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NON ENZYMATIC AND ENZYMATIC ANTIOXIDANT STATUS OF WISTAR RAT ERYTHROCYTE AFTER DIETARY ZINC DEFICIENCY

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ABSTRACT: Zinc deficiency is prevalent worldwide. The present study deals with the effect of dietary zinc deficiency on erythrocytes of Wistar rats. Male Wistar rats (40-50 g) were divided into 3 groups: control (ZC) fed 100 µg/g zinc; pair fed (PF) fed 100 µg/g zinc but the diet given was based on the diet consumed the previous day and zinc deficient (ZD) fed 1 µg/g zinc. The experiment was carried out for 2, 4 and 6 weeks. Erythrocyte cell suspension revealed significant increase in catalase activity and decrease in glutathione concentration after zinc deficiency. A significant ($P<0.05$) increase in erythrocyte lysate superoxide dismutase, glutathione peroxidase and glutathione-s-transferase was observed while glutathione reductase decreased significantly after zinc deficiency. The decreased glutathione reductase coordinates with decreased glutathione concentration making erythrocytes prone to oxidative stress while increased superoxide dismutase, catalase, glutathione peroxidase and glutathione-s-transferase reflects generation of excess free radicals not only due to auto-oxidation of haemoglobin but also due to loss of osmotic fragility leading to oxidative stress indicative of perturbation of erythrocytes on account of dietary zinc deficiency.

INTRODUCTION: Prevalence of nutritional deficiency is predicted to be high in the developing countries [1] with India being one of them [2]. Approximately more than half of the world's population is related to trace element deficiencies which have lead to morbidity and mortality. Mild to moderate zinc deficiency is likely to be prevalent throughout the world although severe zinc deficiency is considered rare [3, 4]. It is nutritionally an essential element for human-beings, animals and plants, an absolute requirement in genetic make-up of every cell [5].

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Reactive oxygen species are continuously being generated within the red blood cells on account of high oxygen tension in arterial blood and haeme Fe^{2+} content. Generation of oxidizing radicals such as $0_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ would lead to oxidative stress[6]. Reactive oxygen species mediated cellular damage effects the membrane integrity accounting for loss of osmotic fragility [7] as well as loss of cellular homeostasis with subsequent activation of apoptotic signaling pathway [8].

Superoxide dismutase (SOD, EC1.15.1.1) a dimeric metalloenzyme catalyzes the dismutation of superoxide anion ($O_2^{\cdot-}$) into molecular oxygen and hydrogen peroxide which occurs rapidly (~10^5 $M^{-1}S^{-1}$ pH 7) [9]. Correlation between SOD activity and life span of erythrocyte has been deduced by Grzelack *et al*; [10] Devi *et al*., [11] reported an increased Cu-Zn-SOD activity in acute lymphocyte and non lymphocyte leukemia's which

reflects its role in protecting erythrocyte from oxidative stress. Catalase (EC1.11.1.6) a heme protein present in peroxisomes and inner mitochondria converts $O_2^{\cdot-}$ which traverses through the membrane into H_2O_2 thereby protecting red blood cells as haemoglobin is a catalyst for peroxidative reactions. Giulivi *et al*., [12] reported ~2×10^{-10} mol/L H_2O_2 in erythrocytes. Al-Naama *et al*., [13] showed increased antioxidant activities (SOD and CAT) as well as weak correlation with malondialdehyde level involving impairement of antioxidant defense system in erythrocytes of sickle cell anemic patients. GSH a vital antioxidant functions as a radical scavenger involved in reduction of superoxide anion against continuous oxidation of haemoglobin as well as oxidative stress damage [14] and is responsible for maintaining the red blood cell integrity under certain conditions[15] with concentration of 99% in red blood cell [16] and 20 µM in plasma [17].

Glutathione peroxidase (GPx, EC1.11.1.9) catalyses the reduction of hydrogen peroxide and various organic hydroperoxides using GSH protecting not only the cells but also DNA, protein and lipids from oxidative damage [18]. A positive correlation has been reported between SOD in erythrocytes and GPx-1 whole blood [19] and plasma GPx and free MDA concentration [20]. Glutathione reductase (GR, EC 1.6.4.2) catalyses the reduction of glutathione disulphide (GSSG) at the expense of $NADPH.H^+$. GR deficiency has been related to several disease states [21], reduced erythrocyte life span *etc*. [22, 23] Glutathione-s-transferases (GST, EC 2.5.1.18) catalyzes the nucleophilic addition of GSH to electrophilic centres of a wide variety of potentially toxic compounds protecting the cells from the deleterious effects of reactive oxygen as well as nitrogen species (RONS) and maintaining the cell structure and function [24]. The present study evaluates the effect of dietary zinc deficiency on Wistar rat erythrocyte non enzymatic-glutathione and enzymatic-catalase, superoxide dismutase, glutathione peroxidase, glutathione-s-tranferase and glutathione reductase.

MATERIALS AND METHODS:

Synthetic Diet: The synthetic experimental diet was prepared based on ICN Research Diet protocol (1999). The composition of synthetic basal diet: Egg white (180g), corn starch (443g), sucrose (200g), corn oil (100g), cellulose (30g), choline chloride (2 g), A17-76 salt mixture (35 g) , A1N-76 C vitamin antibiotic mix (10 g), DL-methionine (7 g), D-Biotin (20 mg). Zinc contents of the basal diet from each lot was estimated at 213.9 nm in air-acetylene flame on GBC 902 double beam atomic absorption spectrophotometer and zinc concentration was adjusted to 1.00 µg/gm and 100 µg/gm by addition of appropriate amounts of zinc sulphate.

Experimental Protocol: Pre-pubertal (30-40 days of age) male Wistar rats (40-50g) were divided into three groups (thirty animals each for 2, 4 and 6 week) (i) Zinc control (ZC) group fed with diet containing 100 µg/gm zinc (ii) Pair fed (PF) group fed with 100 µg/gm zinc diet but the amount of feed given was equal to the feed consumed by zinc deficient group during the previous day. This group was taken into account so as to study the starvation effects due to reduced intake of diet as well as stress effects. Demineralized water was provided *ad libitum* (iii) Zinc deficient (ZD) group were fed zinc deficient (1.00 µg/gm) diet and provided demineralized water *ad libitum*. The animal care and handling was carried out according to the guidelines set by the CPCSEA Committee (No.1678/GO/Re/s/12/CPCSEA dated 16.06.17).

The experiment carried out for 2, 4 and 6 weeks was approved by Departmental Animal Ethics Committee, University of Rajasthan, Jaipur, India. The animals were housed individually in polypropylene cages with stainless steel grills. The polypropylene cages, grills and water bottles were washed with detergent solution, de-mineralized water and finally rinsed in 1% EDTA solution prepared in de-mineralized water so as to avoid contamination and subsequent removal of zinc from cages, grills and bottles. Animals from deficient group were autopsied after 2, 4 and 6 weeks under anaesthesia while supplementation groups were autopsied after 4 weeks. Blood samples from the animals of all the groups were collected by cardiac puncture using heparinized syringe and processed for erythrocyte cell suspension and erythrocyte lysate.

Preparation of Erythrocyte Cell Suspension and Lysate: Red blood cells were washed thrice with PBS (pH 7.4) and erythrocytes were separated from

the blood, haemolysed in hypotonic lysing buffer and centrifuged at 27,000 g for 30 min at 4 ºC in Sigma high speed centrifuge using 18015 rotors and membrane was processed by conventional method till free of haemoglobin [25].

Biochemical Estimations: Erythrocyte cell suspension and erythrocyte lysate was obtained using Sigma refrigerated high speed laboratory centrifuge (10,000 rpm). Superoxide dismutase (SOD) [26]; catalase [27]; glutathione peroxidase (GPx) [28, 29]; glutathione-s-transferase (GST) [30] and glutathione reductase (GR) [31]. Absorbance was read on GBC 911 UV-spectrophotometer, Australia and Systronics UV-vis spectrophotometer 169.

Statistical Analysis: Data are expressed as mean ± SEM. Further, analysis was carried using One Way Analysis of Variance (ANOVA) and if the difference was found significant post-hoc test (Duncan's Multiple Comparison Test) was carried out and P<0.05 was considered significant. Sigma stat 3.5 software was utilized for analysis (Systat Software Asia Pacific Ltd., Bangalore, India).

RESULTS: Catalase activity in erythrocyte cell suspension increased significantly (P<0.05) after 2, 4 and 6 weeks of zinc deficiency when ZD groups were compared with their respective controls and pairfed groups **Table 1**.

TABLE 1: CATALASE ACTIVITY AND GLUTATHIONE CONCENTRATION IN WISTAR RAT ERYTHROCYTE CELL SUSPENSION AFTER 2, 4 AND 6 WEEKS OF ZINC DEFICIENCY. (MEAN ± SEM)

Groups	Catalase (Kat.f)	Glutathione (µg GSH /mg protein)
2ZC	0.118 ± 0.002	19.175 ± 0.369
2PF	0.126 ± 0.005 a*	14.876 ± 0.376 a*
2ZD	0.364 ± 0.004b*c*	14.373 ± 0.526 b*
4ZC	0.129 ± 0.003	28.754 ± 0.277
4PF	0.186 ± 0.001a*	13.604 ± 0.355 *
4ZD	0.493 ± 0.02b*c*	12.630 ± 0.513a b*c*
6ZC	0.132 ± 0.002	31.732 ± 0.379
6PF	0.298 ± 0.002a*	10.514 ± 0.161a*
6ZD	0.618 ± 0.03b*c*	8.265 ± 0.024 b*c*

ZC *vs.* PF = a, PF *vs.* ZD = b, ZC *vs.* ZD = c. Significant (P<0.05) = *. Multiple comparison of means were performed separately for 2, 4 and 6 weeks sub groups.

Decreased (P<0.05) GSH concentration was evident after 2, 4 and 6 weeks of zinc deficiency when groups were compared **Table 1**. Superoxide dismutase activity in erythrocyte lysate increased significantly after 2, 4 and 6 weeks of zinc deficiency when ZD groups were compared with their respective controls and paired groups **Table 2**. Significant (P<0.05) increase was observed in GPx and GST activities after zinc deficiency **Table 2**. Decrease was significant (P<0.05) in erythrocyte lysate GR activity after 2, 4 and 6 week of zinc deficiency when ZD groups were compared with their respective control groups as well as PF groups **Table 2**.

TABLE 2:

TABLE 2: BIOCHEMISTRY OF WISTAR ERYTHROCYTE CELL LYSATE AFTER 2, 4 AND 6 WEEKS OF ZINC DEFICIENCY. (MEAN ± SEM)

Groups	Superoxide dismutase (units / mg lysate protein /h)	Glutathione peroxidase (µM $NADPH.H^+$ oxidized /mg lysate protein /min)	Glutathione reductase (µM $NADPH.H^+$ oxidized /mg lysate protein /min)	Glutathione-s-transferase (µM $NADPH.H^+$ oxidized /mg lysate protein /min)
2ZC	1.657 ± 0.066	0.00516 ± 0.001	1.129 ± 0.067	0.898 ± 0.064
2PF	1.591 ± 0.052a*	0.00985 ± 0.002	0.7879 ± 0.058a*	1.143 ± 0.051 a*
2ZD	2.089 ± 0.025 b*c*	0.0158 ± 0.004c*	0.418 ± 0.072b*c*	1.735 ± 0.084 b*c*
4ZC	1.17 ± 0.002	0.00818 ± 0.003	1.615 ± 0.057	1.129 ± 0.027
4PF	1.24 ± 0.009a*	0.0112 ± 0.003	0.646 ± 0.122 a*	1.985 ± 0.061 a*
4ZD	1.55 ± 0.016 b*c*	0.0317 ± 0.002b*c*	0.340 ± 0.158 b*c*	2.849 ± 0.043b*c*
6ZC	1.18 ± 0.004	0.029 ± 0.014	1.819 ± 0.032	1.218 ± 0.012
6PF	1.37 ± 0.009a*	0.038 ± 0.004	0.475 ± 0.002 a*	2.536 ± 0.018a*
6ZD	1.77 ± 0.21b*c*	0.071 ± 0.002b*c*	0.116 ± 0.018 b*c*	4.973 ± 0.078 b*c*

ZC *vs.* PF = a, PF *vs.* ZD = b, ZC *vs.* ZD = c. Significant (P<0.05) = *. Multiple comparison of means were performed separately for 2, 4 and 6 weeks sub groups.

DISCUSSION: Erythrocytes are sensitive to reactive oxygen species (ROS) generated constantly even during normal physiological events as auto-oxidation leads to generation of superoxide[32]. Although ROS are removed by antioxidant defense mechanism but an imbalance may lead to modification either to the membrane or macro-molecules [14, 24, 33]. Superoxide dismutase is a major antioxidant enzyme in erythrocyte where $O_2^{\bullet-}$ are continuously generated by auto-oxidation of haemoglobin. Zinc deficiency in the present study enhanced SOD activity which can be correlated

with increased resistance towards toxicity/ damaging effects of superoxide radical. Cooperative action of $O_2^{\bullet-}$ and H_2O_2 would generate a potent oxidant $OH^{\bullet}$ involving reduction of Fe III to Fe II [34] leading to enhanced osmotic fragility [7] and ultimately cellular injury. Contrary to our observation decrease in erythrocyte SOD activity in 4 week old Sprague-Dawley rats fed zinc deficient diet for 10-16 days was reported [35] whereas Song *et al*., [36] could not record any variation in zinc deficient rats compared to zinc adequate / control rats. Since H_2O_2 (generated from $O_2^{\bullet-}$ by SOD) serves as a substrate for Fenton reaction to generate highly reactive $OH^{\bullet}$, catalase and glutathione peroxidase enzymes functions as ROS scavenging enzymes by limiting the accumulation of H_2O_2 or organic peroxides [37, 38].

The concentration of glutathione decreased significantly in the present study. Cells deprived of GSH are more prone to oxidative stress even under normal circumstances, since the ability to scavenge or detoxify ROS/FOR is impaired [39] and depletion of GSH levels in erythrocytes has been associated with oxidant burden [40]. Enhanced oxygen free radical is associated with decreased plasma GSH: GSSG ratio which may be one of the factors responsible for disease states in humans [41]. Decreased food intake, intermittent diarrhea [42, 43] leading to decreased protein concentration in erythrocytes of zinc deficient and pair fed groups might be one of the reasons for decreased GSH. Furthermore, oxidation of GSH/GSSG by GPx and GST for scavenging ROS indicative of intracellular redox imbalance has also been correlated to its decreased concentration [44]. Decrease in GSH may probably be due to decrease in its formation as one of its requirements is GR which has also decreased in the present study. Depletion of GSH may have adverse consequences as it weakens the defense against oxidative stress which may have deleterious effect leading to dysfunction of the cell.

Glutathione peroxidase-1 (GPx-1) is found exclusively in erythrocytes while GPx-3 is a seleno-protein [45, 46] with membrane stability (cellular as well as subcellular) dependent on this enzyme. Knock out experiments with catalase or GPx-1 genes in mouse showed that GPx-1 reduces organic peroxides [47]. The present study demonstrates increased GPx activities after zinc deficiency. Increased GPx activity has been reported in erythrocyte after zinc deficiency [48] and after transport stress (GPx-1; beef-cattle Sahin *et al*., [49]). Similarly, increased GPx-1 activities have been reported from acute lymphocytic and non lymphocytic leukemia [11]. GPx-4 reduces the phospholipid hydroperoxides in normal red cell/mammalian cells [50] but is dependent on glutathione. Deficiency would potentiate oxidative damage to the erythrocytes. Thus, the two FOR / ROS scavenging enzymes-catalase and GPx responsible for eliminating H_2O_2/organic peroxides probably function in a coordinated manner to overcome the oxidative stress in erythrocytes after zinc deficiency. Contrary to this, Muller *et al*., [51] reported that H_2O_2 generated in human erythrocytes are removed only by catalase.

Glutathione reductase and glutathione-s-transferase decreased in a duration dependent manner after dietary zinc deficiency in the present study. The decreased glutathione-s-transferase activity can be correlated to decreased consumption of GST cofactor-GSH which can account for depletion of intracellular GSH with resultant decreasing levels of relative Erythrocyte-GST activity. Authors[52, 53] have reported inactivation of erythrocyte GST on account of oxidative stress. The decrease in erythrocyte glutathione reductase has been reported when zinc deficiency was compared with the control [48]. Glutathione reductase has a significant role in protecting haemoglobin, erythrocyte enzymes and membrane from oxidative damage by increasing the level of GSH [54]. The decreased GR activity coordinated well with reduced level of GSH in the present study which might have led to increased oxidative stress to erythrocytes of dietary zinc deficiency and increased concentration of GSSG.

CONCLUSION: Dietary zinc deficiency for a period of 2, 4 and 6 weeks potentiated non enzymatic and enzymatic antioxidants of erythrocytes which may be one of the causative factors for dysfunction. If oxidant molecules are not denatured by antioxidant mechanism then erythrocyte destruction would occur. This is indicative that microelement zinc in diet is crucial for the normal function of mammalian erythrocytes.

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