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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (22): 2589-2593, 2008
ISSN 1028-8880

Effect of Zinc Ion on Peroxidase Activity of Serum in Cow

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Abstract: In this study, for clarifying some possible mechanism of zinc toxicity, the effect of increasing amounts of Zn^{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 0.2-20 mM Zn^{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 30 mM Zn^{2+}. When the preincubation of serum and Zn^{2+} was prolonged to 30 and 60 min, the enzymatic activity decreased more rapidly with increasing metal concentration. Extended exposure of the enzyme to lower concentrations of the metal brought about the same effect as shorter exposure to higher metal concentrations.

Key words: Zinc toxicity, time dependent, enzyme inhibition

INTRODUCTION

Zinc is an essential trace element necessary for sustaining all life. It is required for the action of more than 200 metallo enzymes such as; amino peptidases, alkaline phosphatase, carboxypeptidases, superoxide dismutase, thymidine kinase, carbonic anhydrase and several dehydrogenases (Sousa *et al*., 2007). Zinc plays an important role in polymeric organization of macromolecules like DNA and RNA, protein synthesis, cell division and metabolism (Smith and Akinbamizo, 2000). In addition, Zinc has been reported to influence the process of spermatogenesis (Ebisch *et al*., 2003; Wong *et al*., 2002), controls sperm motility (Wroblewski *et al*., 2003), stabilizes sperm membrane (Kendall *et al*., 2000), preserves the ability of sperm nuclear chromatin to undergo decondensation and modulates sperm functions (Suruki *et al*., 1995). It also required in plants for leaf formation, the synthesis of indole acetic acid (auxin) and anaerobic respiration and is now considered to be neurotransmitters (Hershfinkel *et al*., 2007; Gadallah, 2000).

Even though zinc is an essential element but it is usually identified as toxic, or even highly toxic, at elevated level (Gazaryan *et al*., 2007; Dineley *et al*., 2003; Christopher, 2004; Religa *et al*., 2006). Several episodes of zinc toxicity in cattle and sheep have been described. Sources of zinc included the accidental addition of zinc oxide to cattle feed, galvanized wire and troughs, heavy use of zinc-containing fertilizers and fungicides, milk replacer containing large amounts of a zinc supplemented home-made molybdenum licks (Sharpe and Livesey, 2005). Excess zinc has been implicated in the formation of osteochondrosis lesions in horses (Jeffcott, 1991), pancreatic injury and lesion in the proventriculus in birds (Samour, 2000), central lobular necrosis of the liver in pigs (Plumlee, 2004), hemolysis and hepatocellular necrosis in canine and feline (Bexfield *et al*., 2007), hepatic degeneration and lesions in the pancreas in bovine (Radostits *et al*., 2000). Earlier studies have led to the conclusion that elevated Zn^{2+} levels promote and/or exacerbate oxidative cell damage by an enhanced production of highly toxic oxygen species (Bishop *et al*., 2007) and it has been shown that antioxidant defense enzymes play a key role in the protection against metal and 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 metals (Richards *et al*., 1998; Fieldes and Gerhardt, 1998). However, extreme stress conditions may affect the activity of the detoxification enzymes themselves. For clarifying some possible mechanism of zinc toxicity in animals, the effect of increasing amounts of Zn^{2+} ion on peroxidase activity was investigated *in vitro* in serum of cow at this study.

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MATERIALS AND METHODS

This study was performed during March 2007 to June 2008 in Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.

Chemicals: O-dianisidine dihydrochloride was obtained from Sigma Chemical Co. (Deisenhofen, Germany) Hydrogen peroxide (30% solution); $ZnSO_4$ and all the other chemicals used in this study were obtained from Merck (Darmstadt, Germany) and were of reagent grade.

Sampling: The blood samples were collected from cows by jugular venepuncture. After 30 min blood was centrifuged at 2000 x 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/cm (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 $ZnSO_4$ (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 Zn^{2+} ions, appropriate amounts of $ZnSO_4$ stock solution were mixed with 0.1 M citrate buffer; the final volume was always 3 mL and the concentration of Zn^{2+} ions varied from 0.2 to 30 mM. The pH was readjusted to 5.0 whenever required. Serum (50 μL) was added and incubated with the Zn^{2+} ions for 5, 30 and 60 min 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

Figure 1 shows standard curve of HRP activity. Standards peroxidase kinetic follows a linear order for peroxidase activity.

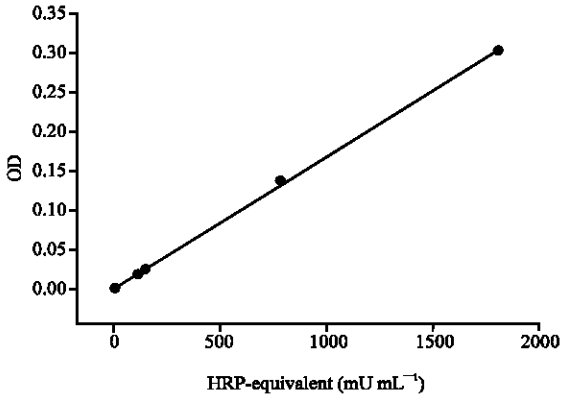

Fig. 1: Standardization of peroxidase activity assay

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

	Serum peroxidase activity (mU mL^{-1}) after		
$[Zn^{2+}]$ (mM) concentration	5 min preincubation	30 min preincubation	60 min preincubation
0.0	1713±5	1713±5	1713±5
0.2	1713±7	1713±8	1610±6
0.4	1713±4	1695±8	1575±3
1	1627±4	1524±3	1490±5
2	1490±7	1404±7	1353±5
5	1387±6	1284±3	1216±4
10	1079±5	959±7	736±3
20	479±4	411±5	325±3
30	Complete inhibition		

The results are expressed as Mean±SD

The effect of Zn^{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 and 60 min preincubation of the serum with the metal ion. Incubation of serum with Zn^{2+} (0.2-20 mM) resulted in inhibition of the serum peroxidase activity. As shown in Table 1, the amount of inhibition of peroxidase activity depended on the length of incubation of the serum with the metal ion and on Zn^{2+} concentration. Incubation for 5 min at room temperature of serum with up to 0.4 mM Zn^{2+} not have any effect on the peroxidase activity. In the presence of 1-20 mM Zn^{2+}, serum peroxidase activity was inhibited compared to the control and decreased rapidly with increasing metal concentrations. For example, at 1, 10 and 20 mM Zn^{2+}, serum peroxidase activity decreased from 1713±5 mU mL^{-1} for the control to 1627±4, 1079±5 and 479±4 mU mL^{-1}, respectively. The enzyme was completely inhibited after 5 min preincubation in 30 mM Zn^{2+}. When the time of preincubation of serum and Zn^{2+} was increased to 30, 60 min, serum peroxidase activity decreased more rapidly with increasing metal concentration and the

enzyme was inhibited at lower metal concentrations. For example, at 0.4 mM Zn^{2+} after 5 min preincubation serum peroxidase activity was 1713±4 mU mL^{-1}, but after 30 and 60 min preincubation serum peroxidase activity reduced to 1695±8 and 1573±3 mU mL^{-1}, respectively. At 10 mM Zn^{2+} after 5, 30 and 60 min preincubation serum peroxidase activity was reduced from 1079±3 to 959±7 and 736±3 mU mL^{-1}, respectively. On the other hand, the same value of peroxidase activity was found for various combinations of Zn^{2+} concentration and incubation time. For example, serum peroxidase activity dropped from 1713±5 mU mL^{-1} for the control to about 1610±6 mU mL^{-1} either after a 60 min preincubation in 0.2 mM Zn^{2+} or after a 5 min preincubation in 1 mM Zn^{2+}. Another example shows the value of peroxidase activity dropping to about 1490±5 mU mL^{-1} either after 60 min preincubation in 1 mM Zn^{2+} or 5 min preincubation in 2 mM Zn^{2+}. Thus, even though serum peroxidase appeared to have a rather low affinity for Zn^{2+}, extended exposure of the enzyme to lower concentrations of the metal brought about the same effect as shorter exposure to higher metal concentrations.

Earlier studies showed the same results for the effect of cadmium and nickel ions on serum peroxidase and HRP enzymatic activity, respectively. Like Zn^{2+}, Cd^{2+} and Ni^{2+} would inhibit peroxidase activity in a time and concentration dependent manner (Tayefi-Nasrabadi *et al*., 2006, 2008; Keyhani *et al*., 2005) and extended exposure of the enzyme to lower concentrations of the metals brought about the same effect as shorter exposure to higher metals concentrations. The enzymatic activity remained active over a broader Cd^{2+} concentration range compared to Zn^{2+}. These results suggest that probably two distinctive binding sites exist for Zn^{2+} and Cd^{2+} on serum peroxidase.

Figure 2 shows the remaining activity of cow serum peroxidase as a function of Zn^{2+} concentration after 5, 30 and 60 min preincubation of the enzyme with the metal. As shown in Fig. 2 for each preincubation time, the decrease in peroxidase activity followed a different pattern. Incubation in 1 mM Zn^{2+} reduced the peroxidase activity by 5% after 5 min preincubation, by 11% after 30 min preincubation and by 13% after 60 min preincubation. However, incubation in 10 mM Zn^{2+} reduced the peroxidase activity by 37% after 5 min preincubation, by 44% after 30 min preincubation and by 57% after 60 min preincubation. Incubation in 20 mM Zn^{2+} reduced the peroxidase activity by 72% after 5 min preincubation, by 76% after 30 min preincubation and by 81% after 60 min preincubation. By considering of long biological half-life of metal in body of animals (Gupta, 2007), it suggested that the damage caused by exposure to excess zinc ion concentration 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 zinc ion, prolonged incubation will affect the enzymatic activity, leading eventually to complete inactivation.

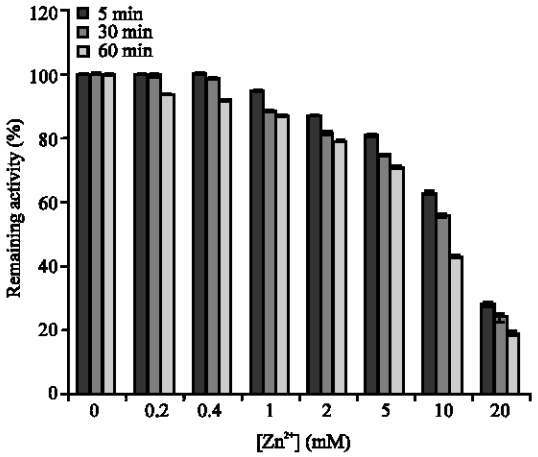

Fig. 2: Remaining cow serum peroxidase activity as a function of $ZnSO_4$ concentration. Serum and $ZnSO_4$ were preincubated for 5, 30 and 60 min prior to addition of o-dianisidine and H_2O_2. The activity was measured without Zn^{2+} was taken as 100%. Each experiment was performed at least three times and data are expressed as average±SD

As mentioned earlier, excess zinc has been implicated in the formation of various injury and lesions in animals. The mechanism by which zinc toxicosis cause these lesions and injury has not been well understood. Some possible explanations include; inhibition of specific red blood cell enzyme, direct damage to the erythrocytic membrane, mitochondrial dysfunction, susceptibility of erythrocyte to oxidative damage, loss of mitochondrial defenses and interaction between zinc, iron, copper and calcium (Gazaryan *et al*., 2007; Plumlee, 2004).

Earlier studies have led to the conclusion that elevated Zn^{2+} levels promote and/or exacerbate oxidative cell damage by an enhanced production of highly toxic oxygen species (Bishop *et al*., 2007; Pong *et al*., 2002). The results presented here indicated that zinc ion at elevated level inhibit serum peroxidase activity by time and concentration dependent manner. Inhibition of peroxidases due to zinc ion could be considered as a reason for enhanced production of H_2O_2 as a highly toxic oxygen species and thereby causing of several pathological processes.

CONCLUSION

In conclusion, present results show that the damage caused by exposure to excess of zinc ion 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 zinc ion, extended exposure of the enzyme to lower concentrations of the metal brought about the same effect as shorter exposure to higher metal concentrations. Inhibition of animal's antioxidant defense enzymes such as peroxidases by elevated level of zinc ion could be considered as an important parameter responsible for enhanced production of highly toxic oxygen species such as H_2O_2 and causing of several pathological processes in animals.

ACKNOWLEDGMENT

This study was supported by Research Council of University of Tabriz (Project No. 27/49297).

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