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# Effect of dietary selenium deficiency on the in vitro fertilizing ability of mice spermatozoa

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**Abstract** Selenium is an essential micronutrient for mammals, being integral part of antioxidant system. The aim of the study was to evaluate the effect of selenium deficiency on in vitro fertilization (IVF) capacity of spermatozoa and on oxidative stress in these cells. Male C57BL/6N mice were maintained on selenium-deficient or selenium-sufficient diets (0.02 or 0.2 ppm of selenium as selenomethionine, respectively) for 4 months. Liver glutathione peroxidase activity measurements were used to confirm selenium deficiency. Sperm quality and IVF capability among both groups were evaluated. To assess oxidative damage, lipid peroxidation as malondialdehyde production was determined in spermatozoa as well as the testes. Ultrastructural analyses of spermatozoa nuclei using transmission electron microscopy were also performed. The percentage of eggs fertilized with sperm from selenium-deficient mice was significantly decreased by approximately 67%. This reduced fertilization capacity was accompanied by increased levels of lipid peroxidation in both the testes and sperm, indicating that selenium deficiency induced oxidative stress. Consistent with this finding, spermatozoa from selenium-deficient animals exhibited altered chromatin condensation. Deficiency in dietary selenium decreases the reproductive potential of male mice and is associated with oxidative damage in spermatozoa.

**Keywords** Selenium deficiency · In vitro fertilization · Oxidative damage · Mice spermatozoa · Reproduction

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## Introduction

Selenium is an essential trace element for mammals and is an integral part of antioxidant systems. Selenium, which commonly replaces sulfur in proteins, is normally incorporated into the body through selenoamino acids (L-selenomethionine, L-selenocysteine, selenium–methylselenocysteine), taken up mainly from cereals, plants, and meat. It has been reported that SeMet can substitute for Met during translation, since $tRNA^{Met}$ is incapable of discriminating between these two amino

acids in the growing peptide chain (Schrauzer 2000). This process has been described in detail in prokaryotes (Böck 2000), whereas in mammals it is increasingly becoming better understood (Hatfield and Gladyshev 2002).

An important aspect of selenium is its nutritional significance as lower levels of selenium in the body decreases the expression of the selenoproteins and thereby impairs selenium's biological functions resulting in nutritional deficiency of the element. Estimated intakes of selenium by US residents exceed the recommended dietary allowance value of 55 μg/day for healthy adults (NAS 2000); however, the selenium intake of some Europeans is as low as 35 μg/day (Broadley et al. 2006) or dramatically as low as 17 μg/day or ≤10 μg/day, respectively, in other geographic areas such as Africa and China (Benemariya et al. 1993; Moreno-Reyes et al. 1998).

Selenium has potential relevance to the reproductive system, as evidenced by reports of selenium deficiency causing impaired reproductive abilities in both sexes (Harrison et al. 1984). In females, disturbances caused by selenium deficiency remain unclear, whereas in males, impaired spermatogenesis has been reported in several species including pigs (Edwards et al. 1977), rats (Wu et al. 1979), and mice (Wallace et al. 1983). Selenium deficiency has been associated with impaired sperm motility, flagellar defects, and structural alterations in the sperm midpiece, which normally contains mitochondria embedded in a keratinous matrix called the mitochondrial capsule (Ursini et al. 1999; Olson et al. 2004).

Selenium plays an important role in cellular antioxidant defenses, since it forms the catalytic site of antioxidant enzymes (mainly as selenocysteine) such as glutathione peroxidase (GPx) and thioredoxin reductase. Thus, selenium deficiency may compromise the functional activity of the GPx and/or thioredoxin systems in sperm, resulting in oxidative damage, alterations of the cauda epididymidis, and impaired spermiogenesis (Olson et al. 2004).

Oxidative stress results when cellular antioxidant defenses become overwhelmed by reactive oxygen species (ROS). In comparison to somatic cells, germ cells are more susceptible to oxidative stress. Spermatozoa, in particular, are highly susceptible to oxidative damage due to high concentrations of polyunsaturated fatty acids and low concentrations of cytoplasmic antioxidants (Jones 1979). ROS positively regulate capacitation and the acrosome reaction, both of which are required for spermatozoa to acquire fertilizing ability (Aitken et al. 1998; O'Flaherty et al. 2005). However, when present in excess, particularly as a result of the $H_2O_2$-generating oxidase system in spermatazoa, ROS attack polyunsaturated fatty acids in the sperm plasma membrane, initiating lipid peroxidation cascades (Aitken 1994; Aitken and Krausz 2001; Kaur and Bansal 2004) that might be responsible for reducing fertility.

Although there are several studies that show the importance of selenium levels in male reproduction, the direct influence of selenium deficiency on in vitro fertilization (IVF) has not yet been quantified. In the current study, we investigated the effect of selenium deficiency on the reproductive capability of male mice and its relationship with oxidative damage.

## Materials and methods

### Chemicals

Bovine serum albumin fraction V (BSA), butylated hydroxytoluene (BHT), desferrioxamine (DFA), hyaluronidase, human chorionic gonadotropin (hCG), lactic acid, sodium pyruvate, and thiobarbituric acid (TBA) were from Sigma Chemical Co. (St. Louis, MO, USA). Pregnant mare serum gonadotropin (PMSG; Folligon) was from Intervet, International B.V. (Boxmeer, Holland). Trichloroacetic acid (TCA) and formaldehyde were from J.T. Baker (Phillipsburg, NJ, USA). Spurr's resine was from Electron Microscopic Sciences (Fort Washington, PA, USA). All other chemicals used were of analytical grade or better.

### Animals

Four-week-old male C57BL/6N mice and five-week-old female C57BL/6N mice were obtained from Cinvestav-IPN, México animal house. All animal procedures were approved by Cinvestav Animal Care and Use Committee in compliance with international guidelines for use and care of laboratory animals. The animal room was kept on a 12/12-h light/dark cycle at 22±1°C with a humidity of 50±5% filtered air (filter efficiency, 95%). Noise levels did not exceed 85 db. The animals were housed in polycarbonate cages (43×27×

15 cm, Nalgene, Rochester, NY) in groups of six animals per cage. The C57BL/6N strain was selected since it is commonly used in fertilization and dietary studies (Kamjoo et al. 2002; Fenton and Hord 2006).

## Diets

The mice were fed for 4 months with one of two diets, prepared in pellets by Bio-Serv Co. (Frenchtown, NJ, USA) and were given free access to distilled water. The base diet was composed of 14.60% protein, 4.90% fat, 2.20% fiber, 5.08% ash, 5.90% humidity, and 67.21% carbohydrates. The base diet was supplemented with L-(+)-SeMet (Sigma Number S3875), to achieve final concentrations of 0.2 mg Se/kg (selenium sufficient group or Se–Suf) or 0.02 mg Se/kg (selenium deficient group or Se–Def). Sufficient dietary selenium levels were selected based on NAS (2000) recommendations. Selenium concentration for each diet was evaluated by the supplier Bio-Serv Co. using hydride atomic absorption spectroscopy. The certificate of analysis showed concentrations of 0.021±0.009 and 0.19±0.013 mg Se/kg for Se–Def and Se–Suf diet, respectively.

## Gamete retrieval

### *Sperm isolation and assessment of sperm parameters*

The sperm parameters assessed included sperm concentration, viability, and progressive motility. These parameters were evaluated according to World Health Organization guidelines (2001). Briefly, the mice were killed by cervical dislocation. The testis–epididimus–vas deferens complex were dissected, spermatozoa were obtained by flushing the lumen of the vas deferens and cauda epididymis with 1 ml of M-16 medium supplemented with 3 mg/ml of BSA at 37°C. Media M-16 was comprised of 100-mM NaCl, 25-mM $NaHCO_3$, 5.5-mM glucose, 2.6-mM KCl, 1.56-mM $Na_2HPO_4$, 0.5-mM sodium piruvate, 1.8-mM $CaCl_2$, 0.5-mM $MgCl_2$, and 20-mM sodium lactate, pH 7.4 (Whittingham 1971). All media were prepared with deionized water (Milli-Q Plus water system, Millipore).

Spermatozoa motility was assessed in ten random fields as percentage of motile cells using optical microscope. The spermatozoa viability was determined using tripan blue exclusion. Sperm concentration was measured in a hemocytometer and expressed as million/ml of suspension. From each sample, three aliquots (100–200 cells each) were separately counted.

### *Sperm capacitation*

Spermatozoa concentration was adjusted to $10\times10^6$ cells/ml, and 200 μl of sperm suspension was processed for transmission electron microscopy (see below). For capacitation, remaining suspension was incubated for 2 h in cell culture medium M-16 supplemented with BSA at 37°C in a high-humidity incubator under a 5% $CO_2$–95% air atmosphere.

### *Egg recovery*

Eggs were obtained from immature female mice C57BL/6. Superovulation was induced by i.p. injection of 10 IU PMSG and 48 h later, 10 IU hCG. Approximately 14–16 h after hCG injection, the animals were killed by cervical dislocation. The uterine ovary–salpinge–horn complex was dissected and suspended in M-16 medium. In each oviduct, the ampulla was punctured, and the cumulus–egg complex was extruded and placed in 0.1% (*w*/*v*) hyaluronidase in M-16 medium for 7 min at 37°C to remove cumulus cells. Then, cumulus free eggs were pooled and washed with M-16 medium, to remove hyaluronidase; approximate 50 eggs by female were obtained. Only mature eggs showing polar body and with zone intact were used to fertilization assay.

### *Insemination of zone-intact eggs*

In vitro fertilization was carried out in 40 random eggs resuspended in 200 μl M-16 medium and seeded in a standard slide with two polished spherical depressions of approximately 0.5–0.8 mm depth (VWR International). The eggs were then inseminated with 10 μl suspension of Se–Suf or Se–Def spermatozoa ($1\times10^5$ cells), which had been previously capacitated. Gametes were coincubated for 20 h at 37°C in a high-humidity incubator under a 5% $CO_2$–95% air atmosphere. The eggs were examined for the presence of the two-cell-stage embryos as an indication of successful fertilization. The cells were fixed in 3% formaldehyde–PBS (*v*/*v*) and observed by phase contrast microscopy. Three independent experiments with different batches of cells, each one in duplicate were performed.

### Glutathione peroxidase (GPx) activity assay

In a previous report, we found that 24 h cumulative urinary selenium excretion in mice fed for 2 months with the same diets used herein clearly showed that levels of dietary selenium supplementation were directly proportional to levels of selenium urinary excretion (0.006±0.0048 and 0.035±0.010 μg/day for Se–Def diet and Se–Suf diet, respectively; Vega et al. 2007).

The activity of GPx was measured by the Paglia and Valentine (1967) method, using a Ransel kit (RANDOX, CA, USA). Briefly, the liver was removed and a representative piece was rinsed in saline solution and homogenized in phosphate buffer (pH 7.4). The homogenates was centrifuged at 10,000×*g* for 30 min at 4°C. The supernatant fraction of each sample was removed, 20 μl of supernatant were added to a mixture containing phosphate buffer (0.05 M, pH 7.2), ethylenediamine tetraacetic acid (4.3 mM), GSH (4 mM), glutathione reductase (≥0.5 U/l), NADPH (0.28 mM), and cumene hydroperoxide (0.18 mM). GPx catalyzes the oxidation of GSH, using cumene hydroperoxide as a substrate. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with the concomitant oxidation of NADPH to NADP. Decreases in NADPH were monitored by measuring absorbance at 340 nm with a spectrophotometer (Vitalabe Eclipse, The Netherlands). Three independent experiments were carried out, each one in duplicate.

### Lipid peroxidation assay

The amount of thiobarbituric acid reactive substances (TBARS) was used as an index of lipid peroxidation (Buege and Aust 1987). Briefly, testes homogenates or sperm suspensions ($2\times10^6$ spermatozoa/ml) were added to a mixture containing 1 ml of 0.5% TBA, 5 μl of 3.75% BHT in methanol, and 5 μl of 1.5 mM DFA. The samples were heated in a boiling water bath for 20 min and then cooled. Absorbance at 532 nm was then measured. Three independent experiments, each one by duplicate, were performed.

### Transmission electron microscopy

Sperm samples were fixed with 3% (*v*/*v*) glutaraldehyde in PBS buffer for 1 h at room temperature. Samples were then postfixed in 1% osmium tetroxide in PBS buffer for 1 h. The cells were rinsed in PBS, dehydrated through a graded ethanol series, and embedded in Spurr's resin. Resin blocks were thin-sectioned and double-stained with uranyl acetate and lead nitrate. The samples were examined using a JEM-1200 EXII transmission electron microscope at 60 keV (Jeol LTD; Tokyo, Japan). From each sample, three thin sections were separately analyzed (~40 cells each).

### Data analysis

Comparisons of group means were performed using paired or unpaired two-tailed *t* tests as appropriate. Values of $p<0.05$ were considered significant. All data are expressed as mean ± standard deviation (SD). All analyses were performed using the statistical software Stata 8.0 (Stata Corp., College Station, TX, USA).

## Results

### Selenium deficiency effects on mice body weight

Mice with Se–Def levels in diet exhibited lesser food consumption (~19%) versus those with Se–Suf diet (data not shown). In consequence, the body weight of mice fed with Se–Def was lower than animals with Se–Suf dietary (Fig. 1). However, no significant differences were observed in the relative weight of organs as testes (data not shown). No obvious indications of poor health such as, hair loss, low fur quality, or bloated abdomen were observed in mice with selenium deficiency.

### Selenium deficiency negatively affects spermatozoa quality

As shown in Table 1, spermatozoa quality was significantly reduced in mice maintained on a Se–Def diet as compared to their control counterparts. There was a slight, but significant, decrease in sperm concentration in Se–Def animals ($p=0.025$ vs Se–Suf), and sperm motility was reduced in these animals by approximately 40% ($p=0.017$ vs Se–Suf). A trend towards decreased sperm viability was also observed in the Se–Def group ($p=0.057$ vs Se–Suf). These results confirm that selenium is an essential dietary

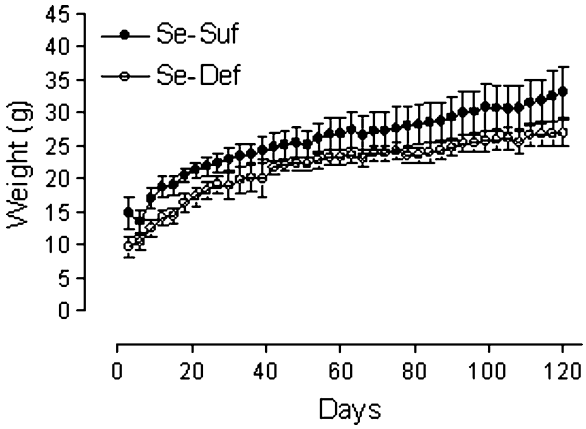


**Fig. 1** Effect of selenium deficiency on body weight. Body weight of mice fed with Se–Def and Se–Suf during 120 days; $n=6$. A significant difference was observed during the time of study ($p<0.01$)

micronutrient required for maintenance of spermatozoa quality.

## Selenium deficiency increases oxidative damage in the testes and spermatozoa

The bioavailability of selenium from selenium-rich SeMet was assessed in selenium-deficient mice by measuring GPx activity in the liver (Beckett et al. 1990; Burk et al. 1981).The liver was selected since it harbors the largest pool of selenium and is one of the main sites of selenium action (Levander 1985). Liver GPx activity was reduced by fourfold in animals maintained on a Se–Def diet ($p<0.0001$ vs Se–Suf; Table 2), confirming selenium deficiency in these animals.

**Table 1** Assessment of sperm parameters in mice fed Se-deficient and Se-sufficient diets

| Sperm parameters | Selenium diet | |
|---|---|---|
| | Deficient ($n=6$) | Sufficient ($n=6$) |
| Concentration ($10^6$/ml) | 14.7±0.47* | 17.0±0.8 |
| Motility (%) | 59±17.5* | 97±5.7 |
| Viability (%) | 79±3.9 | 95±0.57 |

Values are mean ± SD

*$p<0.05$ vs Se-sufficient diet

**Table 2** Selenium deficiency effect on liver glutathione peroxidase (GPx) activity

| Selenium diet | GPx activity (U/g protein) |
|---|---|
| Deficient ($n=6$) | 155.47±42.52* |
| Sufficient ($n=6$) | 633.60±121.12 |

Values are mean ± SD

*$p<0.05$ vs Se-sufficient diet

Lipid peroxidation was also evaluated, as an oxidative damage marker, in the testes and sperm (Table 3). Lipid peroxidation levels in Se–Def spermatozoa and testes were increased by 62-fold ($p=0.008$ vs Se–Suf) and 2-fold ($p=0.036$ vs Se–Suf), respectively.

## Selenium deficiency altered in vitro fertilization capability of spermatozoa

A well-known marker assessment of fertility is the IVF test, which measures the ability of sperm cells to fertilize eggs. Assessment of the fertilization capacity of Se–Def and Se–Suf sperm using the IVF test revealed that the former less efficiently fertilized zone-intact eggs (Figs. 2 and 3). Approximately 80% of eggs were fertilized by Se–Suf spermatozoa, whereas only 25% were fertilized by Se–Def spermatozoa (Fig. 2). Figure 3a shows an inseminated egg with Se–Suf spermatozoa, which is in division process, whereas Fig. 3b shows an inseminated egg with Se–Def spermatozoa, which was not fertilized. This result clearly shows that selenium deficiency adversely affects sperm fertilization capability in mice.

## Selenium deficiency alters chromatin condensation in spermatozoa

Ultrastructural evaluations of Se–Suf and Se–Suf spermatozoa nuclei were performed via transmission electron microscopy, and representative images are shown in Fig. 4. Nuclei of all Se–Suf spermatozoa exhibited a normal morphology characterized by homogenous electron-dense chromatin bound by an intact nuclear envelope (Fig. 4a). This normal appearance was visibly altered in nuclei of Se–Def spermatozoa. Nuclei showed heterogeneous electron-dense chromatin, which was frequently associated with a disrupted nuclear envelope (Fig. 4b).

**Table 3** Effects of selenium deficiency on lipid peroxidation in the spermatozoa and testes

| Selenium diet | TBARS concentration | |
|---|---|---|
| | Spermatozoa (nmol/2×$10^6$ sperm) | Testes (nmol/g tissue) |
| Deficient ($n$=6) | 93.13±5.2* | 0.0107±0.0017* |
| Sufficient ($n$=6) | 1.52±1.0 | 0.0067±0.0014 |

Values are mean ± SD

*$p$<0.05 vs Se-sufficient diet

## Discussion

Recent advances in the understanding of male infertility have implicated oxidative stress as a major causative factor (Aitken et al. 1998). The present study was carried out to evaluate the effect of selenium deficiency on the oxidative damage of the testes and sperm and its consequences on of the IVF of male mice. Here, we demonstrate that spermatozoa quality is significantly reduced as a result of selenium deficiency. Consistent with this result, others have reported that selenium deficiency is associated with flagellar defects in spermatids and epididymal spermatozoa as well as with a reduction in the number of post-meiotic germ cells, spermatids, and spermatozoa (Shalini and Bansal 2006). These effects were attributable to the decreased expression, of both levels mRNA and protein of cJun and cFos in the testicular germ cells. These proteins comprise the transcription factor AP1, which regulates cellular growth and differentiation and also exerts a regulatory role in steroidogenesis and spermatogenesis (Shalini and Bansal 2006). It has recently been shown that selenium deficiency also induces the expression of NFκB, which is known to positively regulate iNOS expression (Shalini and Bansal 2007).

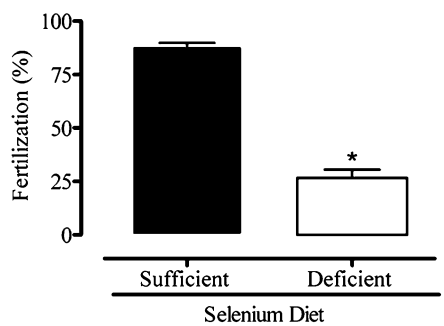


**Fig. 2** Effect of selenium deficiency on fertilization. Spermatozoa isolated from mice maintained on Se–Suf or Se–Def diets were tested for their ability to fertilize zone-intact eggs. A total of 102 eggs were examined, in each experimental group, over the course of three independent experiments; *asterisk*, $p$<0.001

Alterations in cellular redox status toward oxidative conditions may occur as a result of over production of ROS or a deficiency in antioxidant systems. Lipid peroxidation is considered the main indicator of oxidative stress-induced loss of cellular function (Storey 1996). One of the most recognized products of lipid peroxidation are TBARS, which were increased in testes as a result of selenium deficiency (Kaushal and Bansal 2007). Results of present study show a notable increase of lipid peroxidation in mice sperm (Table 3).

Selenium forms the catalytic center of antioxidant enzymes such as cytosolic GPx or membrane bound GPx (PHGPx). GPx plays an important role in the defense against oxidative damage by catalyzing the reduction in a variety of hydroperoxides, using glutathione as the reducing substrate (Brigelius-Flohé et al. 2002). PHGPx undergoes physical modification and acquires alternate biological functions during sperm maturation. PHGPx exists as a soluble peroxidase in spermatids but persists in mature spermatozoa as an enzymatically inactive, oxidatively cross-linked, insoluble protein (Ursini et al. 1999). In rats, this selenoenzyme is present in the mature sperm head and in tail midpiece mitochondria and it is capable of oxidizing reduced sperm protamines (Godeas et al. 1997). PHGPx is highly expressed in the nuclei of late spermatids, where the reorganization and condensation of DNA take place.

In the present study, we found that selenium deficiency caused a 67% decrease in sperm fertilization capability. This decrease in fertility might result from oxidative damage to the plasma membrane, which would lead to a total loss of the membrane fluidity and integrity. This, in turn, would be expected to reduce the number of spermatozoa available to compete in the membrane fusion events associated with fertilization

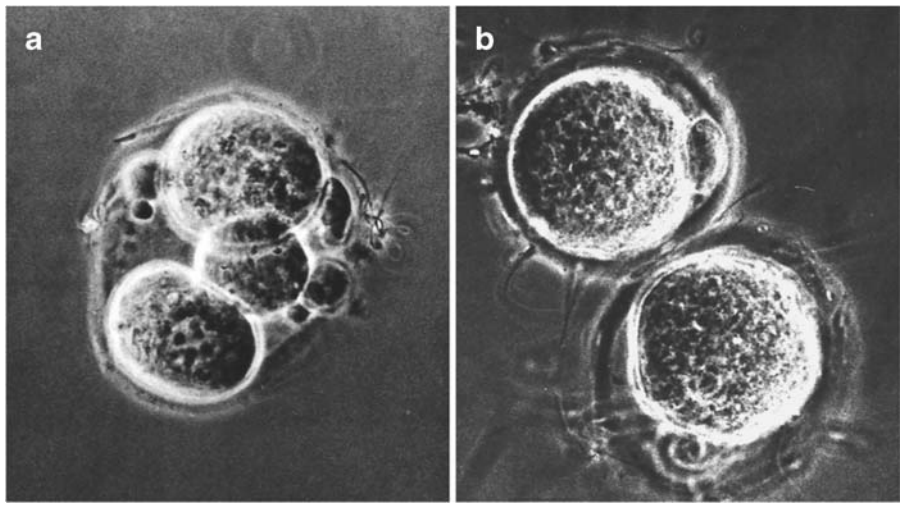


**Fig. 3** Phase contrast microscopy of in vitro fertilization by selenium deficient spermatozoa. Zone-intact eggs were incubated with spermatozoa from mice maintained on Se–Suf or Se–Def or diets. Representative images are shown (×400). **a** Se–Suf spermatozoa were clearly able to inseminate eggs, as shown by fertilized egg in process of division. **b** Two eggs incubated with Se–Def spermatozoa showed no evidence of fertilization. A total of 102 eggs were examined, in each experimental group, over the course of three independent experiments

(Aitken 1994; Levander 1985). In agreement with this, human spermatozoa that have been exposed to very high levels of oxidative stress exhibit a sudden decline in both the quality of sperm movement and sperm–egg fusion (Aitken and Fisher 1994).

In addition to inflicting oxidative damage to the sperm plasma membrane, ROS are also know to attack DNA, inducing strand breaks and oxidative base damage in rodent and human spermatozoa (Huges et al. 1996; Kumar et al. 2002). Accordingly,

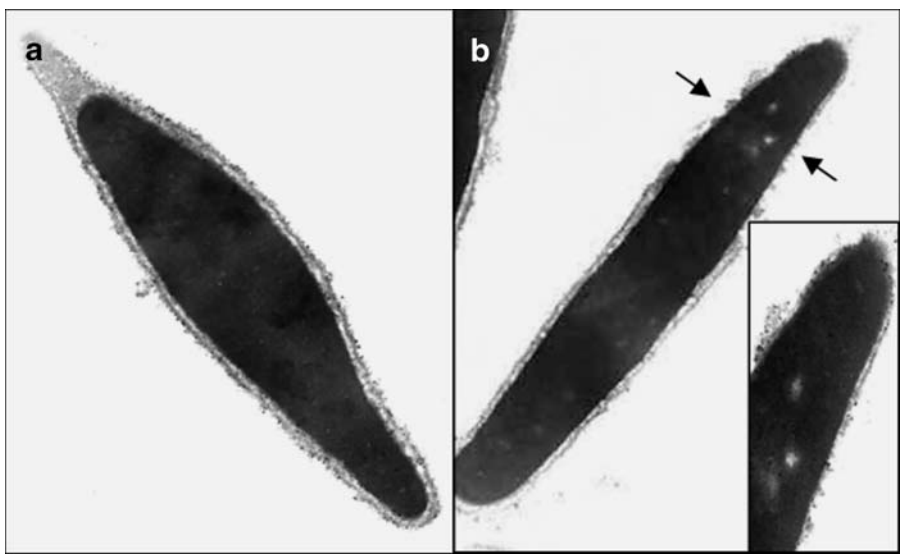


**Fig. 4** Effect of selenium deficiency on the ultrastructure of spermatozoa nuclei. Spermatozoa from mice maintained on **a** Se–Suf or **b** Se–Def diets were examined by transmission electron microscopy (×15,000). Nuclei of spermatozoa from selenium-sufficient animals were uniformly electron-dense, and the nuclear envelope was intact. On the other hand, nuclei of spermatozoa from selenium-deficient animals exhibited heterogeneous electron dense density, and the nuclear envelope was frequently disrupted (*arrows*). *Inset* in **b** shows a portion of the nucleus under higher magnification (×20,000)

it has been demonstrated that a lipid peroxidation occurs concurrently with oxidative damage to testicular DNA (Kumar et al. 2002).

DNA strand breaks and denaturation are inversely correlated with the fertilizing potential of spermatozoa (Aravindan et al. 1997). Here, impaired fertility was associated with alteration on chromatin condensation, as seen by electron microscopy. The process of chromatin condensation seems to be crucial not only for maturation of sperm cells, but also for fertility and genesis of offspring. It has been suggested that nuclear PHGPX plays a role in chromatin condensation and in the protection of sperm DNA against oxidative damage (Pfeifer et al. 2001). In vitro experiments with murine sperm showed that a condensed sperm nucleus with a stable matrix is needed to ensure normal embryonic development (Ward et al. 1999). Our results suggest that selenium deficiency may elicit alteration on chromatin condensation via oxidative damage. The assessment of sperm chromatin is relevant because it is completely reorganized during the late stages of spermatogenesis when histones, small proteins rich in cysteine residues, are replaced by protamines. Thus, additional studies examining some markers of DNA integrity will be required to evaluate the effects of selenium deficiency on embryo development.

In the present work, we showed an important effect on the fertilization capability of spermatozoa due to a severe Se deficiency in these animals, however it is important to establish that selenium deficiency is relatively rare in healthy well-nourished individuals. It can occur in individuals with severely compromised intestinal function, or those undergoing total parenteral nutrition. Alternatively, people dependent on food grown from selenium-deficient soil are also at risk.

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