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EFFECT OF ZINC ON LIPID PEROXIDATION AND ANTIOXIDANT ENZYMES IN HEPATIC AND BRAIN TISSUES OF CHICK EMBRYOS

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Chick embryos were treated with different concentrations (25 and 75 μmoles/kg egg wt.) of zinc on the 14th day of embryonic development. The levels of thiobarbuturic acid reacting substances (TBARS), glutathione (GSH) and activity levels of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase were measured in both hepatic and brain tissues after different time intervals (24 h, 72 h and 120 h) of zinc exposure. Increased levels of TBARS were observed after 24 h of zinc treatment and thereafter (72 h and 120 h) the levels were decreased in both the tissues. Significant induction was observed in antioxidant enzyme activities in both the tissues after 24 h and 72 h when compared to 120 h. However, the GSH levels were increased at 24 h and 72 h and thereafter decreased in both the tissues at 120 h. The elevated levels of antioxidant enzymes at 24 h and 72 h may be responsible for the reduction of TBARS at 72 h and 120 h in developing chick embryos.

Keywords: Zinc; TBARS; Antioxidant enzymes; Liver; Brain; Chick embryo

INTRODUCTION

Zinc an essential trace element is known to be intimately involved in various biological functions ranging from collagen metabolism and bone formation, the complement system and macrophage functions.[1] Zinc and cadmium are major environmental contaminants of air, water and soil especially in the areas of heavy automobile traffic, near smelters or in areas where oil is burned for heating purposes.[2] The homeostatic mechanism evidently fails

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at higher levels of zinc intake leading to zinc intoxication.[3] In humans zinc toxicity causes dehydration, electrolyte imbalance, stomach pain, nausea, lethargy and muscular incoordination.[4]

Free radicals will be generated during enzymatic and non-enzymatic lipid peroxidation in biological systems. However uncontrolled lipid peroxidation is very much detrimental to the cell. Hence the reduction of lipid peroxides has been claimed to play a key role in the control of lipid peroxidation in living systems.[5] Lipid peroxidation is a causative reaction in cellular deterioration in the aging process. Lipid peroxidation also plays a key role in cell damage by environmental pollutants and in a variety of pathological conditions.[6] A possible role of lipid peroxidation has been reported in various biological systems exposed to heavy metals such as lead,[7] mercury, cadmium[8] and selenium.[9] Chvapil *et al.*[10] reported that zinc functions as a stabilizer of biomembranes and biostructures. Hence in the present study the effect of lethal doses of zinc on lipid peroxidation system in developing chick embryos was investigated.

MATERIALS AND METHODS

Freshly laid Bobcock strain zero day old eggs were procured from the Government Veterinary college, Tirupati, Andhra Pradesh. They were placed in an egg incubator, the temperature of which was already maintained at 37°C with 65% relative humidity. The eggs were rotated manually once a day during incubation. Injections of different concentrations (25 and 75 μmoles/kg egg wt.) of zinc ($ZnSO_4 \cdot 7H_2O$) dissolved in 10 μl distilled water were given to 14 day old chick embryos. Control embryos were injected with the same amount of distilled water. Injections were given through a small hole directly into the yolk sac and the hole was covered with surgical tape to prevent infection. A minimum of 25 embryos were used in each group. Embryos were sacrificed after different time intervals of zinc exposure to collect hepatic and brain tissues for the determination of lipid peroxidation and antioxidant enzyme activities.

Chick embryos were dissected and tissues were removed quickly, weighed and chilled in ice cold 0.9% NaCl. The level of lipid peroxidation was measured using thiobarbuturic acid reaction as described by Ohkawa *et al.*[11] Tissues were collected and homogenized in 3 volumes of 70 mM phosphate buffer pH 7.4 containing 0.25 M sucrose, 10 mM EDTA, and 1% Triton X-100. Post-mitochondrial supernatants were prepared by centrifuging the homogenates at 12,000 rpm for 10 min and immediately used for the assay

of antioxidant enzymes. Superoxide dismutase (SOD) activity was measured by the method of Misra and Fridovich.[12] Catalase activity was determined by the method of Beer and Sizer.[13] Glutathione peroxidase (GPx) activity was determined by continuous monitoring of NADPH oxidation in a recycling assay as described by Wendel.[14] Glutathione-S-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate as previously described by Habig *et al.*[15] Glutathione reductase (GR) activity was assayed as described by Carlberg and Mannervik.[16] The glutathione (GSH) levels were measured according to the method of Griffith.[17] The *in vitro* lipid peroxidation was estimated using TBA reaction as described by Gelman and Michaelson.[18] The protein content was determined by the method of Lowry *et al.*[19] using bovine serum albumin as a standard. Statistical evaluation was done by the Student's *t*-test.

RESULTS AND DISCUSSION

In recent years toxicity of zinc has been examined extensively in mice[20] and rabbits,[21] but very little attention was given to studies of its effect on the embryonic system. The induction of membrane peroxidation by producing free radicals is known to be involved in pathogenesis caused by exposure to zinc.[22] Lipid peroxidation was measured as an index of the formation of thiobarbuturic acid reacting substances. The values for hepatic and brain TBARS after zinc treatment are shown in Figure 1. The TBARS in controls decreased with age in both the tissues. Zinc treatment increased the production of TBARS in a dose-dependent manner at 24 h and declined at 72 h and 120 h post-treatment in both tissues. The hepatic TBARS were reduced to 26 and 47 percent compared to 34 and 50 percent in brain tissues at 120 h with 25 and 75 μmoles of zinc treatment respectively. Our results are in agreement with those of Richard *et al.*[23] who also observed diminished TBARS during zinc treatment.

In liver and brain, the SOD activity increased with zinc treatment in a dose-dependent manner at 24 h and 72 h (Table I). In hepatic tissues 3- and 4-fold induction was observed with 75 μmoles of zinc after 24 h and 72 h of treatment respectively. In brain tissues about 2- and 5-fold induction was observed when compared to control values with 25 and 75 μmoles respectively of zinc at 72 h post-treatment. This may be due to an initial burst in antioxidant protective mechanism to cope with an initial flux of free radicals presumably generated by excess of zinc. However at 120 h after

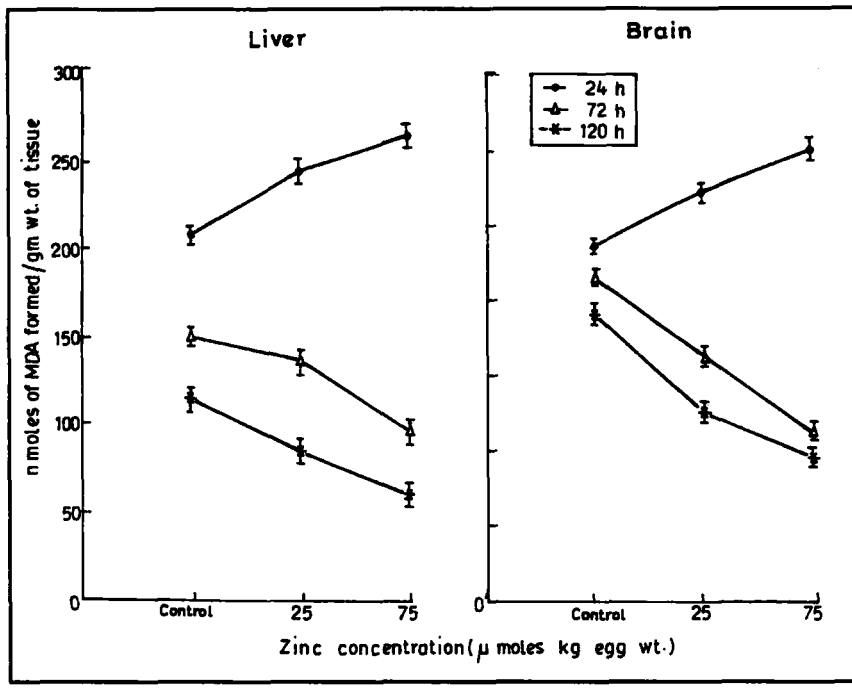


FIGURE 1 *Effect of Zn on lipid peroxides in chick embroys.* Chick embryo liver and brain tissues were removed quickly, weighed and chilled in cold saline. Tissue homogenates were prepared in a ratio of 1 g wet tissue to 9 ml of 1.15% KCl. The assay mixture contain 0.1 ml of 10% tissue homogenate, 0.2 ml of 8.1% SDS and 1.5 ml of 0.8% of TBA. The mixture was made up to 4.0 ml with distilled water and boiled at 95°C for 60 min. After cooling, 1 ml of distilled water and 5 ml of mixture of *n*-butanol and pyridine (15 : 1 v/v) were added and shaken vigorously. Centrifuged at 4000 rpm for 10 min, then the absorbance of the organic layer was read at 532 nm. A standard curve of TMP (1,1,3-tetramethoxypropane) was prepared in a similar condition and used in calculations. Amount of LPO were expressed as n moles MDA/gm wt of tissue.

treatment the rate of induction in SOD activity was decreased but significantly higher than controls. The above results are in agreement with those of Steinebach and Wolterbeek,[24] who also observed significant induction in SOD activity during Zn treatment.

In hepatic and brain tissues catalase activity was increased to 4- and 5-fold with 25 and 75 μmoles of zinc respectively at 24 h post-treatment (Table II). After 72 h of treatment 2.6- and 5.3-fold induction was noticed with 25 and 75 μmoles of zinc respectively. Increased activity of catalase is an indication of the production of H_2O_2 during the metal administration. Catalase

TABLE I Effect of zinc on SOD activity in hepatic and brain tissues of chick embryos

Treatment with zinc (μmoles/kg egg wt.)	*SOD activity* (units/mg protein)		
	24 h	72 h	120 h
Hepatic Tissues			
Control (−Zn)	11.0 ± 0.9	15.0 ± 0.9	7.8 ± 0.5
25	24.0 ± 1.5 *	21.6 ± 1.8 *	8.4 ± 0.6
75	33.0 ± 1.7 *	60.6 ± 5.0 *	11.5 ± 0.9 *
Brain Tissues			
Control (−Zn)	6.6 ± 0.5	4.4 ± 0.4	4.0 ± 0.2
25	10.0 ± 0.9 *	8.4 ± 0.6 *	4.4 ± 0.3 #
75	13.5 ± 1.0 *	20.6 ± 1.8 *	5.0 ± 0.3 *

Values are average of four separate experiments; mean ± S.D., $n = 10$ in each group. * $P < 0.001$; # $P < 0.02$ vs control.

TABLE II Effect of zinc on catalase activity in hepatic and brain tissues of chick embryos

Treatment with zinc (μmoles/kg egg wt.)	*Catalase activity* (units/mg protein)		
	24 h	72 h	120 h
Hepatic Tissues			
Control (−Zn)	10.5 ± 1.0	9.8 ± 0.6	9.3 ± 0.5
25	40.3 ± 3.0 *	11.6 ± 1.0 **	12.7 ± 1.0 *
75	50.2 ± 3.8 *	19.4 ± 1.2 *	15.2 ± 1.1 *
Brain Tissues			
Control	5.0 ± 0.3	4.8 ± 0.2	4.9 ± 0.3
25	5.2 ± 0.4	12.5 ± 0.9 *	11.9 ± 0.8 *
75	7.0 ± 0.4 *	25.8 ± 1.8 *	26.8 ± 1.5 *

Values are average of four separate experiments; mean ± S.D., $n = 10$ in each group. * $P < 0.001$; ** $P < 0.01$ vs control.

along with SOD protects cell by scavenging free radicals.[25] The rate of induction was gradually decreased at 120 h of post-treatment in hepatic tissues whereas it was not altered in brain tissues.

Among the various mechanisms studied against free radical damage, Se-dependent pathways involving GPx are the best understood.[26] Total glutathione peroxidase activity increased significantly in both hepatic (Figure 2) and brain (Figure 3) tissues of experimental animals. Total GPx activity was increased in brain tissue at 24 h of post-treatment to 1.5- and 4.0-fold with 25 and 75 μmoles of zinc concentration respectively. In general, GPx ameliorates oxidative damage and especially breaks the chain of auto-catalysis that follows superoxide activity. Thus the elevated levels of GPx at 24 h and 72 h in both the tissues offers protection against free radical damage, whereas at 120 h it had gradually decreased to 1.2 fold with

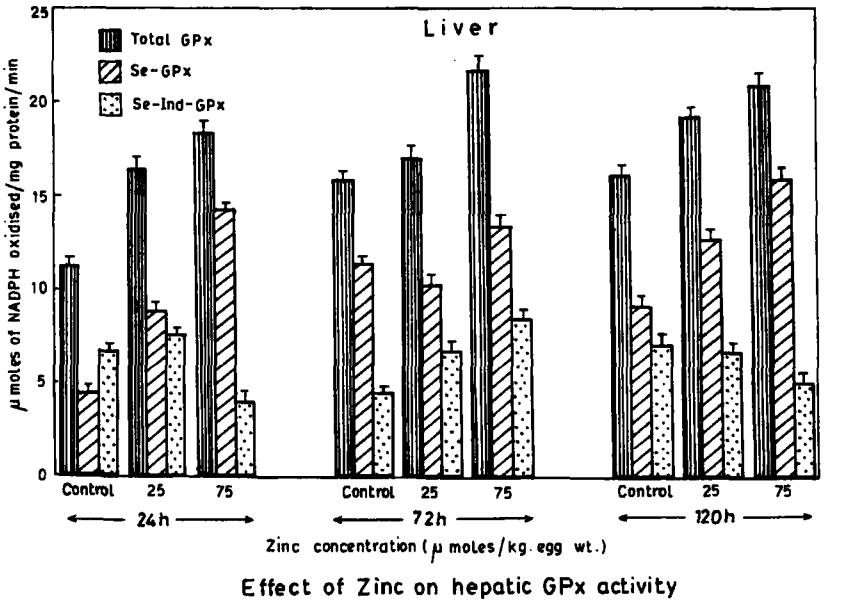
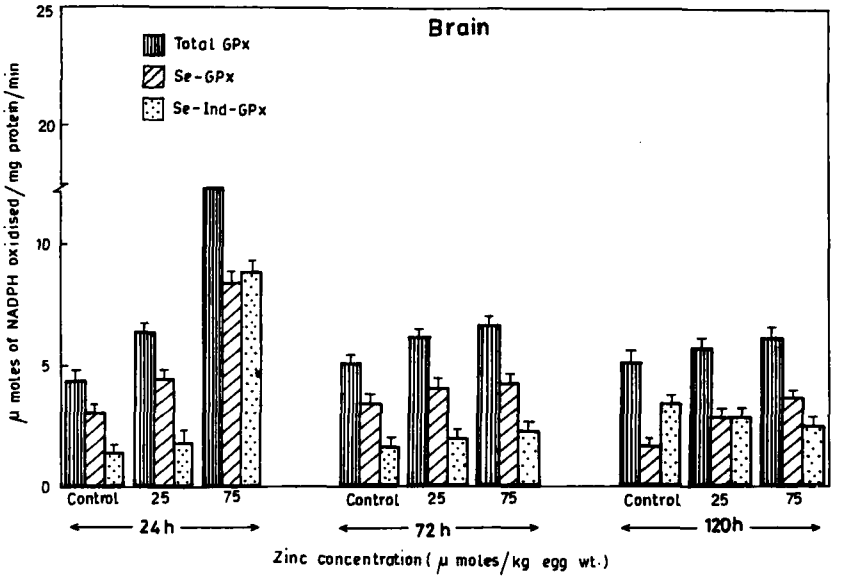


FIGURE 2 *Effect of Zn on GPx activity in chick embryo liver.* Each 100 μl of buffer, GSSG reductase, GSH and NADPH were transferred into a 1 ml cuvette. Enzyme solution, 500 μl containing up to 0.3 U/ml was added and incubated at 37°C for 10 min. The reaction was started by the addition of 100 μl of H_2O_2. The linear decrease in NADPH absorption was recorded at 340 nm. Amount of NADPH oxidized was calculated using molar extinction coefficient 6.22×10^3. Activity of GPx was expressed as μmoles of NADPH oxidized per mg protein/min at 25°C. Total GPx activity was determined using cumene hydroperoxide as a substrate. Selenium dependent GPx activity was measured using H_2O_2 and non-Se-GPx activity was calculated by substracting the Se-GPx activity from total GPx activity.

75 μmoles of zinc treatment. Total GPx, Se-GPx, Non-Se-GPx activities were increased at 24 h and 72 h of post-treatment, whereas in brain tissue at 120 h the Non-Se-GPx activity was reduced to 28% with 75 μmoles of zinc concentration. In hepatic tissue at 24 h the Se-GPx activity was increased to 2- and 3.2-fold with 25 and 75 μmoles of zinc respectively. Even though SOD protects against the reaction of superoxide radicals to a greater extent, our results also suggest the minor role of GPx in this protection.[27]

The response of GST and GR activities are similar to GPx. GST levels were increased with increase in age of the embryo (Table III). The GST activity was increased to 1.2- and 1.4-fold with 75 μmoles of zinc at 72 h post-treatment in hepatic and brain tissues respectively. GST catalyses the transformation of a wide variety of electrophilic compounds to less toxic compounds by conjugating them to GSH.[28] The GR activity was also



Effect of Zinc on brain GPx activity

FIGURE 3 *Effect of Zn on GPx activity in chick embryo brain.* See Figure 2 for experimental details.

TABLE III Effect of zinc on GST activity in hepatic and brain tissues of chick embryos

Treatment with zinc (μmoles/kg egg wt.)	*Glutathione-S-transferase activity* (μmoles of CDNB-GSH conjugate formed/mg protein/min)		
	24 h	72 h	120 h
Hepatic Tissues			
Control (−Zn)	51.5 ± 2.5	57.4 ± 2.8	58.0 ± 3.9
25	56.2 ± 2.9 #	62.0 ± 2.5 #	56.0 ± 3.0
75	60.2 ± 2.6 **	68.0 ± 2.6 **	58.0 ± 3.5
Brain Tissues			
Control	45.0 ± 2.5	50.0 ± 2.9	52.0 ± 2.6
25	52.0 ± 2.9 **	62.0 ± 2.6	50.0 ± 2.9
75	57.0 ± 2.5 *	69.0 ± 3.9 *	53.0 ± 2.6

Values are average of four separate experiments; mean ± S.D., $n = 10$ in each group. * $P < 0.001$; ** $P < 0.01$; # $P < 0.02$ vs control.

increased with the age of the embryo (Table IV). In hepatic tissues, the rate of induction was more at 72 h (2.4 fold) when compared to 24 h (2 fold) with 75 μmoles of zinc concentration. In brain tissue the GR activity was increased to 2.4- and 3.6-fold with 25 and 75 μmoles of zinc at 24 h post-treatment respectively. Unlike hepatic tissue, brain tissue showed maximum

TABLE IV Effect of zinc on GR activity in hepatic and brain tissues of chick embryos

Treatment with Zn (μmoles/kg egg wt.)	*Glutathione reductase activity* (μmoles of NADPH oxidized/mg protein/min)		
	24 h	72 h	120 h
Hepatic Tissues			
Control (−Zn)	17.0 ± 1.3	19.0 ± 1.5	21.2 ± 1.9
25	19.1 ± 1.2	26.2 ± 2.2 *	22.2 ± 1.7
75	33.2 ± 2.5 *	46.5 ± 4.0 *	20.5 ± 2.0
Brain Tissues			
Control (−Zn)	6.6 ± 0.6	6.7 ± 0.6	6.9 ± 0.7
25	15.8 ± 1.1 *	11.2 ± 1.0 **	7.0 ± 0.6
75	24.0 ± 2.0 *	21.7 ± 2.0	7.2 ± 0.8

Values are average of four separate experiments; mean ± S.D., $n = 10$ in each group. * $P < 0.001$; ** $P < 0.01$ vs control.

TABLE V Effect of zinc on glutathione levels in chick embryos

Treatment with zinc (μmoles/kg egg wt.)	*GSH levels* (μg of GSH/mg protein)		
	24	72	120
Hepatic Tissues			
Control (−Zn)	316 ± 29	444 ± 40	501 ± 46
25	378 ± 30 **	497 ± 42 #	295 ± 22 *
75	814 ± 78 *	561 ± 52 **	262 ± 20 *
Brain Tissues			
Control (−Zn)	110 ± 90	120 ± 0	140 ± 12
25	185 ± 12 *	150 ± 11 **	108 ± 90 **
75	236 ± 20 *	170 ± 13 *	91 ± 80 *

Values are average of four separate experiments; mean ± S.D., $n = 10$ in each group. * $P < 0.001$; ** $P < 0.01$; # $P < 0.02$ vs control.

induction of GR at 24 h. The oxidized glutathione is then reduced by GR and maintains the reduced glutathione levels in the system in a cyclic manner.

The GSH levels were significantly increased by zinc in both tissues at 24 h and 72 h post-treatment (Table V), whereas at 120 h the levels were decreased in both tissues. Maximum induction was observed at 24 h (1.2- and 2.5-fold) when compared to 72 h in hepatic tissue with 25 and 75 μmoles of zinc treatment respectively. Similarly in brain tissues also the induction was more at 24 h than at 72 h with 25 and 75 μmoles of zinc treatment respectively. However at 120 h the GSH levels were decreased by 35% and 47% with 75 μmoles of zinc in brain and hepatic tissues respectively. The latter is probably due to progressive increases in GSH efflux.[24]

The *in vitro* lipid peroxide levels were found to be increased by 1.4- and 1.3-fold by the addition of 75 μmoles of zinc to hepatic and brain tissues

TABLE VI *In vitro* effect of zinc on lipid peroxide levels in chick embryos

μmoles *of Zn added*	*Lipid peroxides* (n moles of MDA/g wt. of tissue)	
	Liver	*Brain*
Control (−Zn, −NADPH)	120 ± 10	202 ± 15
Control (−Zn, +NADPH)	131 ± 11	215 ± 17
Control (75 μmoles of Zn, +NADPH)	170 ± 14	268 ± 20

Values are average of four separate experiments; mean ± S.D., $n = 10$ in each group.

isolated from control embryo source (Table VI). Induction of the *in vitro* lipid peroxidation by zinc is a complex phenomenon which involves several unsaturated lipids, catalysts, antioxidants and reducing equivalents. The balance of these factors determine the extent of lipid peroxidation that occurs *in vitro* and referred to as 'lipid peroxide potential' of a tissue extract.[29]

Increased antioxidant enzyme levels at 24 h and 72 h in response to zinc treatment may be responsible for the reduction of TBARS at 72 h and 120 h. This induction could be the defensive adaptation of the system towards the free radical damage to tissues.[30] As far as protection against zinc induced peroxidative damage is concerned, the induction of GPx activity may not be as important as that of SOD. Similar to Se (through GPx), zinc also protects the tissues from peroxidative damage, the enzyme being SOD.

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