)	5 min preincubation	30 min preincubation	60 min preincubation	24 h preincubation
0	1713±5	1713±5	1713±5	1200±8
0.25	1713±5	1713±5	1678±2	804±6
0.5	1713±5	1678±4	1593±7	648±5
1	1557±3	1490±4	1370±8	444±3
5	1356±4	1233±6	1199±7	Complete inhibition
10	1173±5	1027±3	976±8	
25	654±7	565±4	462±2	
30	(*)n.d.	(*)n.d.	Complete inhibition	
50	222±2	Complete inhibition		
100	Complete inhibition			

(*)n.d.: not determined

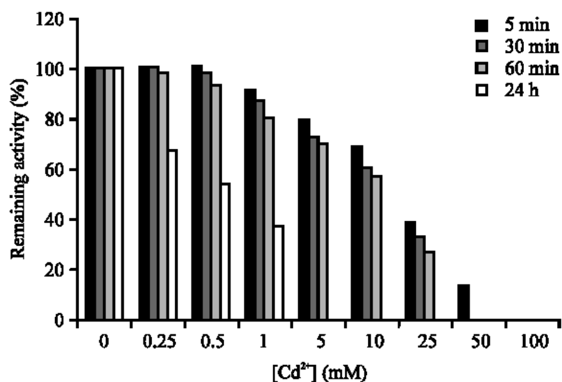

Fig. 2: Remaining cow serum peroxidase activity as a function of $CdSO_4$ concentration.

serum peroxidase activity dropped from 1713±5 for the control to about 1370±8 either after a 60 min preincubation in 1 mM Cd^{2+}or after a 5 min preincubation in 5 mM Cd^{2+}. Another example shows the value of peroxidase activity dropping to about 648±5 either after 24 h preincubation in 0.5 mM Cd^{2+} or 5 min preincubation in 25 mM Cd^{2+}. Thus, even though serum peroxidase appeared to have a rather low affinity for Cd^{2+}, extended exposure of the enzyme to lower concentrations of the metal brought about the same effect as shorter exposure to higher metal concentrations. We also obtained previously the same results for the effect of nickel ion on HRP enzymatic activity. Like Cd^{2+}, Ni^{2+} would inhibit peroxidase activity in a time and concentration dependent manner (Tayefi-Nasrabadi *et al*., 2006; Keyhani *et al*., 2005) and extended exposure of the enzyme to lower concentrations of the nickel brought about the same effect as shorter exposure to higher nickel concentrations.

Results presented in Fig. 2 show the remaining activity of cow serum peroxidase as a function of Cd^{2+} concentration after 5-, 30-, 60 min and 24 h preincubation of the enzyme with the metal. Interestingly for each preincubation time, the decrease in peroxidase activity followed a different pattern.

Serum and $CdSO_4$ were preincubated for 5, 30, 60 min and 24 h prior to addition of o-dianisidine and H_2O_2. The activity was measured without Cd^{2+} ion was taken as 100 %. After 5, 30, 60 min and 24 h preincubation, peroxidase activity was completely inhibited at 100, 50, 30 and 5 mM Cd^{2+} concentration, respectively. Each experiment was performed at least three times and data are expressed as average.

Incubation in 1 mM Cd^{2+} reduced the peroxidase activity by 9% after 5 min preincubation, by 13% after 30 min preincubation, by 20% after 60 min preincubation and by 63% after 24 h preincubation. However, incubation in 5 mM Cd^{2+} reduced the peroxidase activity by 21% after 5 min preincubation, by 28% after 30 min preincubation, by 30% after 60 min preincubation and by 100% after 24 h preincubation. Incubation in 25 mM Cd^{2+} reduced the peroxidase activity by 62% after 5 min preincubation, by 67% after 30 min preincubation, by 73% after 60 min preincubation and by 100% after 24 h preincubation. By considering of long biological half-life of cadmium in body of animals (Ramesh, 2007), it suggested that the damage caused by exposure to heavy metals is often not only dose-dependent, but also time-dependent. Even though detoxifying enzymes such as peroxidases may not show any effect after brief exposure to low concentrations of heavy metals, prolonged incubation will affect the enzymatic activity, leading eventually to complete inactivation.

Previous studies have led to the conclusion that elevated Cd^{2+} levels promote and/or exacerbate oxidative cell damage by an enhanced production of highly toxic oxygen species (Lopez *et al*., 2006; Wang *et al*., 2004; Xie *et al*., 2007). Our results demonstrate that time- and concentration dependent action mechanism of cadmium on inhibition of animals antioxidant defense enzymes such as peroxidases could be considered as important parameter responsible for enhanced production of highly toxic oxygen species and causing of several pathological processes as cancer, renal dysfunction, anemia, testicular necrosis, sterility, arteriosclerosis, central nervous system damage and DNA strand breakage in animals.

CONCLUSION

Our result show that even though cow serum peroxidase appeared to have a rather low affinity for Cd^{2+}, extended exposure of the enzyme to lower concentrations of the metal brought about the same effect as shorter exposure to higher metal concentrations. By considering of long biological half-life of cadmium in body of animals, it suggested that the damage caused by exposure to cadmium is often not only dose-dependent, but also time-dependent. Even though detoxifying enzymes such as serum peroxidases may not show any effect after brief exposure to low concentrations of heavy metals, prolonged incubation will affect the enzymatic activity, leading eventually to complete inactivation.

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