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Morphology and Osmotic Fragility in Wistar Rat Erythrocytes After Zinc Deficiency and Supplementation

KEYWORDS: Zinc; Erythrocyte; Morphology; Osmotic fragility.

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ABSTRACT *Deficiency of zinc affects the cell membrane permeability. The purpose of the study was to assess the effect on erythrocyte morphology and osmotic fragility after 4 and 6 weeks of zinc deficiency and subsequent repletion for a period of 4 weeks. Morphology was adversely affected which enhanced after 6 weeks of zinc deficiency. Moreover, the osmotic fragility which is an indicator of oxidative stress on erythrocytes enhanced wherein 50% was observed at 0.48% (4ZD), 0.60% (6ZD), 0.35% (4ZD-S) and 0.50% (6ZD-S). Supplementation of zinc for 4 weeks did show favorable changes indicating that change in zinc status could lead to loss of membrane architecture with enhanced osmotic fragility.*

Introduction

Zinc, an essential trace as well as redox element needed in miniscule quantities, has innumerable functions in the biological system due to its catalytic and structural aspect in 3000 proteins (Hambidge, 2000; Osei and Hamer, 2008; Maret, 2009). Approximately 10% of the human genome appears to be encoded by zinc proteins (Andreini et al., 2006). It has pervasive role in maintaining genomic stability (Sharif et al., 2012), signaling, apoptosis, reproduction, cell proliferation, neurogenesis, neurotransmission etc. (Bedwal et al., 1991; Fraker and King, 2004; Ho, 2004; Frederickson et al., 2005; Li and Maret, 2009; Plum et al., 2010; Kumari et al., 2011a, b; Roohani et al., 2013; Joshi et al., 2014 a, b). Cellular zinc homeostasis is achieved by ZnT (SLC30a), ZIP (Zrt and Irt-like proteins SLC39a) (Eide, 2004; Liuzzi and Cousins, 2004; Rink and Haase, 2007) as well as metallothionein (Sensi et al., 2003; Cima et al., 2006; Kang, 2006; Maret, 2009, 2011 a, b). In the biological system there is a constant generation of reactive oxygen species (ROS) such as hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet -}$) etc. as well as reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ($ONOO^-$) (Weidinger and Kozlov, 2015). Increased ROS (Clegg et al., 2005) as well as RNS (Ho and Ames, 2002) has been reported after zinc deficiency. Concomitantly, imbalance of antioxidant enzymes due to excessive generation of reactive species leading to oxidative stress has been reported (Nair et al., 2005; Joshi et al., 2014 a, b). Autooxidation of haemoglobin leads to generation of ~ 3% toxic radicals (Winterbourn, 1991).

Deficiency of zinc is prevalent in developing and underdeveloped countries (Lindenmayer, 2014). Wessells and Brown (2012) reported 17.3% of this population in the world is at risk of developing zinc deficiency. Zinc deficiency may either be a) dietary i.e. primary type on account of inhibitors such as phytate, calcium etc or low zinc content in food (Bedwal et al., 1991; Brown et al., 2001) or b) secondary type due to impairment of intestinal absorption and/or increased loss of zinc through intestine, genetic disease, stress, increased body requirements etc (Yanagisawa, 2008; Prasad, 2013; Pfaender and Grabrucker, 2014). Studies revealed that concentration of zinc in human blood as well as serum is 800 ± 200 and 190 to 130 µg/dl respectively (Goldfrank and Flomengaum, 2006) and 24pM of free zinc in human erythrocytes (Simmons, 1991). Healthy mammalian RBCs are disc shaped (discocyte) with a flexible membrane having a high surface-to-volume ratio but under conditions of stress, various deformations are induced altering its general shape (Liu et al., 1990; Silva et al., 2010; Ford, 2013). Sensitivity to changes in osmotic pressure on erythrocytes defines osmotic fragility (Kwari et al., 2011) and is considered as an indirect method of assessment of oxidative stress (Chihuailaf et al., 2002; Sharma et al., 2009). In the present study an attempt has been made to assess the Wistar erythrocyte morphology and osmotic fragility after zinc deficiency and zinc supplementation.

Materials and Methods

Colony bred male Wistar rats (30-40 days of age- pre-pubertal age; 40-50g wt.) were used for the experiment were divided into Zinc control (ZC), Pairfed (PF) and Zinc deficient (ZD). The synthetic experimental diet was prepared based on ICN Research Diet protocol (1999). 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 ppm and 100 ppm by addition of appropriate amounts of zinc sulphate. 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. The experiments were carried out for 4 weeks (Group 1) and 6 weeks (Group 2). Few animals from each group were autopsied after 4 weeks and 6 weeks under light ether anaesthesia. Remaining animals from 4 (Group 3) and 6 (Group 4) weeks zinc deficiency were supplemented with 100 µg /g zinc and autopsied af-

Volume : 6 | Issue : 10 | October 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

ter 4 weeks. Blood samples from the animals of all the groups were collected by cardiac puncture using heparanized syringe and processed. Blood smears were prepared, dried, fixed and stained using Leishmann's stain (Rankem, India). Morphological analysis was carried out using Leica microscope at 400 X.

Red blood cells separated from whole blood (Marks et al., 1960) were washed thrice with PBS (pH 7.4) and buffy coat consisting mainly of leukocytes were separated from erythrocytes by aspiration. Packed red blood cells were collected, haemolysed in hypotonic lysing solution and centrifuged for 30 minutes at Sigma high speed centrifuge using 18015 rotor. The process was repeated so as to obtain red cell membranes free from Hb (Dodge et al., 1963; Steck and Kant 1974). It was then processed / stored at -20^0 C till assayed. Erythrocyte ghost membrane was homogenized in Remi tissue homogenizer and the homogenate was used for estimation of osmotic fragility. The absorbance was measured on Systronics Spectrophotometer 169 (Serial No. 827) Ahmedabad. A 25 µl blood collected from the heart was added to a series of 2.5 ml saline solution ranging from 0.00 - 0.80 g /100ml in 5mM phosphate buffer, pH 7.4. After gentle mixing and incubation for 15min at room temperature it was centrifuged at 500 X g for 10 min and the absorbance of the supernates was measured at 540 nm. The percent haemolysis was calculated using formula (Faulkner and King, 1970) :

$$\frac{\text{Optical density of test}}{\text{Optical density of distilled water (No NaCl)}} \times 100 = \text{Percent haemolysis}$$

Statistical analysis

Data were expressed as Mean ± SEM. Further, analysis was carried using One Way Analysis Of Variance (ANOVA) and if the difference was found to be significant then post-hoc test (Duncan's Multiple Comparison Test) was carried out and $P<0.05$ was considered significant.

Results

Zinc deficiency induced alterations on the morphology of the red blood cells after 4 weeks (Fig 3) compared to the pair fed group (Fig. 2) and control (Fig. 1). Comparison between control (ZC) and pair fed group (PF), few erythrocytes exhibited changes in their morphology. The severity of the alterations was more prominent after 6 weeks of zinc deficiency (Fig. 9). At some places an usual feature was observed in the form of clustering of erythrocytes (Fig. 10). Alteration in morphology was also evident in pair fed group (Fig. 9). Supplementation of zinc to zinc deficient groups revealed normal morphology of blood cells although the number of normal erythrocytes were few in group 3 (Figs 4,5 & 6) as compared to group 4 (Figs. 11,12 &13).

Complete haemolysis (100%) was observed in 0.0 g/100 ml NaCl (i.e. distilled water). The osmotic fragility in zinc deficient groups (4 and 6 weeks) increased significantly ($P< 0.05$) with increase in NaCl (%) concentration when compared with their respective controls. 50% osmotic fragility was obtained at 0.25% NaCl concentration (4ZC and 6ZC), 0.35% (4PF), 0.48% (4ZD), 0.38% (6PF) and 0.60% (6ZD). Supplementation experiments revealed 50% osmotic fragility at 0.25% NaCl concentration (4ZC-S, 6ZC-S), 0.30% (4PF-S), 0.35% (4ZD-S), 0.35% (6PF-S) and 0.50% (6ZD-S) (Tables 1 & 2).

Discussion

Erythrocyte bilayer membrane and cytoskeleton are capable of maintaining the integrity of the cell. Protein-protein and protein-phospholipid interactions associate membrane cytoskeleton with cytoplasmic face of the membrane (Bennett, 1985). Increase in oxidative stress has the ability not only to alter the proteins but also can enhance proteolytic degradation (Cheng and Li, 2007; Lü et al., 2010; Pajares et al., 2015). Degradation of proteins may affect the permeability of red cell membrane causing cell hemolysis (Lubin and Chiu, 1982; Celedon et al., 2001) indicating membrane instability. It is well known that spectrin tetramers interact with protein 4.1 through glycophorin and ankyrin through band 3 (Pasternack et al., 1985; Xiu-L et al., 1996; Machnicka et al., 2014). Band 3 is involved with anion exchange across the cell membrane and permeability would be affected if band 3 is degraded. Xia et al. (1999) reported that –SH content of band 3 decreased after zinc deficiency and is inversely related to osmotic fragility.

Free radicals produced due to deficiency of zinc with decrease in antioxidant concentration caused adverse effects on the cytological membrane of erythrocytes (Avellini et al., 1995; Adenkola and Ayo, 2009). Erythrocyte membrane being rich in polyunsaturated fatty is the prime target of rective oxygen species (ROS) which would account for membrane perturbation causing enhanced haemolysis (Brzezinska-Slebdoziiska, 2003). Zinc deficiency affects the RBC membrane lipid composition and enhances the susceptibility to lipid peroxidation (Chen et al., 2002). The degradation of membrane cytoskeleton along with oxidative stress may have contributed towards deformity of the membrane in the present study wherein the osmotic fragility of the erythrocyte membrane appears to be enhanced. However, supplementation of zinc did reveal that the damage was not permanent as normal erythrocyte morphology appeared along with decreased osmotic fragility since zinc binds with high affinity to the cysteine residues.

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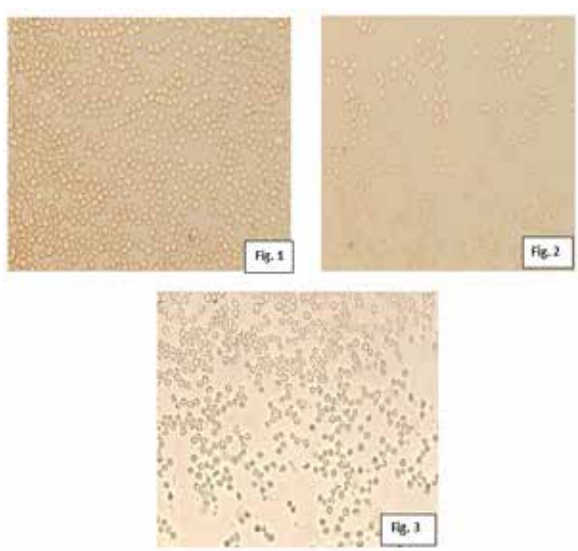
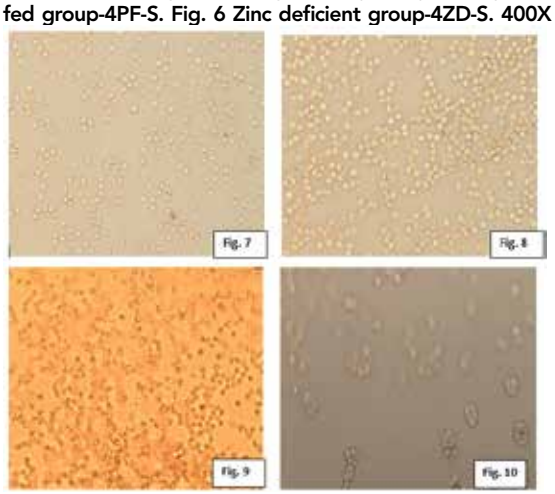


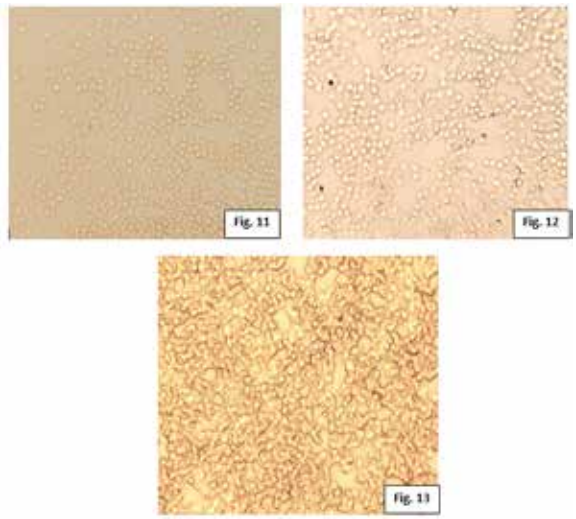
Group 1: Effects on the morphology of Wistar rats erythrocytes after 4 weeks of zinc deficiency. Fig.1 Control group erythrocytes - 4ZC. Fig. 2 Pair fed group-4PF. Fig. 3 Zinc deficient group-4ZD. 400X

Volume : 6 | Issue : 10 | October 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Group 3: Effects on the morphology of Wistar rats erythrocytes after 4 weeks of zinc supplementation to 4 weeks of zinc deficient. Fig.4 4ZC-S group erythrocytes. Fig. 5 Pair fed group-4PF-S. Fig. 6 Zinc deficient group-4ZD-S. 400X



Group-2: Effects on the morphology of Wistar rats erythrocytes after 6 weeks of zinc deficiency. Fig.7. 6ZC group erythrocytes. Fig. 8 Pair fed group-6PF. Figs. 9 & 10 Zinc deficient group- 6ZD. 400X



Group 4: Effects on the morphology of Wistar rats erythrocytes after 4 weeks of zinc supplementation to 6 weeks zinc deficient Fig.11. 6ZC group erythrocytes. Fig. 12 Pair fed group-6PF. Figs. 13 Zinc deficient group-6ZD. 400X.

Table 1: Osmotic fragility of Wistar rat erythrocyte after 4 and 6 weeks of zinc deficiency
(Mean ±SEM)

NaCl concentration (gm/100 ml)	4ZC	4PF	4ZD	6ZC	6PF	6ZD
0	100	100	100	100	100	100
0.1	88.66±0.98	93.0±0.44a*	95.83±0.40 b*c*	94.83±0.16	96.50±0.34a*	97.33±0.21c*
0.15	78.16±0.79	85.83±0.30a*	93.16±0.16 b*c*	81.5±0.34	86.0±0.63a*	96.16±0.16 b*c*
0.2	65.66±0.49	77.33±0.33a*b*	84.83±0.16 b*c*	64.33±0.71	74.33±0.21a*	95.0±0.26 b*c*
0.25	50.16±0.16	66.5±0.34a*	76.83±0.87 b*c*	50 ±0.73	70.0±0.96a*	88.16±0.40 b*c*
0.30	46.0±0.25	64.16±0.54a*	73.16±0.16 b*c*	45.50±0.50	66.33±1.05a*	87.16±0.40 b*c*
0.35	40.16±0.31	50.33±0.42a*	66.00±0.25 b*c*	39.83±0.87	52.66±0.21a*	82.66±0.21 b*c*
0.38	37.16±0.31	43.0±0.26a*	60.55 ±0.22 b*c*	35.50±0.34	50.16±0.65a*	77.33±0.33 b*c*
0.40	34.5±0.34	37.33±0.42a*	56.33±0.33 b*c*	22.83±0.70	43.33±0.61a*	71.83±0.40 b*c*
0.48	30.16±0.65	32.50±0.34a*	50.33±0.56 b*c*	19.50±0.34	33.50±0.50a*	64.50±0.34 b*c*
0.50	26.33±0.42	25.16±0.16a*	46.16±0.16 b*c*	14.50±0.50	24.50±0.95a*	68.16±0.16 b*c*
0.60	20.33±0.21	20.16±0.31	33.16±0.16 b*c*	9.33 ±0.42	13.5±0.72 a*	50±0.26 b*c*
0.70	14.67±0.33	18.16±0.31	26.5±0.34c*	5.0±0.00	8.33±0.21a*	45.83±0.70 b*c*
0.80	10.0±0.00	11.83±0.16a*	14.83 ±0.16 b*c*	2.0±0.00	6.66±0.21a*	34.33±0.21 b*c*

ZC Vs PF = a * P < 0.05 Significance level
ZC Vs ZD = b
PF Vs ZD = c
Note : Multiple comparison of means were performed separately for 4 and 6 weeks sub groups

Table 2: Osmotic fragility of Wistar rat erythrocyte after 4 weeks of zinc supplementation to zinc deficient groups (4 & 6 weeks) (Mean ±SEM)

NaCl Concentration (gm/100 ml)	4ZC-S	4PF-S	4ZD-S	6ZC-S	6PF-S	6ZD-S
0	100	100	100	100	100	100
0.1	88.50±1.33	95.50±0.34a*	97.33±0.21c*	96.83±0.65	93.83±0.17a*	94.33±0.33c*
0.15	73.33±0.21	82.83±0.16a*	88.66±0.21 b*c*	73.50±1.65	80.67±0.21a*	84.67±0.21 b*c*
0.2	60.66±1.14	77.66±0.21a*	75.83±0.16c*	58.50±1.63	75.83±0.17a*	82.50±0.22 b*c*
0.25	50.83±0.98	66.50±0.22a*	67.5±0.34 b*	50.5 ±0.34	64.5±0.22a*	76.67±0.21 b*c*
0.3	47.833±0.16	50.00±1.125a*	64.5±0.34c*	47.33±0.21	57.33±0.21a*	73.67±0.21 b*c*
0.35	34.66±0.21	47.66±0.21a*	50.5±1.057 b*c*	42.50±0.34	50.33±0.80a*	65.66±0.21 b*c*
0.38	29.50±.0.34	43.83±0.16a*	45.66±0.21 b*c*	35.16±0.31	42.67±0.21a*	62.50±0.34 b*c*
0.40	24.66±0.21	31.83±0.16a*	40.66±0.21 b*c*	29.83±0.17	39.67±0.21a*	58.67±0.71 b*c*
0.48	19.50±0.34	27.83±0.16a*	34.83±0.16 b*c*	24.83±0.17	35.50±0.22a*	53.50±0.42 b*c*
0.50	14.50±0.22	21.83±0.16a*	30.16±0.60 b*c*	20.83±0.16	32.66±0.21a*	50.5±0.50 b*c*
0.60	9.33±0.21	11.83±0.16a*	12.83±0.54c*	15.83±0.17	28.67±0.21a*	43.67±0.21 b*c*
0.70	8.50±0.22	9.83±0.31	10.33±0.21	4.17±0.17	14.67±0.21 a*	32.67±0.21b* c*
0.80	6.5±0.22	5.33±0.21a*	4.83±0.16c*	2.167 ±0.167	7.167 ±0.40 a*	23.5 ± 0.34 b*c*

ZC Vs PF = a * P < 0.05 Significance level
ZC Vs ZD = b
PF Vs ZD = c
Note: Multiple comparison of means were performed separately for 4 and 6 weeks sub groups

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