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Autometallographic Study after Dietary Zinc Insufficiency: Wistar Rat Ventral Prostate

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ABSTRACT:
Zinc is an essential biological trace element with acquired zinc deficiency being common and spread worldwide affecting all age groups. The study investigates the effect of low dietary zinc on Wistar rat ventral prostate. Prepubertal male Wistar rats (35-50 g) were grouped into, negative control (standard feed), zinc control (100 µg Zn /g), pair fed (100 µg Zn /g but diet given was equal to the diet taken by zinc deficient group the previous day) and zinc deficient (1.00 µg Zn /g) for 4- and 6- weeks. Localization of zinc nanocrystal using autometallographic technique after 4 weeks of deficiency decreased ranging from moderate to faint in columnar epithelial cells, glandular cell layer, intra-luminal secretion, secretory vesicles with uneven dispersion in degenerated fibromuscular stroma as against the control groups. Further decrease in localization of free zinc ion was detected after 6 weeks of deficiency. Decline in zinc localization was also evident in pairfed groups accounting for stress and starvation due to limited food intake. These observations were further supported by decline in ventral prostate total zinc level as assessed by atomic absorption spectrophotometer. Depletion of zinc in ventral prostate would alter the internal environment milieu / prostatic secretions leading to increased risk for infertility.

Keywords:
Zinc deficiency, ventral prostate, autometallography.

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INTRODUCTION

Nutritional trace element zinc deficit is extensively prevalent not only in most Latin America and Caribbean countries (Cediel et al., 2015) but also in Sub-Saharan Africa, South Asia (Wessells and Brown , 2012),other developing countries with India being one of them (Chiplonkar et al., 2003) and is emerging as a worldwide problem. Globally, zinc insufficiencies among adults, children and neonates were reported and have become a major challenge due to its diverse biological functions (Ahsan et al., 2021; Kageyama et al., 2022; Duffy et al., 2023; Molenda and Kolmas, 2023; Bellini et al., 2024; Chen et al., 2024).Some of the clinical manifestations occurring due to zinc deficiency has been recognized (Hussain et al., 2022; Stiles et al., 2024). Efflux and influx of zinc is regulated by transporters - ZIP as well as ZnT (Eide, 2016; Stiles et al., 2024) and significant association with metallothioneins

(Kimura and Kambe, 2016). Human prostatic fluid has ~500 mg Zn/mL fluid (Costello and Franklin. 1998). Total zinc in normal rat ventral prostate detected was in the range of 0.103 – 0.202 µg/ mg protein (Joshi et al., 2014 a). Primarily derived from the prostate gland, seminal plasma zinc may indicate the function of the prostatic secretary (Khan et al., 2011). Another important aspect of zinc in prostate is to inhibit mACO2 activity - a feature required for appropriate citrate accumulation in prostate epithelial cells (Costello et al., 1997; Xue et al., 2019). Inadeqate zinc lead to deterioration of ventral prostate (Joshi et al., 2014 a, b) which would have an impact on prostate function. The study assesses autometallographic localization of zinc nanocrystal in Wistar rat ventral prostate and total organ zinc after dietary zinc deficiency.

MATERIALS AND METHODS

Synthetic Diet

Basal diet as prepared in accordance with the composition of American Institute of Nutrition AIN -76 semi-purified diet (ICN Research Diet,1999) given by ICN Nutritional Biosciences, Life Sciences Group based on studies of Bieri et al., (1977) and Bieri (1980). The composition was prepared as follows: Egg white-180 gm, Corn oil- 100 gm, Corn starch- 443gm, Sucrose-200 gm, Cellulose-30 gm, Choline Chloride-2 gm, AIN-76 Salt mixture -35, AIN-76C Vitamin antibiotic mixture-10gm ,DL-methionine-7 gm.

Zinc contents of the synthetic / basal diet from each lot were estimated on GBC 902 double beam Atomic Absorption Spectrophotometer (Australia) at 213.9 nm in air-acetylene flame and zinc concentration was adjusted to 1.0 ppm and 100 ppm by addition of appropriate amounts of zinc sulphate.

Experimental protocol

Forty eight colony bred pre-pubertal male Wistar rats (30-40 days of age, 40-50 gm) were divided into four groups(each group comprised of 6 animals in a 4- and 6 week sub group): (1) Zinc control (ZC) - synthetic diet (100 ppm zinc diet) and tap water provided *ad libitum* (2) Pair fed - 100 ppm zinc diet but the amount of the feed given was equal to the feed consumed (average) by zinc deficient group the previous day. Tap water was provided *ad libitum.* This group was run so as to study starvation effects caused by reduced intake of diet and stress and (3) Zinc deficient (ZD) - 1.00 ppm diet and demineralized water was provided *ad libitum.* Individually animals were housed in polypropylene cages with stainless steel grills. The polypropylene cages, grills and bottles were washed daily with detergent solution, demineralized water and finally rinsed in 10% EDTA solution prepared in demineralized water so as to avoid any contamination and subsequent removal of zinc from the cages, grills and bottles.

Ethical approval , The experiments were set for 4- and 6- weeks and approved by Department Animal Ethics Committee, Department of Zoology, University of Rajasthan, Jaipur, India and Committee for the Purpose of Control and Supervision of Experiments on Animals (No. 1678/GO/Re/S/12/CPCSEA dated 16.06.17). After completion of experiments, the animals were anesthetized by intraperitoneal (I.P) injection using sodium thiopentone (20 mg/kg body weight, Thiosol sodium, Neon Laboratories Ltd. Mumbai, India) ventral prostate removed, weighed on electronic balance and processed for autometallography and trace element zinc studies.

Autometallography – Zinc nanocrystal localization protocol

Sodium selenite (Na_2SeO_3) was administered to normal and experimental animals intraperitoneally (10 mg /kg). After a survival period of 2 hrs, animals were transcardially perfused with 400 ml 25 glutaraldehyde in 0.1M Sorenson buffer, pH 7.4.Ventral prostate was excised quickly and refixed in 2% glutaraldehyde in 0.1M Sorenson buffer pH 7.4, dehydrated, cleared and embedded in epon resin below 45 ^{0} C. Semithin sections (2µ) were cut on ultratome (AIIMS, New Delhi) and placed on Farmer's solution pretreated glass slides and dried for 1 hr on heating plate at 45 ^{0}C. These slides were coated with 0.5% gelatin and allowed to air dry for 30 min. and dipped in AMG developer (unrefined crystalline gum arabic, citrate buffer, hydroquinone, silver lactate) (Danscher, 1981) for 60 minutes. Slides

were then washed with deionized water, 5% sodium thiosulphate solution for 5 minutes, rinsed in running tap water at 40^0C for 20 minutes, dehydrated, mounted in DPX and examined under light microscope (Su et al., 1997).

Organ total zinc element study

Ventral prostatic zinc was estimated at 213.9 nm respectively in an air acetylene flame with 0.5 nm slit width, background correction and an integration time of 3.0 sec. in Varian fast sequential AA240FS (Australia) atomic absorption spectrophotometer. Known quantities of ventral prostate of different groups was digested in diacidic solution (HNO_3: $HClO_{4,5:1}$) and then diluted with distilled water. The homogenous preparation was aspirated into Varian fast sequential AA240FS (Australia) atomic absorption spectrophotometer in air acetylene flame and atomized.

Statistical Analysis,

Data expressed as mean ± SEM. One Way Analysis of Variance (ANOVA) was carried out separately for 4- and 6- week zinc deficient groups followed by post- hoc test (Tukey's Multiple Comparison test) if the difference was found to be significant. Data were analyzed using Graph Pad Prism Version 7.0e. $P < 0.05$ was considered to be significant.

RESULTS

Zinc control (Figs. 1 & 4) groups of 4- and 6-week experiment revealed moderate localization of zinc nanocrystal in columnar glandular epithelium, secretory vesicles and basal cells, prominent nanocrystals in the nucleus with nucleoli as well as basement membrane, homogenous distribution of nanocrystal in intra-luminal secretion and high in basement membrane and fibromuscular stroma**.** In 4 week pair fed group, the localization of zinc nanocrystal was moderate in and around the nucleus although less than controls, faint to moderate in secretory vesicles, moderate and even localization in intra-luminal secretion, high in basement membrane and fibromuscular stroma (Fig.2) whereas after 4 weeks zinc deficiency moderate to faint localization of zinc nanocrystal within as well as around the columnar epithelial cell nuclei, moderate in prostatic epithelial glandular cells layer and basement membrane, faint to moderate localization of nano-crystal in intra- luminal secretion, decreased and faint granules in secretory vesicles and uneven dispersion in degenerated fibromuscular stroma was detected (Fig. 3).

Ventral prostate of 6 weeks pair fed group revealed moderate to faint zinc nano-crystals in the nucleus of columnar epithelial cells, faint granules in secretory vesicles, localization of nanocrystal was moderate in prostatic epithelial cell layer and basal membrane, distinct even distribution in intra-luminal secretion with aggregation of AMG Zn-S nano-crystal in few tubular lumen and moderate granules localized in fibromuscular stroma (Fig.5). After 6 weeks of zinc insufficiency moderate to faint zinc nano-crystal were detected within and around the nuclei of columnar epithelial cells, faint granules in secretory vesicles and basal cells, moderate to faint AMG Zn-S detected in basal membrane, few aggregation of AMG Zn-S grains detected in the intra luminal secretion and uneven dispersion of faint zinc granules in fibromuscular stroma (Fig. 6). Further, there was evident decrease in gradation of localization of free zinc ion from ZC to PF, ZC to ZD and PF to ZD.

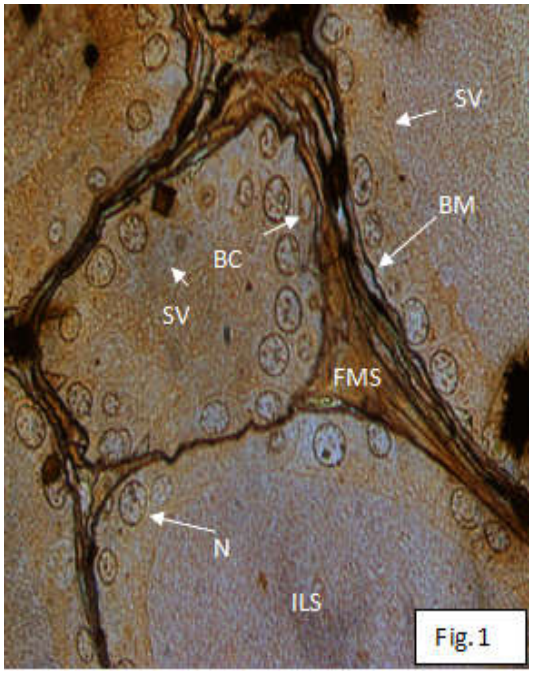

Figure 1: Microphotograph of autometallographic localization of zinc in epoxy embedded ventral prostate of 4 weeks zinc control pre-pubertal Wistar rat exhibiting prominent zinc nanocrystals in the nucleus(N) as well as nucleolus of columnar epithelial cells, moderate in secretory vesicle (SV) and basal cells (BC), high zinc granules localization in basement membrane (BM), homogenous distribution in intra-luminal secretion (ILS) and intense AMG Zn-S nanocrystal in fibromuscular stroma (FMS). 1000x.

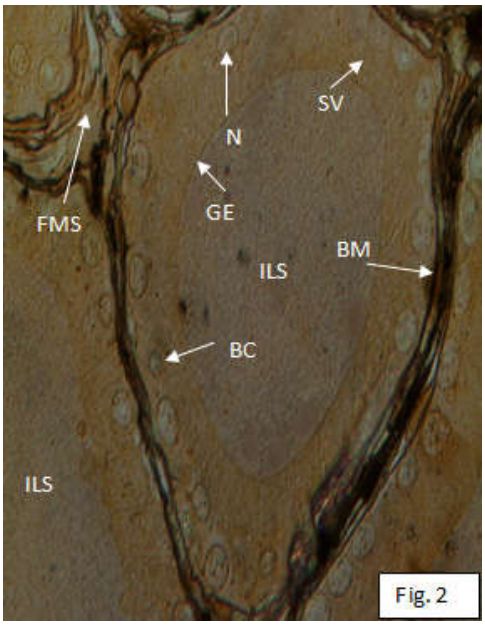

Figure 2: Microphotograph of autometallographic localization of zinc in epoxy embedded ventral prostate of 4 weeks zinc pair fed (PF) pre-pubertal Wistar rat showing zinc nanocrystals in the nucleus (N) and prostatic glandular epithelial cells layer (GE), faint to moderate localization in secretory vesicles (SV) evenly dispersed in intraluminal secretion (ILS), with high zinc localization in basement membrane (BM) and fibromuscular stroma (FMS).1000x.

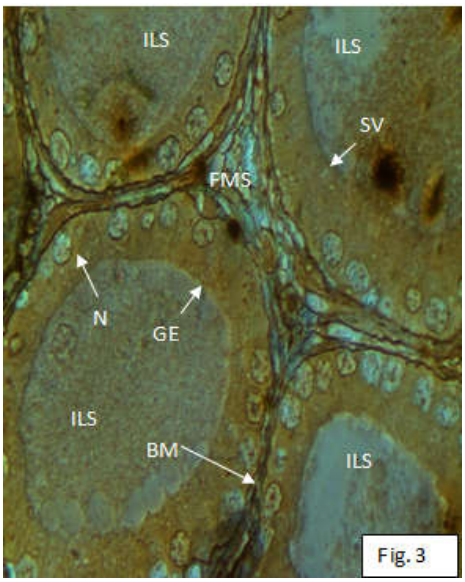

Figure 3: Microphotograph of autometallographic localization of zinc in epoxy embedded Wistar rat ventral prostate of 4 weeks zinc deficient group exhibiting moderate to faint localization of zinc nanocrystal within and around the nuclei (N) of columnar epithelial cells, moderate in prostatic epithelial glandular cells layer (GE), moderate AMG-S granules in basement membrane (BM), faint to moderate localization of nano-crystal in intra- luminal secretion (ILS), decreased and faint granules in secretory vesicles (SV) and uneven dispersion in degenerated fibromuscular stroma (FMS).1000x.

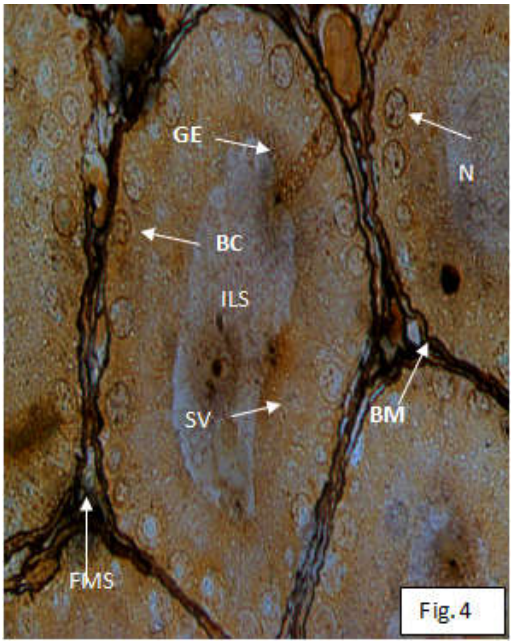

Figure 4: Microphotograph of autometallographic localization of zinc in epoxy embedded ventral prostate of 6 weeks zinc control (ZC) pre-pubertal Wistar rat exhibiting prominent zinc nanocrystals in the nucleus(N) and moderate localization in cytpoplasm of columnar epithelial cells, moderate in prostatic glandular epithelium layer (GE), moderate zinc granules localized in secretory vesicle (SV),basement membrane (BM) and basal cells (BC),even distribution in intraluminal secretion (ILS) and high localization of AMG zinc nanocrystal in fibromuscular stroma (FMS). 1000x.

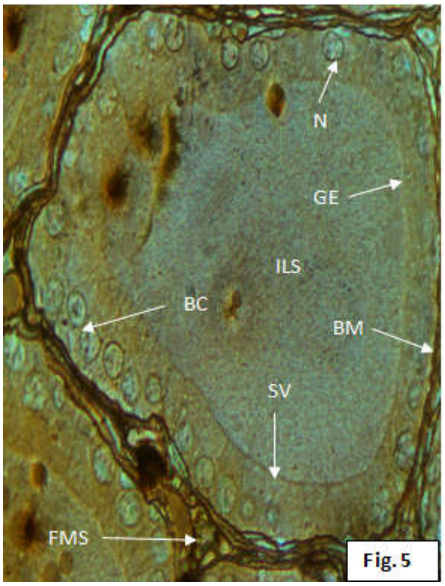

Figure 5: Microphotograph of autometallographic localization of zinc in epoxy embedded ventral prostate of 6 week pair fed (PF) pre-pubertal Wistar rat showing few moderate/faint zinc nano-crystals in the nucleus(N) of columnar epithelial cells, faint granules in secretory vesicles(SV), localization of nano-crystal was moderate in prostatic glandular epithelial cells layer (GE) and basal membrane(BM), distinct, even and faint distribution in intra-luminal secretion(ILS) and moderate localization in fibromuscular stroma (FMS).1000x.

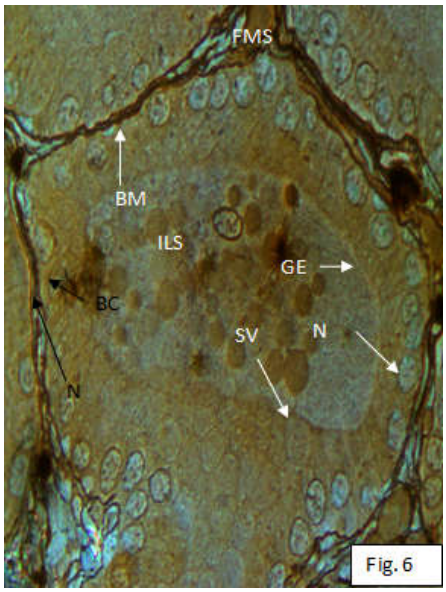

Figure 6: Microphotograph of autometallographic localization of zinc in epoxy embedded ventral prostate of 6 weeks zinc deficient (ZD) pre-pubertal Wistar rat exhibiting moderate to faint localization of zinc nanocrystal within and around the nuclei (N) of columnar epithelial cells, faint intensity granules localized in secretory vesicles and basal cells, moderate to faint AMG Zn-S detected in basal membrane (BM),few aggregation of AMG Zn-S nanaocrystal detected in the intra luminal secretion (ILS) and uneven faint dispersion of zinc nanocrystal in fibromuscular stroma (FMS).1000x.

Total zinc

Significant (P< 0.05) decrease in total zinc concentration of ventral prostate was recorded in zinc deficient groups (4ZD and 6 ZD) after comparison with NC and ZC as well as PF. Pair fed groups of 4-and 6-weeks exhibited significant (P<0.05) reduction as against control (NC and ZC groups) (Table 1).

Table 1: Zinc concentration in ventral prostate (µg /gm) of Wistar rat after 4 - and 6 - weeks of dietary zinc deficiency (Mean ±SEM)

Groups	4 weeks	6 weeks
ZC	0.2161 ±0.0021	0.3193 ± 0.0015
PF	0.1208 ± 0.0016 a*	0.1226 ± 0.0010 a*
ZD	0.0266±0.0017 b*c*	0.0183 ±0.0011 b*c*

Where * P<0.05 Significant, a =ZC Vs PF, b =PF Vs ZD, c = ZC Vs ZD
Multiple comparison procedures were performed for 4- and 6 weeks experimental groups.

DISCUSSION

Prostate has a significant role in molecular pathways involved in ejaculation, sperm activation as well as capacitation (Gilany et al., 2015) with Zn^{2+} present within the cytoplasm of prostatic epithelium (Baltaci and Mogulkoc, 2012). Zinc nanocrystal localization of variable intensity in ventral prostate of control group indicates the occurrence of ionized zinc and provides evidence of zinc ions physiological function. The zinc-enriched secretory cells in the prostate are part of glandular cells system that use zinc ions to assemble macro-molecules for excretion. The glandular cells secrete zinc ions continuously into the acinar lumen and intercellular canaliculi (Sørensen et al., 1997). Using indirect immunogold labeling method with anti-MT, the presence of metallothionein was affirmed in the rER, secretory vesicles, secretory products and sub epithelial connective tissue of the prostate (Bataineh et al., 1986). Their presence at these regions implies that MT associates zinc intracellularly as well as extracellularly, where it probably may play a role in zinc metabolism as well as storage. Zinc ions deposits in pair fed groups (4 – and 6 – weeks) were less compared to controls. This indicates that stress and starvation had an impact on zinc ions with consequent loss of zinc. Intensity of AMG Zn-S deposits further reduced with uneven dispersion in fibromuscular stroma. Reduced specific activity can also be correlated to the lost expression of particular zinc uptake transporters which causes the epithelial cells to lose their capacity to collect zinc and consequently their capacity to accumulate citrate as well (Franklin et al., 2005).The reduced specific localization is in agreement with the decreased total zinc organ concentration after deficiency.

"Zinc-accumulating citrate-producing cells" are specialized normal prostate acinar epithelial cells with zinc concentration in the range of ~800 –1500 µM (Costello and Franklin, 2016). The mitochondrial zinc concentration is around 20 times higher in normal prostate epithelial cells than in other cells due to the significantly high cellular zinc content (Liu *et al.,* 1997). Testosterone as well as prolactin coordinate the major events such as: (i) zinc build up in cells and mitochondria is increased due to up regulation of the ZIP1 zinc transporter (ii) citrate exported from mitochondria to the cytosol and then secreted into prostatic fluid and (iii) aspartate transporter, mitochondrial aspartate aminotransferase, pyruvate dehydrogenase E1a and other enzymes upregulated (Costello and Franklin,2016).In humans, peripheral zone acini epithelial cells both hormones regulate zinc uptake as well as accumulation (Liu *et al.,* 1997; Costello *et al.,* 1999; Costello and Franklin, 2002). Low intracellular zinc level has been linked to impairment of zinc transporters including ZnT1, ZnT3-4 and Zip1-3 (Beck *et al.,* 2004; Iguchi *et al.,* 2004). Tubulin depolymerization is induced by low intracellular zinc level which although may signal for activation of NF-κB but its nuclear

translocation gets impeded which inhibits the transactivation of NF-κB-driven genes one of the causative factor affecting cell subsistence (Mackenzie *et al.,* 2002).Decreased zinc levels in serum and prostate reflect low expression of ZIP that mediate zinc inflow in prostatic cells (Huang *et al.,* 2006) as there is degeneration after dietary zinc deficiency. Deficiency of zinc results in accrual of reactive oxygen species in the prostate (Kelleher et al., 2011; Maret, 2013; Karunasinghe, 2022). Signalling pathways within the cells which involves S-nitrosation of metallothionein associates NO and zinc (Kröncke et al., 1994; St.Croix *et al.,* 2002). Additionally, liberation of labile zinc maintained by metallothionein was not able to be maintained at a constant level due to NO increase (St.Croix *et al.,* 2002).High level of NO would cause oxidative stress (Joshi *et al.,* 2014 a) with generation of NO radical which in turn causes damage of Zn-S clusters and liberate zinc from numerous proteins pertinent in changing expression of gene. Subsequently, decline in regulation of metallothionein occurs due to zinc deficiency and this interferes with the varying Zn^{+2} pool known to interact with nitric oxide. In addition, superoxide radicals and NO may interact resulting in redox imbalance (Nair et al., 2005; Bedwal et al., 2009;Joshi et al., 2014a).Decreased hormonal level after dietary zinc deficient has also been recorded (Singh et al., 2023). Dietary zinc deficiency caused diminution in ventral prostate total zinc concentration which correlates with localization studies and would impede prostatic functions. This may lead to infertility if the deficiency duration is enhanced.

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Conflicts of interest: There is no conflict between the authors.

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