Original Article

Molecular basis for the effects of zinc deficiency on spermatogenesis: An experimental study in the Sprague-dawley rat model

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ABSTRACT

Introduction: The objective of this study is to investigate the molecular mechanisms underlying the effects of zinc deficiency on spermatogenesis in the Sprague-Dawley (SD) rat.

Materials and Methods: Three groups of eight adult male SD rats were maintained for 4 weeks on a normal diet as control, zinc deficient diet and zinc deficient diet with zinc supplementation of 28 mg zinc/kg body weight respectively. Using standard techniques, the following parameters were compared between the three groups of experimental animals at the end of 4 weeks: (a) Serum zinc, magnesium (Mg), copper (Cu), selenium (Se) and cadmium (Cd), (b) serum sex hormones, malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPX), (c) interleukin-4 (IL-4), tumor necrosis factor-alpha (TNF-α), Bcl-2, Bax and caspase-3 expression in the testes, (d) assessment of apoptosis of testicular cells using electron microscopy and (e) testicular volume and histology using the orchidometer and Johnsen score, respectively.

Results: The zinc deficient group showed a reduction of testicular volume, serum concentrations of Zn, Cu, Se, Mg, SOD, GPX, IL-4, Bcl-2 and testosterone ($P < 0.05$), as well as increased levels of serum Cd, MDA and tissue TNF-α, Bax, caspase-3 and apoptosis of the germ cells ($P < 0.05$) compared with control and zinc supplementation groups.

Conclusion: Zinc deficiency is associated with impaired spermatogenesis because of reduced testosterone production, increased oxidative stress and apoptosis. These findings suggest that zinc has a role in male reproduction.

Key words: Apoptosis, oxidative stress, sex hormones, spermatogenesis, zinc

INTRODUCTION

Zinc (Zn) is critical to the function of over 300 enzymes. Many zinc dependent enzymes such as RNA polymerases, alcohol dehydrogenase, carbonic anhydrase and alkaline phosphatase depend on zinc as a cofactor. Zinc is essential for the structural formation of many biologically active molecules (e.g. copper-zinc dismutase). It has also been shown to influence both apoptosis and protein kinase C activity. Some investigators have demonstrated that zinc is key to membrane integrity[1] and the nucleus and chromatin are thought to have high concentrations of zinc. Chester *et al.*[2] have also demonstrated that zinc is very essential in DNA synthesis, cell proliferation and immunocompetence.

In both *in vitro* and *in vivo* models, Zn supplementation prevents apoptosis induced by a variety of agents such as umor necrosis factor-alpha (TNF-α), the Fas ligand, and chemotherapeutic agents and cells grown under conditions of Zn deprivation/deficiency.[3,4] Intracellular agents, which regulate apoptosis include proteases, phosphatases, kinases, products of lipid metabolism and the cations Ca^{2+} and Zn. The

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possible protective effect of Zn against apoptosis has been attributed to its inhibition of a Ca^{2+}- and Mg^{2+}- dependent endonuclease, thereby preventing DNA fragmentation, a terminal step and hallmark of apoptosis.[4,5]

One marked effect of zinc deficiency in developing humans and animals is hypogonadism.[6] The mechanisms underlying zinc deficiency-associated alterations in testicular development and function include impaired testicular steroidogenesis, free radical-mediated damage to some components of the developing male rats that results in reduced testicular growth.[5-7] Such a testis is characterized by a high ratio of 2-thiobarbituric-reactive substances (TBARS), an indicator of lipid peroxidation, and low glutamine synthetase activity, indicator of protein oxidation; and a high concentration of 8-oxo-2'-deoxyguanosine, a marker of DNA oxidation. Furthermore, severe zinc deficiency has been shown to adversely affect sperm integrity and also modulate fatty acid composition by interrupting essential fatty acid metabolism.[8] It has also been suggested that zinc deficiency-associated testicular malfunction is often preceded by altered membrane fatty acid composition.[8] Recently, Yamaguchi *et al*.[9] suggested that zinc is an essential trace element for the maintenance of germ cells, the progression of spermatogenesis and the regulation of sperm motility.

Zn and copper (Cu) are components of superoxide dismutase (SOD) and selenium (Se) is part of glutathione peroxidase (GPX), which are two important enzymatic antioxidants.[1-3] It is known that most elements interact in many ways and change in one may also be reflected by deviations in the others. Element interactions are crucial for an understanding of the mechanisms involved in environmental exposure to inorganic elements. Magnesium (Mg), zinc, and chromium are mineral elements required in modest amounts to maintain health and optimal physiologic function and perform important roles in regulating whole-body metabolism, including energy utilization and work performance. Magnesium, a ubiquitous element that plays a fundamental role in many cellular reactions, is involved in over 300 enzymatic reactions in which food is catabolized and new chemical products are formed. Examples include glycogen breakdown, fat oxidation, protein synthesis, adenosine triphosphate synthesis, and the second messenger system. Magnesium also serves as a physiologic regulator of membrane stability and is involved in neuromuscular, cardiovascular, immune, and hormonal function. On the other hand, it is well-known that many toxic effects of cadmium (Cd) action result from interactions with essential elements, including zinc.[5] These interactions can take place at different stages of absorption, distribution in the organism and excretion of both metals and at the stage of Zn biological functions. Exposure to Cd leads to disturbance in Zn in the organism on one hand, while dietary Zn intake has an important effect on Cd absorption, accumulation and toxicity on the other. The Zn status of the body is important in relation to the development of Cd toxicity. Numerous data show that increased Zn supply may reduce Cd absorption and accumulation and prevent or reduce the adverse actions of Cd, whereas Zn deficiency can intensify Cd accumulation and toxicity.[3,5,9] Because of the close interactions between these trace elements, we intend to measure the serum levels of the following trace elements; Zn, Cu, Se, Mg, Cd as well as levels of SOD and GPX.

From the above, it is evident that zinc is not only a cofactor in proteins involved in antioxidant defenses, electron transport, DNA repair, and p53 protein expression that is important in the prevention of cell damage, but it is also an essential element in male fertility through various mechanisms.[3,8-11] So far, no study has fully addressed the association between zinc deficiency and the role of Bcl-2 family members in modulating apoptosis during spermatogenesis in Sprague-Dawley (SD) rat. Similarly, changes in the seminiferous tubules on electron microscopy (EM) in zinc deficient animals have received scanty attention in the literature. The aim of this study was, therefore, to investigate the molecular basis for the effects of zinc deficiency on spermatogenesis in the SD rat with regards to its possible effect on the antioxidant system and the Bcl-2 family as well as other regulators of apoptosis. Furthermore, we investigated events at the cellular level in an attempt to better understand some of the morphological and biochemical changes seen in experimental animals fed with zinc deficient diet.

MATERIALS AND METHODS

Experimental animal groups and diets

Three groups of adult male SD rats weighing about 180 g were used for the study: (a) 8 SD rats on a normal diet, as control, (b) 8 SD rats on continuous zinc deficient diet for 4 weeks and (c) 8 SD rats initially fed on zinc deficient diet for 2 weeks and subsequently fed zinc supplementation diet containing 28 mg Zn/kg body weight for another 4 weeks. Rats on a normal diet (control) had diet containing 28 mg zinc/kg. Rats on zinc deficient diets were on diets with < 1 mg zinc/kg. Rats on zinc supplementation diets were initially on zinc deficient diets for 2 weeks before being fed diets containing 28 mg zinc/kg for another period of 4 weeks. The lifespan of SD rats used in our experiments is very short, usually about 24-36 months. Similarly, the spermatogenic cycle in SD rats is about 13 days; consequently, it is possible to see the effect of factors affecting spermatogenesis within 1-2 spermatogenic cycles that is between 13 and 26 days. The rats were kept in a controlled environment of about 23°C, 55% humidity and 12 h of daylight and night, respectively. After randomization, the rats were allowed access to food and water *ad libitum*. The animals were cared for according to guidelines of the Kuwait University Animal Resource Center. The local Ethics Committee gave approval for this study. All measurements and analyses, etc., described in this study were blinded.

The initial testicular volume was estimated by pictorial orchidometry using beads and the weight extrapolated after harvesting the testes at the end of the experimental period. The testes were harvested at the end of 4 weeks of observation in control animals and animals fed zinc deficient diet. In animals fed zinc supplemented diet, the testes were harvested at the end of 6 weeks of observation. Blood was collected from all the rats weekly from the tail vein for biochemical analyses (*vide infra*). At the end of four or 6 weeks, the rats were sacrificed and the testes harvested and immediately divided into three portions and used for histological examination, immunohistochemical staining and EM. The rats were anesthetized by administering sodium pentobarbitone at a dosage of 40 mg/kg of body weight.

Histological examination

A portion of the testis was preserved in 4% formaldehyde solution, processed routinely into paraffin and sections stained with hematoxylin-eosin. Spermatogenesis was assessed using the Johnsen scoring system[12] [Table 1].

Immunohistochemical staining for Bcl-2, Bax and C3

Immunohistochemical studies were carried out on paraffin sections as described by Eguchi *et al*.[13] Briefly, specific polyclonal antibodies and biotinylated rabbit-anti-mouse immunoglobulins were used to produce a distinct granular cytoplasmic staining, evaluated on a scale of 0 (no staining) to 5 (maximum intensity).

Electron microscopy

Preparation of rat testes for EM: A piece of the harvested testis was washed with phosphate buffered saline (PBS) and immersed in glutaraldehyde overnight at 4°C. Thereafter, the testis was cut into 1 mm pieces and washed 3 times in fresh Millonig's PBS to remove traces of glutaraldehyde and fixed in 1% osmic acid and washed in Millonig's PBS. Tissue dehydration was achieved in different concentrations of alcohol (25%, 50%, 70%, 90%, and 100%) and 1% uranyl acetate was added and left for 10 min for *en'block* staining.

Table 1: The effect of zinc deficiency or supplementation on testicular weight and histology (mean±SD)

Parameters	Zinc deficiency *n*=8	Zinc supplementation *n*=8	Control *n*=8
Initial testicular weight	12.4±3.8	12.4±4.2	12.0±5.1
At 4 weeks	7.2±4.3[a]	12.6±2.9[b]	12.7±3.1[a,b]
Johnsen score*	4.4±2.2[c]	9.9±0.2[d]	8.2±0.8[c,d]

[a]*P*<0.05, [b]*P* (NS), [c]*P*<0.001, [d]*P* (NS). *Johnsen score[12] – Scale of 10 to 1; where, 10=Normal spermatogenesis with open lumen, 9=Many spermatozoa with obliteration of lumen, 8=Only a few spermatozoa, 7=No spermatozoa but many spermatids present, 6=No spermatozoa and only a few spermatids, 5=No sperms/spermatids but several spermatocytes, 4=Only a few spermatocytes present, 3=Spermatogonia only germ cells present, 2=No germ cells but Sertoli cells only present, 1=No cells in tubular section. SD=Standard deviation, NS=Not statistically significant

Embedding molds were used to block the tissue for sectioning and left overnight in the oven at 37-60°C. The sections were stained with uranyl acetate and lead citrate for 30 min at 40°C. Sections were examined using standard techniques of transmission electron microscope to study the pattern of apoptosis of the Sertoli cells. Events such as blebbing of the cellular membranes and nuclear condensation of the chromatin were evaluated.

Estimation of serum levels of trace elements and sex hormones

Serum levels of zinc, Mg, Cu, Se, and Cd were estimated by flame atomic absorption spectroscopy (AAS). Flame AAS is a spectro-analytical procedure for the quantitative determination of chemical elements employing the absorption of optical radiation (light) by free atoms in the gaseous state. AAS can be used to determine over 70 different elements in solution or directly in solid samples employed in pharmacology, biophysics, and toxicology research. Flame AAS is used in clinical analysis of metals in biological fluids and tissues such as whole blood, plasma, urine, saliva, brain tissue, liver, muscle tissue, semen, etc., Briefly, serum samples were wet-ashed with 16 mol/L nitric acid, evaporated, and diluted with 0.1 mol/L nitric acid. Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, and testosterone were assayed by radioimmunoassay.

Estimation of serum oxidants and antioxidants

The estimation of serum malondialdehyde (MDA) was carried out as we had previously described.[14] Briefly, serum was incubated in 50 mmol/L HEPES buffer (pH 7.4), 125 mmol/L KCl in a 0.5 mL reaction volume. TBARS were measured before incubation without additions and after 60 min of incubation at 37°C in the presence of 50 μmol/L $FeSO_4$. The incubation was terminated by the addition of 0.1 mL of 40 g/L butylated hydroxytoluene in ethanol, and lipid peroxidation products were evaluated as TBARS, expressed as MDA equivalents.

Total antioxidant, SOD and GPX activities were determined with Randox kit (Randox Labs Ardmore, UK). For levels of serum alpha-tocopherol and retinol we used high performance liquid chromatography technique as previously described by us.[15]

Estimation of serum T helper cytokines, Bcl-2 family and caspase-3 by ELISA

The standards for each cytokine interleukin-4 (IL-4) and TNF-α, was first used to generate a standard curve. IL-4 and TNF-α were measured using ELISA employing the multiple antibody sandwich principle (Quantikine, R and D Systems, Inc., Minneapolis, MN). The quality control parameters of these ELISAs were as follows: Intra-assay coefficient of variation (CV), 4.1-7.5%; inter-assay CV, 4.5-7.5%, respectively. Plates were read by a microplate reader and

absorbance was transformed to cytokine concentration (pg/mL).

Bcl-2, Bax and caspase-3 expression in the rat serum were evaluated by ELISA

The technique used a 96 well kit (Assay Designs assay Inc., Minneapolis, MN) according to the manufacturer's instructions. For each protein a standard curve was initially generated with different concentrations of the standard of Bcl-2, Bax, or caspase-3. The sample size was 100 μL, sensitivity 10.1 pg/mL, and assay duration was 3 h. Yellow antibody was then added to the wells and incubated for another 30 min and the blue conjugate was added and further incubated for 30 min, substrate was pipetted into the well and incubated for further 30 min, and the stop solution added and read on a plate reader at 450 nm. The sample concentrations were derived from the standard curve. Coefficients of variability were 4.9-6.3% for intra-assay and 5.7-17% for inter-assay.

Statistical analysis

Data are shown as mean ± standard deviation (SD) and were analyzed using one-way ANOVA. Fisher's least significance difference test was used to determine differences between group means. $P < 0.05$ was considered as statistically significant.

RESULTS

Animal characterization (histopathology, trace elements, antioxidant status, and hormone profile)

All results shown are for mean ± SD of three experiments and eight animals per experimental group. There was no unusual attrition (mortality) rate in the three experimental groups of animals including those animals fed zinc deficient diet. Table 1 and Figure 1 show changes in testicular weight and Johnsen score of the three experimental groups of animals. The zinc deficient group of rats had a 42% reduction in testicular volume and weight compared to the zinc supplementation group and the controls ($P < 0.02$). As shown by the Johnsen scores, zinc deficiency was associated with depressed spermatogenesis compared with control group ($P < 0.02$) or zinc supplementation group ($P < 0.004$). Zinc supplementation was associated with remarkable improvement of spermatogenesis ($P < 0.05$). Figure 1a-c shows the light microscopic appearance of the cross-section of the rat testis outlining the seminiferous tubules and the interstitial connective tissue, with Sertoli cells and the germ cells in stages 1-6 of germ cell maturation. Zinc deficiency was associated with spermatogenic arrest [Figure 1c].

As shown in Table 2, serum levels of some of the trace elements studied were reduced while others increased. Serum levels of the trace elements that were significantly reduced in the zinc deficient group compared to the zinc supplementation and control groups were zinc ($P < 0.05$), selenium ($P < 0.04$), and magnesium ($P < 0.05$). Serum levels of trace elements that showed an increase in the zinc deficient group compared to the zinc supplementation and control groups were copper ($P < 0.05$) and cadmium ($P < 0.01$). The zinc deficient group also showed significantly increased oxidative stress with significantly higher levels of MDA ($P < 0.01$) and TNF-α ($P < 0.02$), whereas there was significantly reduced total antioxidant capacity, Cu-Zn SOD ($P < 0.05$) and alpha-tocopherol (vitamin E) ($P < 0.01$) but no significant differences in GPX and retinol (vitamin A), compared with the zinc supplementation group.

Table 2 further shows the effect of zinc deficiency or supplementation on sex hormone levels. Zinc deficiency was associated with lower levels of serum testosterone and prolactin compared with control group. Zinc supplementation was associated with statistically significant higher serum levels of testosterone ($P < 0.001$) and prolactin ($P < 0.01$) compared to zinc deficient states. Serum levels of FSH and LH were not affected by zinc supplementation or deficiency.

Changes in cytokine levels

Table 3 shows changes in the serum levels of T helper cytokines at the end of 4 weeks in the 3 groups of

Table 2: The effect of zinc deficiency or supplementation on serum levels of trace elements, antioxidant activity, and sex hormones of experimental animals (mean±SD)

Parameters	Zinc deficiency *n*=8	Zinc supplementation *n*=8	Control *n*=8	*P***
Trace elements (mg/L)				
Zinc	143±21	178±25	180±27	0.01
Copper	141±20	94±18	96±23	0.01
Selenium	4.4±1.8	7.8±2.4	7.5±2.5	0.001
Cadmium	8.4±4.2	2.2±0.8	3.8±2.2	0.001
Magnesium	8. 6±4.1	14.9±5.4	14.4±5.3	0.01
Oxidants				
MDA (nmol/mL)	5.2±2.3	1.7±0.8	1.6±0.9	0.001
TNF-α (pg/mL)	34±6	12±5	14±5	0.001
Antioxidants				
TAC (mmol/L)	2.1±0.9	4.6±1.3	4.2±1.4	0.01
Cu-Zn SOD (mmol/L)	0.6±0.3	1.6±0.5	2.1±0.7	0.01
GPX (mmol/L)	1.4±1.2	1.6±1.2	1.4±1.2	NS
Retinol (μg/L)	0.4±0.3	0.6±0.4	0.7±0.4	NS
α-tocopherol (μg/L)	1.5±0.8	6.2±1.5	5.9±1.7	0.001
Hormone profile				
Testosterone (nmol/L)	4.4±3.2	16.8±4.8	7.8±4.2	0.01
FSH (IU/L)	24±14	22±14	24±14	NS
LH (IU/L)	14±8	12±8	14±10	NS
Prolactin (pmol/L)	324±84	384±110	358±122	NS

**Control versus zinc deficient group. NS=Not statistically significant, MDA=Malondialdehyde, TNF-α=Tumor necrosis factor-alpha, TAC=Total antioxidant activity; Cu-Zn SOD=Copper-zinc superoxide dismutase, GPX=Glutathione peroxidase, FSH=Follicle-stimulating hormone, LH=Luteinizing hormone, α-tocopherol=Alpha-tocopherol, SD=Standard deviation

experimental animals. Zinc deficiency was associated with significant increases in TNF-α (5-fold, $P < 0.001$), but almost 6-fold decrease in the T helper 2 cytokine IL-4 ($P < 0.001$). Conversely, zinc supplementation led to significant reduction in TNF-α but conversely, significantly enhanced expression of IL-4 > 7-fold ($P < 0.001$).

Immunohistochemistry

Table 4 and Figure 2 show the immunohistochemical staining for Bcl-2, Bax, caspase-3 for the 3 groups of animals. There was increased expression of Bax and caspase-3 in the zinc deficient group of rats compared to control or zinc supplementation group of rats. Caspase-3 activity was reduced by about 50% in the zinc supplementation group compared with control group, while it was increased by about 100% in zinc deficient group compared with control group ($P < 0.05$). Bcl-2 was highly expressed in the zinc supplementation and the control groups, but least expressed in the zinc deficient group. Figure 2 shows immunohistochemical staining of the testis. Figure 2a shows intense staining with Bcl-2 in mature spermatozoa associated with normal zinc status and with zinc supplementation as shown on Figure 2c, while Figure 2b revealed marked staining with Bax and caspase-3 in association with zinc deficiency.

Electron microscopy

Electron microscopy evaluation as shown in Figure 3a and b revealed apoptosis of both round and elongated spermatids and maturation arrest at different stages of spermatid development with variation in sizes of the nuclei (n) of maturing spermatids in the seminiferous tubules of rats fed zinc deficient diet [Figure 3b] compared to rats fed normal diet [Figure 3a] or those fed with zinc supplementation [Figure 3c]. Furthermore, the germ cells of the seminiferous tubules of rats fed zinc deficient diet showed degeneration of Golgi apparatus and other cellular organelles [Figure 3b]. Figure 3c shows features comparable to those shown on Figure 3a.

Table 3: The effect of zinc deficient diet and zinc supplementation on mean±SD serum T helper cytokine levels in rats

Parameters	Zinc deficient *n*=8	Zinc supplementation *n*=8	Control *n*=8	*P***
TNF-α (pg/mL)				
Initial	10.4±3.8	10.1±3.6	9.9±3.8	NS
At 4 weeks	48.2±15.2	5.6±3.6	9.8±3.9	0.001
IL-4 (pg/mL)				
Initial	6.2±1.9	5.8±1.8	5.8±2.1	NS
At 4 weeks	1.2±0.9	40±17	7±2	0.001

**Control versus zinc deficient group. SD=Standard deviation, TNF-α=Tumor necrosis factor-alpha, IL-4=Interleukin-4, NS=Not statistically significant

Table 4: Immunohistochemical staining for Bcl-2, Bax and caspase-3 in experimental groups or rats (mean±SD)

Diet	Zinc deficient	Zinc supplementation	Control
Bcl-2	+	+++++	+++
Bax	+++++	++	+++
Caspase	+++++	+	++

Staining evaluation: +++++=Intense staining, +=No staining. SD=Standard deviation

DISCUSSION

The fact that Zn causes apoptosis in testicular tissues and regulates spermatogenesis has been known for some time and the effect of Zn on serum testosterone levels has also been shown.[1,3,4,7] In this study, we extend some of these previous basic findings and show other changes of testicular function at the molecular level associated with Zn deficiency and supplementation. The reduction of testicular weight in the zinc deficient group of rats in the present study indicates that zinc deficiency is associated with a reduction of the total germ cell mass since it makes up about 40-75% of the testicular volume.[6,7] This study has also demonstrated two important effects of zinc on SD rat spermatogenesis. As shown by the Johnsen scores, zinc deficiency is associated with impaired spermatogenesis, increased apoptosis of the testicular cell sub-population, especially the germ cells. On the other hand, zinc supplementation is associated with correction of the impairment caused by zinc deficiency, thus resulting in a higher Johnsen score and evidently, normal spermatogenesis. The effect of zinc deficiency on serum levels of trace element appear mixed. As shown in Table 2, serum levels of some of the trace elements studied were reduced while others were increased. Serum levels of the trace elements that were significantly reduced in the zinc deficient group compared to the zinc supplementation and control groups were zinc ($P < 0.05$), selenium ($P < 0.04$), and magnesium ($P < 0.05$). Serum levels of trace elements that showed an increase in the zinc deficient group compared to the zinc supplementation and control groups were copper ($P < 0.05$) and cadmium ($P < 0.01$). Oteiza *et al*.[5] showed previously that cadmium-induced testicular damage can be ameliorated by zinc supplementation in rats.

We have also demonstrated in this study that zinc deficiency is associated with decreased Bcl-2 and increased Bax and caspase-3 activities. Bax (Bcl-2 associated x protein) and Bcl-2 are apoptosis regulating proteins of the Bcl-2 family. The ratio of Bax to Bcl-2 determines survival or death following an apoptotic stimulus.[16,17] There is evidence from the study by Helal *et al*.[17] that apoptosis is mandatory in the three testicular cell sub-population in the prenatal and postnatal period to maintain the relative balance of Sertoli: Germ cell ratio. It is tempting to speculate that severe zinc deficiency at these periods and during prepubertal or early pubertal stages of maturation could result in hypogonadism from decreased production of testosterone.[18] The mechanisms of zinc deficiency-associated alteration in testicular development

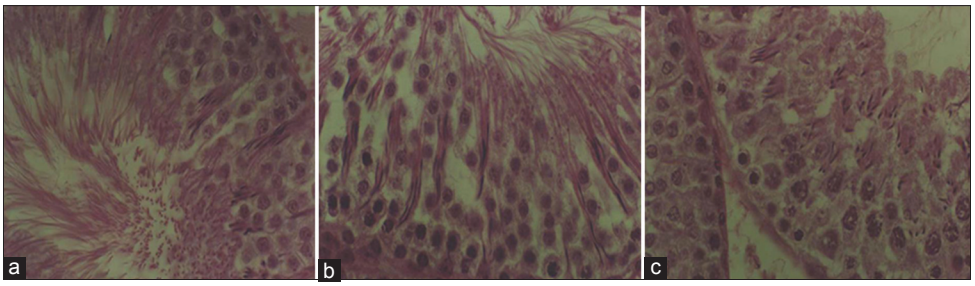

Figure 1: Light microscopy (hematoxylin and eosin staining) of a cross section of the seminiferous tubule (×100) (a) normal seminiferous tubule with spermatogonia near the basement membrane, and spermatocytes, round and elongated spermatid and mature spermatozoa in the lumen after spermiogenesis (release of mature spermatozoa into the lumen). (Johnsen score = 10) (b) normal spermatogenesis as in (a) after zinc supplementation (Johnsen score = 10) (c) scarcity of spermatozoa in the lumen consistent with spermatogenic arrest associated with zinc deficiency (Johnsen score = 6)

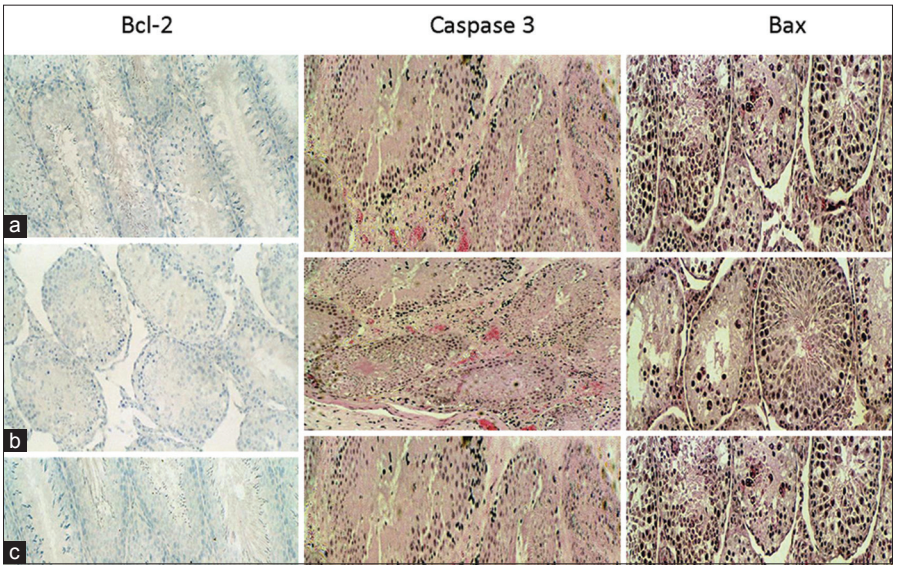

Figure 2: Immunohistochemical staining with Bcl-2, caspase-3 and Bax (×250) of rats fed normal diet (a), zinc deficient diet (b) and zinc supplementation diet (c). These staining reactions show the following: *Intense staining with Bcl-2 of mature spermatozoa *Intense staining with Bax (black) and caspase-3 (red) consistent with increased apoptosis of the early germ cells such as spermatogonia, spermatocytes and spermatids associated with zinc deficiency *intense staining with Bcl-2 of mature spermatozoa in the seminiferous tubule of rats fed with zinc supplementation diet

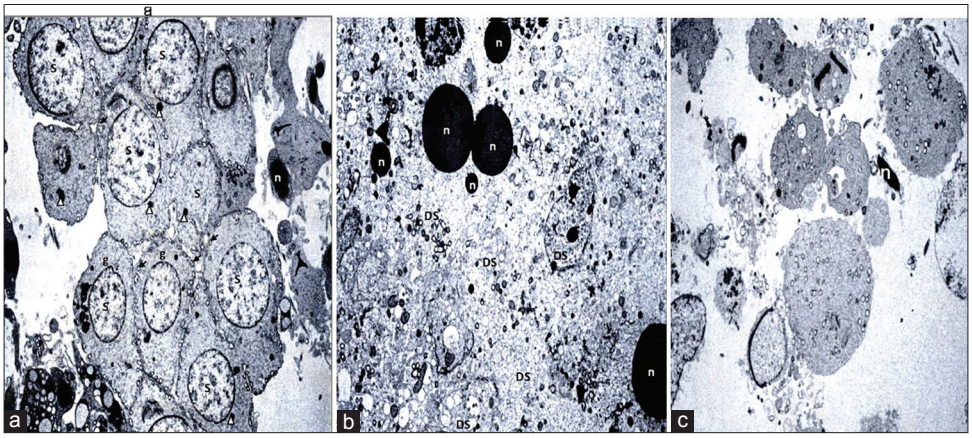

Figure 3: Electron microscopy of seminiferous tubules in normal rats (a), rats fed zinc deficient diet (b) and rats fed zinc deficient diet with zinc supplementation (c). (×4000) (a) typical pattern of normal round spermatids with peripheral mitochondria (arrows), a characteristic Golgi complex (g) lysosomes (arrowheads) and nuclei (n) of maturing spermatids are observed in the seminiferous tubule of normal rat

have been outlined by Hamdi *et al*.[7] They include impaired testicular steroidogenesis and free radical-mediated damage of selected testicular cells. Decreased testosterone level in the zinc deficiency group of the present study, is strong evidence that zinc is involved in the regulation of production of testosterone, as zinc deficiency may cause

apoptosis of the Leydig cells. Testosterone is an essential hormone for spermatogenesis. Therefore, zinc deficiency may be associated with apoptosis of cells in early stages of spermatogenesis, as well as spermotocyte maturation stage, resulting in hypogonadism in human and animals.[6]

In this study, zinc deficiency was associated with increased oxidative stress as shown by high serum levels of MDA and high TNF-α and low levels of total antioxidant activity, serum SOD and alpha-tocopherol in zinc deficient animals. Agents that indirectly increase oxidative stress by decreasing the ability of cells to resist oxidation or to scavenge reactive oxygen species will also induce apoptosis.[19] This study has demonstrated spermatogenic arrest at the level of round and elongated spermatid on light microscopy, with zinc deficiency. This shows that increased lipid peroxidation affects the outcome of spermatogenesis, as demonstrated by electron microscopic morphological features of apoptosis in the Sertoli cell in association with zinc deficiency and oxidative stress.

The effect of zinc deficiency on T helper cytokines involves a reprogramming of the immune system. Zinc deficiency activates CD^{4+} T cells to produce distinctive T helper 1 cytokines like IL-2, IL-8 and TNF-α[20] as shown in this study, in which zinc deficiency was associated with increased expression of TNF-α but decreased expression of IL-4, a T helper 2 anti-inflammatory cytokine. The proinflammatory cytokines give rise to endothelial injury and sperm dysfunction. In humans, zinc deficiency has been associated with several abnormalities of cellular immunity like reduced lymphocyte proliferation in response to mitogen with selective decrease of T^{4+} helper suppressor T lymphocytes and natural killer cells.[21] IL-2 production is also impaired. These impairments are reversed by zinc administration.[22]

Zinc supplementation was associated with reversal of the adverse effects of zinc deficiency, with reduction of oxidative stress, increased serum and testicular homogenate testosterone, Bcl-2 protein and Bcl-2/Bax ratio, but decreased Bax and caspase-3. This is in agreement with other studies[23-26] that have demonstrated that zinc supplementation increased Bcl-2 mRNA and Bcl-2 protein and Bcl-2/Bax ratio, but decreased caspase-3 activity. Perry *et al*.[6] have suggested that caspase-3 is a novel target of Zn inhibition in apoptosis and has a regulatory role for Zn in modulating the upstream apoptotic machinery, through inhibition of a Ca^{2+}- and Mg^{2+}-dependent endonuclease[25] and prevents sperm DNA fragmentation. Thus, our findings are in agreement with those of Hockenbery *et al*.[26] who showed that there was a direct association between Bcl-2 and antioxidant status.

Finally, as shown by EM, rats fed zinc deficient diet had marked apoptosis of both round and elongated spermatids and maturation arrest at different stages of spermatid development with varied sizes of nuclei of maturing spermatids in the seminiferous tubules compared to rats fed normal diet. Furthermore, germ cells of the seminiferous tubules of rats fed zinc deficient diet showed degeneration of Golgi apparatus and other cellular organelles. These changes can therefore be used to explain part of the general morphological as well as biochemical changes seen with animals fed zinc deficient diet. Clinical studies examining a possible role for zinc therapy in a group of men with male infertility are warranted.

We wish to acknowledge that there are limitations of our study. These include the following. (a) Rats on zinc supplementation diets were initially on zinc deficient diets for 2 weeks before being fed diets containing 28 mg zinc/kg for a period of 4 weeks. May b, we would have seen more profound changes due to zinc deficiency if the animals were fed zinc deficient diets for 4 instead of 2 weeks. (b) Dietary Zn deficiency is known to cause anxiety and increase cortisol levels in rats and increased stress and anxiety can affect spermatogenesis. We did not measure cortisol levels or assess the degree of anxiety in the experimental animals studied. However, we did not observe any undue anxiety or stress in any groups of the animals. The animals were treated in a humane way in accordance with the regulations of our animal research center. (c) Lastly, possible interactions between serum Zn and melatonin levels have been described.[6,9] Serum melatonin level is affected by the amount of exposure to sunlight. The experimental animals were exposed to 12 h of day and night. It is possible that this may affect the melatonin levels of the experimental animals. Unfortunately, we did not measure serum melatonin levels in the experimental animals. However, as all experimental animals were exposed to the same amount of light per day, we want to believe that this may not affect the outcome of our study.

CONCLUSIONS

Zinc has a role in spermatogenesis through complex apoptotic and multiple signal transduction pathways. Zinc deficiency is associated with impairment in almost all stages of spermatogenesis through oxidative stress, apoptosis and reduced testosterone production. These findings suggest that zinc has a role in male reproduction.

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