Short-term zinc deficiency in diet induces increased oxidative stress in testes and epididymis of rats

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In order to determine the effects of Zinc deficient diet on oxidative stress in testis and epididymis, various parameters viz: total proteins, lipid peroxidation, hydroperoxides, antioxidant capacity and enzymatic activities are evaluated in rats fed on zinc deficient diet for 2, 4 and 6 weeks. Total proteins, water and lipid soluble antioxidant capacity decreased while lipid peroxidation (TBARS) and hydroperoxides concentration increased in testes, caput and cauda epididymis except in 2ZD (testes) where hydroperoxides revealed a significant decrease. GSH decreased in testes and caput and cauda epididymis. GPx and y-GT activities increased in testes and caput and cauda epididymis of zinc deficient rats. Further, GST increased in testes but exhibited decreases after 2 and 4 weeks and an increase after 6 weeks in caput and cauda epididymis of zinc deficient rats. GR activities decreased in testes but it increased in caput and cauda epididymis of zinc deficient rats. Thus, zinc deprivation results in increased sensitivity to oxidative stress. All these may have been as a consequence of increased ROS generation and/or decreased zinc dependent antioxidant processes.

Keywords: Antioxidant capacity, Epididymis lipid peroxidation, GSH, GST, Hydroperoxides, Testes, Zinc deficiency

Zinc is an essential nutrient for growth, reproduction, development, differentiation, immune and antioxidant functions, gene expression, DNA synthesis, hormone synthesis, storage and release of neurotransmitters, memory, visual processes and apoptosis1-6. It is required in more than 300 enzymes for both catalytic and structural roles whereas in zinc fingers or similar structures, it provides scaffold that organizes protein subdomains for the interaction with DNA or other proteins7,8. Studies suggest that reduced zinc availability affects membrane signaling systems and intracellular second messenger that coordinate cell proliferation in response to IGF-1. Deficiency of zinc leads to impaired testicular steroidogenesis9,10, altered protein patterns in caput epididymis11 and free radical mediated damage to some of the cell components12,13. Oxidative stress has been suggested to be an early effect of zinc deficiency rather than a simple reflection of zinc deficiency-induced tissue pathology14. Differential distribution of the element-specific fluorophore-Zinquin and its loss by treatment of spermatozoa with TritonX-100 suggested its (Zn) association with plasma membrane and is possibly involved in maturation of spermatozoa during passage through epididymis15. In spermatozoa of rats and bulls, in addition to aforesaid functions, zinc is thought to be bound to -SH groups of cysteine amino acids of proteins of the outer dense fibers of the spermatozoa tail16-18. Spermatozoal zinc is suggested to protect an inherent capacity of decondensation thereby helping to extend the life span of ejaculated sperm19.

Biological membranes contain high concentration of polyunsaturated fatty acids (PUFA) that are vulnerable to oxidative stress. Lipid peroxidation of PUFA molecules of plasma membrane of spermatozoa is largely responsible for defective spermatozoa and male infertility20,21. Lipid peroxidation causes leakiness of sperm plasma membrane, which in turn, loses its fluidity and integrity22, damages midpiece and axonemal complex leading to loss of motility and decreased capacitation23. There is no substantial literature that accounts for the influence of zinc deficiency on antioxidant parameters of male reproductive organs. The objective has been to provide a significance of zinc and for that lipid peroxidation, hydro-
zymes have been analyzed.

Materials and Methods

Colony bred Wistar rats (30 days old) used in the present study were fed a synthetic diet with either 100 ppm zinc or <1 ppm zinc for 2, 4 and 6-weeks. The synthetic diet was prepared according to Wallace et al. Animals (90) were divided into following three groups of 30 each: ZC (control; with 100 ppm zinc in the diet), ZD (zinc deficient; <1 ppm zinc the diet) and PF (pairfed; 100 ppm zinc control diet but the amount of the diet was equal to the amount consumed by zinc deficient group the previous day). All the animals were provided with demineralized water ad libitum.

Animals were autopsied after 2, 4 and 6-weeks under light ether anaesthesia. Testes and epididymis of both caput and cauda were excised, cleaned of extraneous tissues, weighed and processed for following biochemical estimations: total protein, lipid peroxidation, lipid hydroperoxides, antioxidant capacity and antioxidant enzymes have been analyzed.

A significant (P<0.01) decrease in GSH concentration in testes of 2ZD and 2PF as compared to 2ZC group and an increase in testes of 2ZD as against 2PF group have been observed. Decreased concentration was evident in 4 and 6 weeks ZD groups as compared to their respective ZC and PF groups. In caput and cauda epididymis, GSH exhibited significant decrease in the deficient groups of all the three subsets of the experiment as compared to their respective controls (Table 1).

GPx activity displayed significant increase in testes, caput and cauda epididymis from zinc deficient groups (2ZD, 4ZD and 6ZD) as compared to their respective control and pairfed groups. However, enzyme activity decreased (P>0.1) in 2PF and 4PF (testes) and 6PF (testes and cauda) groups when compared with their respective ZC groups (Table 1).

Glutathione reductase (GR) activity decreased significantly in testes of zinc deficient groups as compared to their respective control groups. The comparison of GR activity in testes of ZD groups with respective PF groups demonstrated non-significant decrease. Parallel were the observations with control and pairfed groups of 2 and 4 weeks experiment. However, in 6PF the decrease was highly significant (P<0.001) as compared to 6ZC group. Caput epididymis exhibited an increase in GR activity from 2, 4 and 6 ZD groups when compared to their respective control (ZC) groups (Table 1).

GST activity increased non-significantly in testes of both pairfed and zinc deficient groups as compared
Fig. 1—Effect of zinc deficiency on various parameters [(1) Total protein; (2) Lipid peroxidation; (3) Lipid hydroperoxides; (4) Antioxidant capacity-water soluble; (5) Antioxidant capacity-lipid soluble] in (a) Testes; (b) Caput epididymis and (c) Cauda epididymis in rats.
to their respective control groups except in 6PF where a non-significant decrease was observed. In caput and cauda epididymis, the enzyme activity decreased non-significantly in 2ZD and 4ZD and increased significantly in 6ZD as compared to their respective control groups (ZC). However, an increase in cauda epididymis of 4PF and 6PF groups as against their respective control groups was observed. Similarly, an increase was observed in 6ZD group when compared with 6PF group (Table 2).

Testicular γ-GT activity increased in 2ZD, 4ZD and 6ZD groups as compared to their respective control and pairfed groups. γ-GT activity decreased in caput and cauda epididymis of 2ZD and increased significantly in 4ZD and 6ZD groups as compared to their respective control groups. Comparison of PF group with their respective control groups indicated an increase in γ-GT activity (Table 2).

### Discussion

Zinc is essential for spermatogenesis and spermatozoa maturation and its deficiency leads to atrophy of seminiferous tubules, failure of spermatogenesis and decreases testosterone secretion due to impaired responsiveness of Leydig cells to gonadotrophin but not by hypothalamus-hypophyseal dysfunction. The elemental deficiency has been also implicated in increased susceptibility to oxidative damages in membrane suggesting that the increased oxidative stress may be a small but significant component of the pathology observed in dietary zinc deficiency.

Wistar rats of thirty days of age, fed on zinc deficient diet for 2, 4 and 6 weeks in the present study, displayed a wide range of zinc deficiency symptoms such as scabby lesions on skin and near foot pads, rough/spiny hair coat, loss of hair mostly from snout and head regions (alopecia), reddening of paws and nose-tips, hunched back posture, moderate polydipsia, intermittent mild diarrhoea and retarded growth in terms of body weight gains. The severity of zinc deficiency symptoms increased after second week of experiment. The control groups retained normal morphological features whereas pairfed groups exhibited retarded growth and restlessness. Similar morphological observations have been reported earlier.

Zinc deficiency has been known to impair protein synthesis in humans and animals. Recurrent diarrhoea, frequently observed in zinc deficient rats, has been associated with increased fecal nitrogen and
protein turnover and decreased efficiency of food utilization and energy. This leads to nutrient malabsorption that in turn plays an important role in pathogenesis of zinc deficiency. Total protein concentration decreased in testes, caput and cauda epididymis of zinc deficient group as compared to pairfed and control groups. Similar decreases have been reported in plasma and other tissues such as testis, ovary, liver and kidney. The decreased values have been attributed to impaired protein synthesis as zinc deficiency impairs utilization of amino acids in protein synthesis and increased protein turnover. This induces a negative nitrogen balance in the animal and thus decreases the protein levels.

High levels of PUFA are vulnerable to attacks by reactive oxygen species (ROS) leading to the formation of lipid peroxides that induce cross-linking of proteins. Both undernutrition and zinc deficiency causes three- to four-fold increase in malondialdehyde formation. This suggests for enhanced free radical generation and lipid peroxidation in zinc deficiency. The present study also exhibited an increased lipid peroxidation in testes, caput and cauda epididymis of zinc deficient and pairfed groups as compared to control groups. However, the lipid peroxidation was comparatively high in zinc deficient groups as compared to pairfed/adequate fed or control groups or in other words, the level of lipid peroxides in pairfed was almost intermittent between zinc deficient and control/adequate fed group. Further, iron concentrations have been reported to be high in testes of zinc deficient rats as compared to ad libitum controls. Thus, the increased oxidative damage observed in zinc deficient rats might have been as a consequence of increased ROS generation secondary to tissue iron accumulation and/or reduction in zinc dependent antioxidant process.

The water- and lipid-soluble antioxidant capacities of testes and epididymis decreased in zinc deficient groups as against control and pairfed groups. However, an increase in 2ZD and 4ZD was observed in testes when compared to their respective pair fed groups. The oxidative damage observed in the form of increased lipid peroxidation and decreased water- and lipid-soluble antioxidant capacity of testes and epididymis of zinc deficient rats may have occurred as a consequence of increased ROS generation secondary to tissue iron accumulation (not measured in the present study) and/or decreased zinc dependent antioxidant processes.
The elemental deficiency has also been implicated in increased susceptibility to oxidative damages in membrane suggesting that the increased oxidative stress may be a small but significant component of the pathology that has been observed in dietary zinc deficiency. Little blood supply and a low epididymal temperature (both reduce oxidative stress by limiting pO2 and cell metabolism) characterize epididymis yet the epididymal fluid contains high level of toxic hydroperoxide. Consequently mammalian spermatoza are at the risk during their transit and storage within the epididymis essentially because of their high contents of PUFA. Thus it is necessary for the epididymis to secrete antioxidant for scavenging ROS in order to enhance sperm survival. Natural antioxidant system of the body consists of GSH and the related enzymes. GSH through its thiolic/sulphhydryl group can react with H2O2, O2−, OH−, alkoxy radicals and lipid hydroperoxides and thus serves as a storage and transport form of cysteine, acts as a redox buffer and protects cells from toxicants and ROS generated by normal metabolism. A positive correlation between zinc and GSH contents of platelets in normal and uremic patients and an increase in GSH levels in culture cells on exposure to 110 μM of ZnCl2 have been reported. This increase has been suggested to be due to binding of GSH to Zn2+, lowering GSH concentration and signaling GSH synthesis. Sertoli and interstitial cells, pachytene spermatocytes, round spermatids, spermatozoa and epididymal fluid have high GSH level. GSH is important for sperm penetration and has a significant effect on sperm motility pattern. In the present study, GSH levels decreased significantly in testes, caput and cauda epididymis of zinc deficient as compared to control or pairfed rats. Depletion of GSH in various cells has been related to (i) inhibition of GSH synthesis; (ii) oxidation of GSH to GSSG; and (iii) conjugation by transferase. Zinc deficiency, as well GSH depletion are both responsible for apoptosis. Thus zinc deficiency is one of the factors in reduced levels of GSH in testes and epididymis.

Testes have been reported to have low cytosolic GPx and have been implicated in the metabolism of H2O2 associated with steroid hormone synthesis in Leydig cells. Mouse sperm and epididymal fluid also contain high concentration of GPx and in spermatozoa it has been believed to be necessary for sperm motility. PHGPx mRNA is abundantly and preferentially expressed in soluble form at transition stage of round to elongated spermatids. All these in vivo experiments indicate for gonadotrophin dependent expression of PHGPx in testes. In testicular cells, it has been localized in mitochondria where it is membrane bound and in nuclei where it is bound to chromatin. It has also been reported from mid-piece of mature spermatozoa (at least 50% of the capsule material that embeds in the helix of mitochondria) as an enzymatically inactive and oxidatively cross-linked insoluble protein and in head along with condensed chromatin. PHGPx, in testes, is believed to protect sperm from oxidant or peroxidant damage particularly during periods of rapid proliferation and gametogenesis. Immunogold electron microscopy has indicated the association of PHGPx with chromatin structure in isolated nuclei. A possible interpretation of these data is that PHGPx protective role against DNA peroxidative damage. Sertoli cell phagocytosis increases synthesis of organic hydroperoxide and GPx is responsible for removal of these. An overall decline in testicular PHGPx mRNA transcript has been reported after efferent duct ligation and cryptorchidism and has been associated to germ cell depletion. On the contrary, in the present study, an increase in GPx has been estimated in the testes of zinc deficient as compared to pairfed and control rats. Oteiza et al. observed no variation in GPx activity in testes of zinc deficient rats as compared to pairfed and control rats. Sertoli cell phagocytosis is increased in testicular injury for removal of cell debris and residual bodies. Thus an increased concentration could be required for removal of these. E-GPx interacts with the transiting spermatozoa and protects their cell integrity. Hence, an increased activity has been estimated in epididymis.

Glutathione reductase (GR) activities have been reported to be high in Sertoli cells and low in germ cells. Further, the activities of GR have been reported to increase significantly in testes of rats fed on corn oil diets and this appears to be in response to the lipid peroxidation. Again, the activities of GR were significantly higher (23%) in the testes of zinc deficient animals than in those of ad libitum control. In the present study, in spite of corn oil diet and increased lipid peroxidation (reported elsewhere), GR activities decreased in testes and increased in caput and cauda epididymis of zinc deficient rats as compared to their respective control and pairfed rats. Northern blot analysis of rat GST-cDNA with different testicular cells have shown GSTmRNA
localization in testicular cytosol, Sertoli and peritubular cells from testes of normal rat and Leydig tumor cells.\(^5\). Peltola et al.\(^6\) demonstrated a 31% decrease in GST following administration of testosterone for eight days. A significant decrease in GST activity in both paired and zinc deficient animals and a still further decrease in zinc deficient animals as compared to paired has been reported. The decrease has been correlated to additive effect of food restriction and zinc deficiency.\(^6\) In the present study, significant increases have been observed in GST activities in testes and epididymis, in caput and cauda epididymis of 6-week zinc deficient rats. However non-significant decreases were estimated in caput and cauda epididymis of 2 and 4 week of zinc deficient rats. Reduced GSH is the major reductant for the defense of the cells against toxic xenobiotics and ROS. Its depletion generates cell sensitivity to oxidants and induces an antioxidant response mediated by an increase in xenobiotic-metabolizing enzymes such as GST.\(^7\)

High levels of \(\gamma\)-GT despite germ cell depletion or low levels in immature seminiferous tubules indicate for it being a marker enzyme for Sertoli cell maturation.\(^8\) Hormonal deprivation by hypophysectomy or GnRH-antagonist treatment of immature rats, resulted in marked decrease in testicular \(\gamma\)-GT activity than in the testes of age-matched controls.\(^9\) FSH treatment after 15 days hypophysectomy significantly increased testicular \(\gamma\)-GT above that in testes from hypophysectomy controls in a time- and dose-dependent manner. Thus, the data indicate that FSH regulates \(\gamma\)-GT activity in rat testes and of its Sertoli cell.\(^9\) However, the role of androgen is controversial. Hatier et al.\(^10\) considered \(\gamma\)-GT to be androgen dependent while Caston and Sanborn\(^10\) could demonstrate a small effect of testosterone on \(\gamma\)-GT activity. Moreover, in hepatocytes and in other cell types, xenobiotics (alcohol, quinine etc), hypoxia or radiation-mediated oxidative stress induces \(\gamma\)-GT activity related to GSH depletion.\(^11\) In epididymis, two \(\gamma\)-GT transcripts (mRNA III & IV) are sensitive to oxidant stimuli and their upregulation depend upon the type of ROS generated by oxidizing agent.\(^12\) Hence the observed increase in \(\gamma\)-GT activities in testes and epididymis, in the present study, is corroborated to (i) increased FSH because of lack of feed back from testicular testosterone (ii) decreased level of GSH and (iii) zinc deficiency induced oxidative stress.

References
27 Prieto P, Pineda M & Aguilar M, Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E, Anal Biochem, 269 (1999) 337.
30 Paglia DE & Valentine WN, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, J Lab Clin Metab, 70 (1967) 158.
32 Carlgberg I & Mannervik B, Glutathione reductase, Methods Enzymol, 113 (1985) 484.
34 Tate SS & Meister A, y-Glutamyl transpeptidase from kidney, Methods Enzymol, 111 (1985) 400.


68 Lu C & Steinberger A. Gamma-glutamyl transpeptidase activity in the developing rat testes. Enzyme localization in isolated cell types, Bio Reprod, 17 (1977) 84.

