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EFFECT OF SHORT TERM DIETARY ZINC DEFICIENCY AND ZINC SUPPLEMENTATION ON WISTAR RAT ERYTHROCYTE: BIOCHEMICAL STUDIES

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ABSTRACT: Deficiency of zinc has been reported in developed and developing countries due to low zinc intake, which also accounts for crops grown in low zinc areas. 2 billion people still remain at risk of zinc deficiency affecting all age groups in spite of proven benefits of adequate zinc nutrition. The study evaluates the effect of dietary zinc deficiency on male Wistar rat erythrocytes for 2-, 4- and 6 weeks and 4 weeks zinc supplementation to the deficient groups. A significant decrease in the total protein of erythrocyte ghost membrane and erythrocyte cell suspension was recorded in zinc deficient groups. Increase ($P< 0.05$) in erythrocyte ghost membrane protein carbonyl, protein thiol, and lipid peroxidation (TBARS / induced) was observed. Erythrocyte cell suspension OH radical and hydroperoxide levels increased while α-tocopherol and ascorbic acid decreased in ZD groups. A decrease was observed in ghost membrane zinc concentration in zinc-deficient groups, while there was a gradual increase in copper level of ZD groups. Zinc supplementation studies revealed the same pattern of changes within its group /subgroups. There was an evident alteration in severity pattern when ZD group experiments were compared with dietary zinc supplementation experiments. Zinc deficiency from pre-pubertal age onwards revealed perturbations in the parameters indicative of radical generation and could be one factor contributing to oxidative stress which could lead to erythrocyte dysfunction. Zinc supplementation to zinc deficient group altered the severity to some extent (dose and duration dependent) indicating the essential role of zinc in maintaining the structural and functional integrity of erythrocytes.

INTRODUCTION: Trace element zinc is involved in growth, differentiation, genomic stability, cell division, cell signaling, reproduction, apoptosis, synthesis and secretion of hormones, antioxidant *etc.* [1-10].

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17.3% of the global population is at risk of developing zinc deficiency, with children <5 yrs contributing 1.7% of total deaths [11]. Erythrocytes are susceptible to oxidative stress being rich in polyunsaturated membrane lipids and auto-oxidation of hemoglobin due to endogenous production of reactive oxygen species, which would affect the physicochemical properties [12].

Changes in the primary structure of protein due to lipid oxidation products would lead to changes in the secondary and tertiary structure of proteins. Protein carbonylation is involved in functional

changes of various structural and enzymatic proteins [13] can be due to oxidative attack on cysteine/methionine/lysine/proline/threonine/histidine/ tryptophan /tyrosine etc. and by the secondary reaction of these residues with reactive carbonyl compounds [14, 15]. Oxidation of protein sulfhydryls to mixed disulfides and their reduction back to sulfhydryls is an early cellular response to oxidative stress [16]. Polyunsaturated fatty acid lipid peroxidation may be: (a) enzymatic generating peroxy radicals or (b) may be non-enzymatic requiring molecular oxygen and Fe^{2+} ions [17, 18]. Hydroxyl radical with *in-vivo* half-life of approximately 10^{-9} s [19] usually reacts in its immediate surroundings and with DNA molecule [20]. Under oxidative stress, the consumption of ascorbate can be high, and in order to restore ascorbate levels, systems exist that reduce AFR and/or DHA [21]. Ascorbic acid, a water-soluble chain-breaking antioxidant, acts synergistically with vitamin E protecting plasma membranes from oxidative damage [22]. α-tocopherol may probably protect the red cell from oxidative damage via a free radical scavenging mechanism and as a structural component of the cell membrane [23]. The present study evaluates the effect of dietary zinc deficiency and zinc supplementation on erythrocytes of male Wistar rats.

MATERIALS AND METHODS: The synthetic experimental diet was prepared based on ICN Research Diet protocol (1999). Pre-pubertal (30-40 days of age) male Wistar rats (40-50 gm wt.) 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 experiment was carried out for 2-, 4- and 6 weeks. 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. Supplementation of zinc was carried out for a period of 4 weeks after deficiency. Fifteen animals each from 2-, 4- and 6- weeks zinc-deficient experiments were supplemented with 100 µg /gm zinc diet (Control: ZC-S; Pair fed: PF-S; Zinc supplemented: ZD-S). The experiment design was approved by Institutional Animal Ethics Committee (IAEC), Department of Zoology, University of Rajasthan, Jaipur, India, and Committee for Control and Supervision of Experiments on Animals (CPCSEA) (No.1678/GO/Re/s/12/CPCSEA dated 16.06.17).

Animals from the deficient group were autopsied after 2-, 4- and 6- weeks under anesthesia, while supplementation groups were autopsied after 4 weeks. Blood samples from the animals of all the groups were collected by cardiac puncture using a heparinized syringe and processed.

Preparation of Erythrocyte Ghost Membrane and Erythrocyte Cell Suspension: Red blood cells were washed thrice with PBS (pH 7.4), and erythrocytes were separated from the blood, haemolysed in a 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 hemoglobin [24, 25].

Biochemical Estimation: Total Protein [26], Protein carbonyl [27], Protein sulphydryl [28], Lipid Peroxidation [29], Hydroperoxides [30], Hydroxyl radical [31], α-Tocopherol [32], Ascorbic acid [33, 34] were estimated on Systronics spectrophotometer 169. Total zinc and copper were estimated at 213.9 and 324.8 nm, respectively, with 0.5 nm slit width with background correction and an integration time of 3 sec on GBC-902 double beam atomic absorption spectrophotometer (Australia).

Statistical Analysis: Data are expressed as mean ± SEM. Further, the analysis was carried using One Way Analysis of Variance (ANOVA), and if the difference was found significant posthoc 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: After 2-, 4- and 6- weeks of dietary zinc deficiency significant (P< 0.05) decrease was observed in erythrocyte ghost membrane total protein in zinc-deficient group when compared with their respective pair-fed and control groups. Significant (P<0.05) decrease was evident when 4- and 6-week pair-fed groups were compared with their respective control groups **Table 1**.

Dietary zinc supplementation for a period of 4 weeks revealed an increase in comparison with their respective deficient group's **Table 1** & **Table 2**. However, comparing supplementation groups (ZD-S) (2-4-and 6-weeks) with their respective control and pair-fed group decrease was still evident in **Table 2.**

TABLE 1: WISTAR RATS ERYTHROCYTE GHOST MEMBRANE TOTAL PROTEIN, PROTEIN CARBONYL, THIOL AND LIPID EROXIDATION AFTER 2, 4 AND 6 WEEKS OF ZINC DEFICIENCY (MEAN ± SEM)

Groups	Total Protein (mg / gm)	Protein Carbonyl (n Moles / mg Protein)	Protein Thiol (-SH) Group (n Moles / mg Protein)	Lipid Peroxidation (n Moles / mg Protein)	
				TBARS	Induced
2ZC	2.59 ± 0.02	0.071±0.002	0.413±0.007	0.0933±0.003	0.0615±0.0012
2PF	2.428±0.03	0.079±0.002 a*	0.269±0.005 a*	0.145±0.023 a*	0.0675±0.0041a*
2ZD	1.05±0.03 b*c*	0.126±0.002 b*c*	0.254±0.007 b*c*	0.266±0.0089 b*c*	0.0979±0.0046 b*c*
4ZC	3.041 ± 0.013	0.169 ± 0.002	0.438 ± 0.174	0.116 ± 0.0008	0.114 ± 0.0005
4PF	2.023 ± 0.048 a*	0.274 ± 0.004 a*	0.405 ± 0.002a*	0.133 ± 0.0003 a*	0.232 ± 0.002 a*
4ZD	0.349 ± 0.007 b*c*	0.476 ± 0.005b*c*	0.32 ± 0.002 b* c*	0.250 ± 0.0009 b*c*	0.348 ± 0.0005 b*c*
6ZC	6.018 ± 0.029	0.187 ± 0.004	0.612 ± 0.007	0.158 ± 0.0013	0.218 ± 0.0029
6PF	2.136 ± 0.169 a*	0.339 ± 0.005 a*	0.3392 ± 0.005 a*	0.180 ± 0.0013a*	0.421 ± 0.0045a*
6ZD	0.216 ± 0.023 b*c*	0.612 ± 0.007b*c*	0.1968 ± 0.005 b*c*	0.605 ± 0.0111 b*c*	0.669 ± 0.0107 b*c*

Zc vs. pf = a significant (p<0.05) = * pf vs zd = b zc vs zd =c note: multiple comparison of means were performed separately for2-, 4- and 6 weeks sub groups

Significant (P<0.05) increase in erythrocyte ghost membrane protein carbonyl concentration was evident when ZD groups were compared with their respective pair-fed and control groups. Further, a comparison of pair-fed groups with their respective control groups revealed an increase in **Table 1**.

Supplementation of dietary zinc to zinc-deficient experiments revealed a significant (P<0.05) increase on a comparison between the group's **Table 2**. Comparison of zinc supplementation experiment groups **Table 2** with zinc deficient experiment groups **Table 1** revealed decrease.

TABLE 2: ERYTHROCYTE GHOST MEMBRANE TOTAL PROTEIN, PROTEIN CARBONYL, THIOL AND LIPID PEROXIDATION AFTER 4 WEEKS OF ZINC SUPPLEMENTATION TO ZINC DEFICIENT WISTAR RATS (2-, 4- & 6 WEEKS) (MEAN ± SEM)

Groups	Total Protein (mg / gm)	Protein Carbonyl (n Moles / mg Protein)	Protein Thiol (SH) Group (n Moles / mg Protein)	Lipid Peroxidation (n Moles / mg Protein)	
				TBARS	Induced
2ZC-S	3.91± 0.06	0.073 ± 0.002	0. 554 ± 0.003	0.119 ± 0.006	0.029 ± 0.007
2PF-S	2.96 ± 0.03 a*	0.058 ± 0.004 a*	0.330±0.007 a*	0.135 ± 0.01 a*	0.054 ± 0.003 a*
2ZD-S	2.09 ± 0.05 b*c*	0.094 ±0.006 b*c*	0.276 ± 0.008 b*c*	0.196 ± 0.008 b*c*	0.076 ± 0.004 b*c*
4ZC -S	5.36 ±0.024	0.167±0.002	0.638 ± 0.005	0.131 ± 0.001	0.322 ± 0.134
4PF-S	3.18 ± 0.012 a*	0.233± 0.015 a*	0.596 ± 0.006	0.146 ± 0.003 a*	0.154 ± 0.005 a*
4ZD -S	1.96 ± 0.014 b*c*	0.238 ±0.008 c*	0.412 ± 0.005 b*c*	0.187 ± 0.002 b*c*	0.224 ± 0.004 b*c*
6ZC -S	7.18± 0.02	0.183± 0.0002	0.805 ± 0.003	0.159 ± 0.001	0.196 ± 0.013
6PF- S	3.98± 0.09 a*	0.284± 0.002 a*	0.483 ± 0.003	0.224 ± 0.007 a*	0.310 ± 0.002 a*
6ZD-S	1.76 ± 0.016 b*c*	0.407 ± 0.003 b * c*	0.331 ± 0.003 b*c*	0.402 ± 0.0005 b*c*	0.396 ± 0.016 b*c*

Zc-s vs pf-s = a significant (p<0.05) = *pf-s vs zd –s = b zc –s vs zd-s = c note: multiple comparison of means were performed separately for 4 and 6 weeks sub groups.

Protein thiol (-SH) concentration of erythrocyte ghost membrane decreased significantly (P<0.05) when ZD groups of 2-, 4-and 6-weeks were compared with their respective pair-fed and control groups. Further, a comparison of pair-fed groups with the control group's revealed a significant decrease **Table 1**. Dietary supplementation of zinc for 4 weeks although revealed an increase when supple-mentation experiment groups (ZD-S) were compared with zinc deficient experiment groups

Table 1 and **2** but comparison of ZD-S groups with ZC-S and PF-S till 6 weeks there was still a significant (P<0.05) decrease **Table 2.** Lipid peroxidation (TBARS and Induced) of erythrocyte ghost membrane increased significantly (P<0.05) after 2-, 4- and 6-weeks of zinc deficiency when ZD groups were compared with their respective control and pair-fed groups as well as when pair-fed groups were compared with respective control groups **Table 1.** Supplementation of dietary zinc still revealed a significant increase in all groups **Table 2,** but in comparison with ZD experiment groups **Table 1** there was a decline. After 2-, 4- and 6-weeks of dietary zinc deficiency erythrocyte cell suspension total protein concentration decreased significantly (P<0.05) when ZD groups were compared with their respective pair-fed and control groups. Furthermore, a significant decrease was observed when pair-fed groups were compared with respective controls, except the decrease was non-significant after 2 weeks **Table 3**.

TABLE 3: BIOCHEMISTRY OF WISTAR ERYTHROCYTE CELL SUSPENSION AFTER 2, 4 AND 6 WEEKS OF ZINC DEFICIENCY (MEAN ± SEM)

Groups	Total Protein(mg/gm)	OH radical (n Moles / mg Protein)	Hydroperoxides (FOX) (µM Hydroperoxides /gm)	α – Tocopherol (µg /mg Protein)	Ascorbic Acid (µg /mg Protein)
2ZC	4.476 ±0.04	0.245±0.014	2.357±0.048	4.512 ± 0.072	2.318 ± 0.037
2PF	4.44± 0.04	0.384±0.002 a *	2.863±0.053 a *	3.09 ± 0.09 a *	2.307 ± 0.051
2ZD	3.44±0.162 b*c*	0.444±0.007 b*c*	4.331±0.144 b*c*	2.109 ± 0.062 b*c*	2.156 ± 0.034 b*c*
4ZC	4.208 ± 0.023	0.268 ± 0.003	2.061 ± 0.037	3.251 ± 0.046	2.522 ± 0.074
4PF	3.160 ± 0.063 a*	0.426 ± 0.003 a*	4.188 ± 0.056 a*	2.708 ± 0.045 a*	1.506 ± 0.014 a*
4ZD	1.895 ± 0.042 b*c*	0.615 ± 0.0112 b* c*	8.233 ± 0.071b*c*	2.062 ± 0.025 b* c*	0.946 ± 0.016 b* c*
6ZC	5.594 ± 0.085	0.362 ± 0.142	3.086 ± 0.059	4.020 ± 0.034	3.799 ± 0.098
6PF	2.644 ± 0.034	0.584 ± 0.007 a*	6.111 ± 0.055 a*	1.598 ± 0.023 a*	1.206 ± 0.018 a*
6ZD	0.818 ±.027 b* c*	0.878±0.009 b* c*	12.156 ± 0.043 b* c*	0.848 ± 0.019 b* c*	0.706 ± 0.015 b* c*

Zc vs pf = a significant (p<0.05) = * pf vs zd = b zc vs zd = c note: multiple comparison of means were performed separately for 2-,4 -and 6- weeks sub groups.

Supplementation of dietary zinc for a period of 4 weeks still revealed a significant decrease when ZD-S groups were compared with their respective pair-fed (PF-S) and control groups (ZC-S) **Table 4,** but on comparison with zinc-deficient experiment groups **Table 3** an increase was evident.

TABLE 4: BIOCHEMISTRY OF ERYTHROCYTE CELL SUSPENSION AFTER 4 WEEKS OF ZINC SUPPLEMENTATION TO ZINC DEFICIENT (2-, 4- & 6 WEEKS) WISTAR RATS (MEAN ± SEM)

Groups	Total Protein (mg/gm)	OH Radical (n Moles / mg Protein)	Hyderoperoxides (FOX) (µM Hydroperoxides /gm)	α – Tocopherol (µg /mg Protein)	Ascorbic Acid (µg /mg Protein)
2ZC-S	5.371±0.07	0.216±0.02	1.98±0.04	4.218±0.064	3.196± 0.04
2ZPF-S	5.09±0.16	0.310±0.001a*	2.573±0.026 a*	3.73±0.028 a*	2.832±0.036 a*
2ZD-S	4.32± 0.11b* c*	0.350±0.009b*c*	3.187±0.132b*c*	2.98±0.04 b*c*	2.481±0.011b*c*
4ZC -S	6.015 ± 0.025	0.221 ± 0.004	2.013 ± 0.041	4.087 ± 0.047	3.695 ± 0.023
4PF-S	3.986 ± 0.045 a*	0.345 ± 0.003 a*	2.621 ± 0.031 a*	2.879 ± 0.019 a*	1.921 ± 0.017 a*
4ZD -S	2.524 ± 0.111 b*c*	0.565 ± 0.006 b*c*	5.608 ± 0.168 b*c*	2.294 ± 0.012 b*c*	1.262 ± 0.007 b*c*
6ZC -S	6.808 ± 0.026	0.237 ± 0.004	2.844 ± 0.059	6.564 ± 0.062	4.194 ± 0.053
6PF- S	4.153 ± 0.055 a*	0.468 ± 0.004 a*	4.093 ± 0.044 a*	5.634 ± 0.040 a*	3.088 ± 0.036 a*
6ZD-S	2.765 ± 0.121 b*c*	0.742 ± 0.005 b*c*	8.532 ± 0.154 b*c*	1.667 ± 0.027 b*c*	2.643 ± 0.147 b*c*

ZC-S Vs PF-S = a Significant (P<0.05) = *PF-S Vs ZD –S = b ZC –S Vs ZD-S = c Note: Multiple comparisons of means were performed separately for 4 and 6 weeks subgroups.

Generation of OH radical and hydroperoxides after 2-, 4- and 6-weeks of dietary zinc deficiency in erythrocyte cell suspension was significant (P<0.05). Significance was evident when ZD groups were compared with their respective control and pair-fed groups **Table 3**. Increase (P<0.05) was still evident after supplementation of dietary zinc for 4 weeks **Table 4,** but comparison between

supple-mentation groups with zinc-deficient experiment groups decrease in OH radical concentration was evident. α - Tocopherol and ascorbic acid of Wistar rat erythrocyte cell suspension decreased significantly (P<0.05) when ZD groups (2-,4- and 6-weeks) were compared with their respective pair-fed and control groups as well as when pair-fed groups was compared with their control groups **Table 3**. The decrease was still evident in - groups when ZD-S was compared with PF-S and ZC-S groups **Table 4,** but in comparison with ZD group **Table 3** an increase was observed.

TABLE 5: WISTAR RATS ERYTHROCYTE GHOST MEMBRANE ZINC AND COPPER (µG / MG PROTEIN) AFTER 2, 4 AND 6 WEEKS OF ZINC DEFICIENCY (MEAN ± SEM)

Groups	Zinc (µg / gm protein)	Copper (µg / gm protein)
2ZC	58.67 ± 0.66	22.19±0.09
2PF	50.78 ± 0.34	29.67±0.45
2ZD	48.56±0.22 b*c*	38.52±0.28 b*c*
4ZC	61.66 ± 0.66	34.166 ± 0.31
4PF	54.50 ± 0.76 a*	42.50 ± 0.43 a*
4ZD	41.33 ± 0.55 b*c*	58.50 ± 0.22 b*c*
6ZC	73.50 ± 0.67	45.16 ± 0.31
6PF	46.00 ± 0.52 a*	64.66 ± 0.71 a*
6ZD	33.83 ± 0.60 b*c*	68.83 ± 0.31 b*c*

Zc vs pf = a * p < 0.05 significance level pf vs zd =b; zc vs zd = c note: multiple comparison of means were performed separately for 2-, 4- and 6 weeks sub groups.

Erythrocyte ghost membrane trace element zinc decreased significantly (P<0.05) after 2-, 4- and 6-weeks of dietary zinc deficiency when ZD groups were compared with respective control and pair-fed groups. Copper, another trace element, increased significantly (P<0.05) **Table 5**. Supplementation of dietary zinc to zinc-deficient experiment groups still revealed a decline in zinc concentration when ZD experiment groups were compared with ZC-S groups **Table 6,** but comparison with zinc-deficient experiment groups **Table 5** an increase was evident. Copper concentration remained enhanced even after dietary zinc supplementation for 4 weeks **Table 6,** but a comparison of supplementation experiment groups with zinc-deficient experiment groups revealed a decline in **Tables 5 & 6**.

TABLE 6: WISTAR RATS ERYTHROCYTE GHOST MEMBRANE ZINC AND COPPER (µG / MG PROTEIN) AFTER 4 WEEKS OF ZINC SUPPLEMENTATION TO ZINC DEFICIENT (2-, 4 - & 6 WEEKS) WISTAR RATS (MEAN ± SEM)

Groups	Zinc (µg / gm Protein)	Copper (µg / gm Protein)
2ZC-S	68.76 ±0.71	28.88±0.73
2PF-S	59.653±0.91a*	33.45 ±0.92
2ZD-S	55.188±0.54b*	36.15 ±0.36b*
4ZC -S	70.015± 0.306	38.257 ± 0.394
4PF-S	58.247 ± 0.359 a*	43.540 ±0.447 a*
4ZD -S	52.042 ± 0.354 c*	46.387 ± 0.388 b* c*
6ZC -S	82.424±0.420	47.832 ± 0.285
6PF- S	50.445 ± 0.187 a*	50.505 ± 1.206
6ZD-S	41.422 ± 0.254 b* c *	53.358 ± 0.178 b* c *

Zc-s vs pf-s = a significant (p<0.05) = * pf-s vs zd –s = b; zc –s vs zd-s = c note: multiple comparison of means were performed separately for 4 and 6 weeks sub groups.

DISCUSSION: Erythrocyte's structural integrity and its other physiological properties are dependent on its membrane. Erythrocyte ghost membrane total protein, cell suspension total protein showed a significant reduction in their values in zinc-deficient animals when compared to their respective pair-fed and control groups. Zinc deficiency markedly affects protein to varying degrees. Marreiro *et al.*, [35] in their review reported altered expression of zinc transporters Zip-7 and ZnT-7 found in several organelles - endoplasmic reticulum, Golgi apparatus etc. rendering them non-functional during zinc deficiency. Erythrocyte membrane proteins due to oxy radicals are covalently modified by lipid peroxidation products which enhance its hemolysis – a phenomenon

observed during zinc deficiency [8]. RBC structural shape is affected by alteration in the structure of lipids, band 3 as well as spectrin [36,] which is associated with erythrocyte properties. Studies on haemolysis in hemoglobinopathy patients reported membrane damage due to combined spectrin, band 3 with denatured hemoglobin [37]. The reduction in total protein content appears as zinc has a prominent role to play in protein synthesis and its deficiency lowers the transcriptional and translational capacity leading ultimately to low levels of protein synthesis [38]. Increased protein levels in PF groups compared to ZD groups probably accounts for upregulation of stress genes. Similar increase has been reported in organs [5, 7].

Supplementation studies for 4 weeks exhibited an increase in the total protein content when compared with 2-, 4- and 6- weeks zinc-deficient groups suggesting that deficiency-induced changes for the protein machinery were not severe and irreversible and the protein status could be brought back to the normal physiological range indicating: (a) homeostatic balance was attempted to obviate the injury caused by zinc deficiency and (b) duration of zinc deficiency has a significant impact as it may be a critical point for the onset of events that encompasses zinc deficiency. Proteins are sensitive to reactive oxygen species and rapidly undergo oxidation. The accumulation of oxidized protein and protein carbonyl is complex involving ROS and /or secondary oxidation products of the macromolecules that would impair the cells and affects the balance between pro-oxidant and antioxidant activities [39].

Increase in reactive carbonyl compounds as a result of oxidative and non-oxidative reactions lead to increased chemical modification of proteins, defect in detoxification of carbonyl compounds, and consequently oxidative stress generation [40, 41]. Decreased protein concentration, *i.e.*, inhibition of protein synthesis, would enhance the possibility of oxidized protein to induce oxidative stress. The enhanced level of protein carbonyl in the present study contributes to oxidative stress leading to cellular dysfunction *via* impacts on cellular metabolism and may even lead to toxicity. This finding supports the change in morphology as well as enhanced osmotic fragility in erythrocytes reported from our laboratory [8]. Supplementation studies revealed a decrease in protein carbonyl content on comparison with zinc-deficient groups (2-, 4- & 6- weeks). Maintaining low carbonyl content, the erythrocyte would be able to maintain sufficient protein synthesis essential for regulating cell structure as well as enzymatic processes. Similar decline in protein oxidation after zinc supplementation has been reported [20].

Under physiological and pathophysiological conditions, cysteines (-SH group) have an essential role in protein function and are susceptible to irreversible damage by oxidative conditions. Oxidation of cysteine would lead to misfolding of protein, affecting protein function [42]. In the present study, the decline in –SH concentration after zinc deficiency (2-, 4- and 6-weeks) was observed which reflects a decrease in binding affinity of zinc to membrane –SH group as against enhanced affinity towards cysteine. Decline in erythrocyte SH concentration (band 3 protein) from 75 nmol/mg protein to 68 nmol/mg protein was observed after 21 days of zinc depletion [43] . Even pair-fed groups showed a decrease in comparison to their respective control groups.

The SH concentration of membrane, which decreased after zinc deficiency reversed after zinc supplementation, suggests that zinc plays a major role in protecting membrane –SH group oxidation. Lipid peroxidation and induced lipid peroxidation activities of the erythrocyte ghost membrane revealed a significant increase. Erythrocyte membrane has band 3 transmembrane protein which not only interacts with phospholipids at its surface but has a significant role in anion transport. Due to accumulation of malondialdehyde: (a) anion transport as well as function of band 3 associated enzymes, namely glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase, are affected and (b) polyunsaturated fatty acids of the cellular membrane are degraded disrupting the membrane integrity ultimately leading to cell membrane damage [44, 45].

Enhanced lipid peroxidation due to oxidative damage of membrane lipids and consequent cross-linking on account of free radical formation due to impaired detoxification function would account for the change in morphology and increased osmotic fragility as reported earlier [8] as well altered

glutathione concentration, catalase, superoxide dismutase, glutathione peroxidase, glutathione-s transferase, and glutathione reductase activities [46] which could be an important cause of destruction of the cell membrane. Erythrocytes of zinc-deficient rats appear to be susceptible to oxidative stress. Supplementation studies for a period of 4 weeks revealed a significant increase between various groups indicating that lipid peroxidation production was relatively high, although decrease was observed compared with zinc-deficient experiment.

Hydroperoxides are the major primary product formed during the process of lipid peroxidation. Hydro-peroxides concentration in erythrocyte cell suspension revealed a significant increase after 2, 4, and 6 weeks of zinc deficiency. Comparison of data after zinc supplementation revealed a significant increase in both the deficient groups (4- and 6- weeks), although the concentration decreased when the supplementation group was compared with the deficient (4- and 6-weeks) group. Hydroperoxides may disastrously affect the cell membrane directly through (a) degradation to highly toxic hydroxyl radical and also (b) by the formation of stable aldehydes [18, 47]. Enhancement of hydroperoxides is indicative of an increase in oxidative stress. Hydroxyl radical, a potent reactive oxygen species in the biological system, increased significantly in the erythrocyte cell suspension after zinc deficiency. The increase contributes significantly to alterations in membrane integrity as well as may account for an increase in oxidative stress, which leads to pathologic states. Supplementation of zinc to zinc-deficient groups (4- & 6- weeks) still exhibited an insignificant increase but on comparing the data with zinc-deficient groups (4- & 6- weeks), there was a decline. Chen [48] reported that high levels of zinc decreased $O_2^{\cdot-}$ and $OH^{\cdot}$ formation in the erythrocytes. Ascorbic acid, considered the strongest reductant for free radicals, decreased significantly after zinc deficiency. Aqueous peroxyl radicals attack the erythrocyte and induce oxidation of proteins as well as lipids which is detrimental to the cell. Subsequently, supplementation studies although revealed decrease but on comparison with deficient (2-, 4- and 6-weeks) groups, there was an evident increase which could be due to an efflux probably from neutrophils, lymphocytes, monocytes, and platelets which has milli molar concentration of ascorbate[49] enhancing the oxidative defense mechanism by probably reducing the ascorbate radical generated not only during oxidative stress as well as by reduction of α-tocopheroxyl radical during lipid peroxidation. Ascorbate can act on catalytic metal ions reducing Fe^{3+} to Fe^{2+} (Fenton reaction), and itself gets oxidized to ascorbate radical. The ascorbate radical so generated could serve as a source for regeneration of extracellular ascorbate from transmembrane ascorbate radical reductase in erythrocyte using either NADH/intracellular ascorbate [50]. This may have been possible due to alteration in osmotic fragility and morphology of erythrocyte after zinc supplementation [8]. Change in osmotic fragility and lowered lipid peroxidation after zinc supplementation can be associated with the increase in ascorbic acid concentration in the present study. Canadan *et al.*, [51] observed the same phenomena wherein supplemented zinc and ascorbic acid improved osmotic fragility and decreased plasma lipid peroxides. α-tocopherol a major lipid-soluble chain-breaking antioxidant, readily reacts with superoxide.

It protects the membrane from damage by trapping the lipid peroxyl radicals [52]. Studies carried out on plasma levels of vitamin E of the adult population in the west revealed normal range from 5 µg/ml – 15 µg/ ml [53]. The decrease in the present study can possibly account for: (a) increased consumption to destroy oxygen free radicals (b) reduction in regeneration of α-tocopherol from tocopheroxyl radical as for its biological function it requires constant regulation (c) decreased concentration of ascorbate and /or (d) ring breakage of tocopherol quinine leading to loss of α-tocopherol. Synergistic action of zinc and α- tocopherol has been reported[54]. Both at low levels appear to cause damage to the cell membrane. Supplementation of zinc for a period of 4-weeks revealed decreased levels when groups were compared, but when these groups were compared with deficient experiment groups, an increase was observed. This can be correlated with decreased lipid peroxidation owing to suppression of free radical formation. The decrease of both vitamins suggests that deficiency of these two vitamins contributes to enhanced susceptibility of erythrocytes to oxidation injury. It is also apparent that ascorbic acid decreased first in comparison to α-tocopherol. Zinc concentration

when observed in the erythrocyte ghost membrane during the experiment decreased significantly after 4- and 6- weeks of zinc deficiency when ZD groups were compared with their respective ZC and PF groups. The decrease was significant even when PF groups were compared with respective ZC groups. The decline probably reflects: (a) reduced expression of Zn^{2+} transporters - ZIP which mediates the influx of $Zn^{2+,}$ and (b) decreased metallothionein level, which maintains the homeostatic balance. Deficiency of zinc has been reported to induce oxidative stress in organs [1].

One of the factors involved for altered zinc homeostasis has been reported to be due to nitrosative stress [55] as oxidative or nitrosative stress have been known to release zinc from proteins containing zinc-fingers and cluster motifs and its re-distribution[56]. Zinc supplementation to deficient groups (2-,4- and 6- weeks) still showed an evident decrease when ZD groups were compared to respective ZC and PF groups as well as when PF groups was compared with respective ZC group. Moreover, in comparison between zinc supplementation and deficient experiments, an increase was observed in supplementation groups. Supplementation studies emphasize the role of zinc as an antioxidant. Decreased NF-kβ activity causing a decline in the gene expression of TNF-α and IL β has been reported after zinc supplementation in HUT [Th_o (T helper) cell line] cell line [57]. Affect the involvement of zinc in reducing oxidative stress. Further, it also decreases the formation of free radicals through its antagonism to redox-active transition metals.

Copper, a structural requirement for the efficient functioning of various critical enzymes, functions both as an antioxidant and pro-oxidant. Although normally bound to proteins, copper may be released and become free to catalyze the formation of highly reactive hydroxyl radical. Authors [17, 18] reported that intracellular concentration of redox-active transition metal to be low with free iron in the range of 0.2-0.5 µM and free Cu^{2+} about a single ion/cell. In the present study, a high level of transition metal copper seems to have initiated the Fenton reaction, which may also have been one of the mechanisms to promote lipid peroxidation. Data obtained from *in-vitro* and culture studies largely indicate the capacity of copper to initiate oxidative damage and interfere with important cellular events [58]. Further, copper cannot exist in ionic form and is readily taken up by metallothionein (MT), forming Cu-MT, which in excess is prone to oxidative stress [59]. The present study revealed an increase in copper concentration which corroborates well with damage to the membrane as evident by increased protein oxidation, lipid peroxidation, hydroperoxide generation, OH radical formation, *etc*. Under situations of zinc deficiency, the balance maintained between both zinc and copper trace elements is affected. Zinc supplementation to zinc-deficient experiment groups declined the concentration of copper, achieving a balance between zinc and copper, probably a subsequent increase in stability of copper bound proteins and decrease in Cu-MT levels.

CONCLUSION: Dietary zinc deficiency after 2-, 4- and 6 weeks affected erythrocyte ghost membrane - total protein, protein carbonyl, protein SH group, lipid peroxidation, trace element - total zinc and copper. Erythrocyte cell suspension –total protein was affected with subsequent generation of OH radicals and hydroperoxides along with a disturbance in the physiological concentration of α-tocopherol and ascorbic acid. Supplementation of dietary zinc to deficient groups altered the profile, which was found to duration dependent. These observations provide evidence that a low level of zinc affects the integrity of erythrocytes which could lead to dysfunction.

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