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Alterations in selenium status influences reproductive potential of male mice by modulation of transcription factor NFκB

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Abstract

Selenium (Se), an essential dietary trace element, is required for the maintenance of male fertility. In order to study its role in spermatogenesis, Balb/c mice with different Se status (Se deficient, group I; adequate, group II and excess, group III) were generated by feeding yeast based Se deficient diet for group I and deficient diet supplemented with Se as sodium selenite at adequate (0.2 ppm) and excess (1 ppm) for group II and III, respectively, for a period of 4 and 8 weeks. Percentage fertility was reduced in group I and III as compared to group II. A significant decrease in Se levels and glutathione peroxidase (GSH-Px) activity were observed in group I animals, whereas increase in GSH-Px activity was seen in group III. Further, significant increase in lipid peroxidation was observed in both Se deficient and excess groups. This indicated that dietary manipulation of Se levels either deficiency or excess leads to increased oxidative stress. Nuclear factor kappa B (NFκB), a well-known redox regulated transcription factor has also been suggested to play a crucial role in spermatogenesis. The expression of both *p65* and *p50* genes (components of NFκB) increased in Se deficient group I mice while the expression of the inhibitory IκBα declined significantly. This indicated activation of NFκB in Se deficiency. We also studied iNOS expression, which is a known target gene of NFκB, by RT-PCR. Significant elevation in the iNOS levels as well as NO levels was recorded. Both enhanced NO levels and NFκB are harmful in the progression of normal spermatogenic cycle. Therefore, present result clearly demonstrates the effect of reduced supply of Se on up-regulation and activation of NFκB in testis and its influence on spermatogenesis.

Spermatogenesis is a complex process, which requires precisely timed signals to regulate the genetic pathways responsible for proliferation and differentiation of cells. Selenium (Se) is an essential dietary trace element, which is required for the maintenance of male fertility (Brown & Arthur 2001; Hill *et al*. 2003). Both testis and epididymis require exogenously supplied Se in order to synthesize a variety of known selenoproteins, whose precise role in spermiogenesis and post testicular sperm maturation are not yet clearly defined. Testicular morphology and functions are affected by severe Se deficiency and this element is also required for testosterone biosynthesis and the formation and normal development of spermatozoa (Watanabe & Endo 1991; Behne *et al*. 1996).

The antioxidant and other beneficial effects of Se have been recognized for quite some time. Se forms an integral part of glutathione peroxidase (GSH-Px) that is involved in the rapid removal of reactive oxygen species (ROS), mainly the peroxides (Ursini *et al*. 1999). On the other hand reports also suggest the possible induction of ROS by higher levels of Se. Selenopersulfide, GSSe- (a selenide), is formed as a result of direct reaction between selenite and glutathione. These selenides

redox cycle with oxygen and generate superoxide (O_2^-) as well as other ROS (Shen *et al.* 2001; Spallholz 2001). ROS are presently being considered as important second messengers in signal transduction, gene expression and cell proliferation (Sen & Packer 1996).

Several advances have been made in understanding the mechanisms by which Se regulates spermatogenesis but role of signaling molecules such as transcription factors has yet not been studied in this regard. Selenium behaves both as an antioxidant and anti-inflammatory agent and has been linked to regulatory functions in cell growth, cytotoxicity and transformation (Bansal *et al.* 1990). It appears to modulate such cellular activities presumably by activity of proteins important for signal transduction (Handel *et al.* 1995; Kim & Stadtman 1997).

Nuclear factor kappa B (NFκB) is a ubiquitous redox sensitive transcription factor (Ginn-Pease & Whisler 1998) that is a major regulator of immune and stress responses (Baldwin 1996; Pahl 1999) and is an interesting candidate to study spermatogenesis since there is a growing evidence that this transcription factor is involved in cell proliferation and apoptosis (Barkett & Gilmore 1999; Pahl 1999). NFκB has been found to be regulated during mammalian spermatogenesis (Delfino & Walker 1998, 1999). NFκB factors are stage specifically controlled and play an important role during the development of sperm cells (Lilienbaum *et al.* 2000).

In most cells, NFκB is present as a dimer of protein components (p65/p50) in a latent/inactive form, bound to inhibitory protein IκB in the cytoplasm. Activation results in phosphorylation of IκB by IκB kinases, subsequently leading to rapid ubiquination and degradation of IκB in the proteasome. Degradation uncovers the nuclear localization signals on the p65/p50 complex resulting in rapid translocation to the nucleus where it binds to specific κB recognition elements in the promoter region of target genes (Baeuerle & Baltimore 1989). One of these target genes is inducible nitric oxide, iNOS (Xie *et al.* 1994).

NO is an important intracellular messenger and regulates various cellular functions such as vasorelaxation and inflammation (Shinde *et al.* 2000). NO leads to generation of several reactive nitrogen oxide species (RNS) such as peroxynitrite (ONOO–), a highly reactive unstable species formed as a result of reaction of NO with superoxide, which spontaneously decomposes to nitric and hydroxyl radical (Drew & Leeuwenburgh 2002). RNS in turn pose a risk for disruption of the cell signaling machinery as they can S-nitrosate thiols to modify key signaling molecules such as kinases and transcription factors (Banan *et al.* 2000; Coleman 2001). NO has been reported to be important in the regulation of male reproductive function and fertility, leydig cell function, myofibroblast contraction and tubular peristalsis (Zini *et al.* 1996; Rosselli *et al.* 1998). Both isoforms [endothelial (eNOS) and inducible (iNOS)] have been detected in the testes (Santoro *et al.* 2001).

Several studies indicate that the effects of Se might be mediated by NFκB (Jozsef & Filep 2003; Maehira *et al.* 2003). Present study was also aimed to investigate the effect of different Se status on spermatogenesis and to examine the possible involvement of NFκB in this process.

Materials and methods

Materials

Sodium selenite and 2,3-diamine naphthalene (DAN) were purchased from Sigma–Aldrich. Primary antibodies used were NFκB-p65 (Santa Cruz) and IκBα (Sigma–Aldrich). Peroxidase labeled anti-rabbit IgG as supplied by Sigma–Aldrich was used as secondary antibody. Rest all other chemicals used in the present study were of analytical grade obtained from Indian labs.

Animals and animal care

Male BALB/c mice of the 20–25 g body weight range were obtained from the Central Animal House, Panjab University, Chandigarh (India) and housed in the departmental animal room in poly propylene cages in 12/12 h light/dark cycle. To make different Se status animals' *viz*. 0.02 ppm, 0.2 ppm and 1 ppm, in different groups (Group I, II and III, respectively), the mice were kept on yeast-based diet (Burk 1987). The yeast-based diet usually contains 0.02 ppm Se and hence animals fed on this diet were considered Se deficient animals (Group I). For the Se supplemented groups, Se was added at 0.2 ppm (Group II; adequate level) and 1 ppm (Group III; excess level) as sodium

selenite. The selenium deficient diet was prepared in the laboratory using inactivated Baker's yeast with the following composition (Sucrose 59.7%, yeast 30%, Corn oil 5%, Salt mixture (USPXIV) 4%, Vitamin mixture 1% and DL-Methionine 0.3%). All the animals were fed with the respective diets for 4 weeks (Group Ia, IIa and IIIa) and 8 weeks (Group Ib, IIb and IIIb).

After completion of the diet-feeding schedule, animals were sacrificed under mild ether anesthesia followed by cervical dislocation. Tissue homogenates were prepared (10%w/v) in 20 mM Tris–HCl (pH 7.4) using mechanically driven Teflon fitted potter elvejham type homogenizer under ice-cold conditions. Homogenates were centrifuged at 9500 *g* for 30 min at 4 °C to get post mitochondrial fraction (PMF).

Selenium estimation

Selenium levels were measured in tissues as previously described (Hasunuma *et al*. 1982). This is based on the principle that Se in tissues on acid digestion is converted into selenous acid which on reaction with aromatic-o-diamines such as 2,3-diamine naphthalene (DAN) leads to the formation of 4-5-benzopiazselenol, which displays brilliant lime green fluorescence. Briefly, 100 mg of tissue (liver or testis) were acid digested in conc HNO_3 on a sand bath at approx. 100 °C in digestion flasks fitted with long air condenser to prevent any Se vapour loss. A known amount of digest was reacted with aqueous solution of DAN (precleaned of impurities with cyclohexane), 4,5-benzopiaselenol product formed was extracted completely with cyclohexane and quantitated on fluorescence spectrophotometer (Perkin–Elmer, USA) using 366 nm as excitation and 520 nm as emission wavelength. Sodium selenite was used as a standard Se for this assay.

Glutathione peroxidase

GSH-Px activity was assayed in PMF of liver and testis by the coupled enzyme procedure with glutathione reductase using hydrogen peroxide as substrate (Lawrence & Burk 1976). The amount of NADPH oxidized during the reaction was quantitated at 340 nm in spectrophotometer (UV 160A, Shimadzu).

Lipid peroxidation (LPO)

Level of malondialdehyde (MDA), index for determining the extent of lipid peroxidation from the breakdown of polyunsaturated fatty acids, was determined in PMF (Wills 1966). TCA precipitated supernatant from PMF was reacted with Thiobarbituric acid. The development of pink colour with the absorption characteristics (maxima 532 nm) as TBA-MDA chromophore was taken as an index of lipid peroxidation. Tetra ethoxy propane (TEP) was used as the standard.

Determination of NO production

NO levels were determined by measuring nitrite, a stable metabolic product (Raddassi *et al*. 1994). In brief, 100 μl Griess reagent (1:1 v/v mixture of 0.1% *N*-[1-naphthyl] ethylenediamine dihydrochloride in double distilled water and 1% sulfanilamide in 2.5% ortho-phosphoric acid solution was added to 100 μl of the PMF in ELISA strips. The plate was kept in dark for 10 min and the pink colour so obtained was read at 540 nm on a scanning multiwell spectrophotometer (ELISA reader, STAT fax 325). The amount of nitrate produced in each well was determined by a standard curve prepared by using sodium nitrate.

Fertility studies

For assessing the fertility, the male mice from the different groups of animals were exposed to normal female mice for 7 days in the ratio of 1:3. The females were examined for occurrence of pregnancy between 14 and 21 day after initial exposure and the mean litter size was recorded. Percent fertility was checked as follows: (Number of females giving birth/ Number of females exposed to mating) $\times$ 100.

RNA isolation

Total RNA was isolated from the testis using TRI-reagent (RNA, DNA, and protein isolation reagent) as per manufacturer's protocol (Molecular Research Center, Inc. Cincinnati, OH) and the quality of isolated RNA was checked on 1.2% formaldehyde agarose gel electrophoresis.

RT-PCR for p50, p65, IκBα and iNOS

PCR analyses were carried out in 50 μl using the QIAGEN one step RT-PCR kit. Optimal oligonucleotide primer pairs for RT-PCR were selected and are listed in Table 1. Primers for PCR were designed using the Clone Manager Software. 3 μg RNA was used for each amplification reaction. After an initial denaturation step of 1 min at 94 °C, 35 amplification cycles were performed. Each cycle included an initial denaturation step at 94 °C for 45 s, annealing at 56 °C for 45 s and extension at 72 °C for 45 s. A final extension step of 5 min at 72 °C was performed in order to complete the PCR reaction. The amplified product was analysed on 1.5% agarose gel electrophoresis and densitometric analysis of the bands was done using Image J software (NIH).

Nuclear and cytoplasmic extract preparation

Briefly, 100 mg of testis was gently homogenized in 0.5 ml of buffer A [10 mM HEPES (pH 8.0), 50 mM NaCl, 500 mM sucrose, 1 mM EDTA, 0.2% triton X-100] in the presence of 1 mM phenyl methyl sulphonyl fluoride (PMSF). After centrifugation at 4800 *g* (2 min) at 4 °C, the supernatant was removed and stored as cytoplasmic extract. The pellet was washed in 500 μl buffer B [10 mM HEPES (pH 8.0), 50 mM NaCl, 25% glycerol and 1 mM EDTA] in the presence of 1 mM PMSF. The nuclear pellet obtained was suspended in 50 μl of buffer C [10 mM HEPES (pH 8.0), 420 mM NaCl, 25% glycerol and 1 mM EDTA] in the presence of 1 mM PMSF and incubated on ice for 30 min with constant shaking. Nuclei were pelleted by centrifugation at 9500 *g* for 10 min at 4 °C. The supernatant was collected and stored as nuclear extract. Protein concentrations of cytoplasmic and nuclear fractions were determined by the method of Lowry *et al.* (1951).

Western blot

50 μg nuclear protein for NFκB p65 (*Rel A)* or 20 μg cytoplasmic extract for IκB was electrophoresed on 10% SDS-PAGE and blotted onto polyvinylidine difluoride (PVDF) membrane. Membrane was blocked overnight with 5% non-fat milk in PBS (pH 7.4) and incubated with primary antibody against NFκB-p65 (Santa Cruz; 1:1000) and IκBα (Sigma–Aldrich; 1:500) separately for 2 h. The membranes were then probed with peroxidase labeled anti-rabbit IgG as secondary antibody (1:1000). Detection was done using DAB and H_2O_2 as substrate. Data are a mean of four independent observations from different animals.

Statistical analysis

The difference between the Means ± SD for control and treated groups were examined by using the Student's *t*-test for unpaired values. Statistical differences of *p*-values at the level of 0.05 or less were considered significant. Densitometric analysis of bands was done by ImageJ software (NIH). Number of independent observations from different animals carried for each analysis is shown in the respective Tables/Figures.

Table 1. Primer pairs used for RT-PCR.

Gene	Primer		Reference No.	Product size
IkappaBα	Sense	5′-CTG CAG GCC ACC AAC TAC AA-3′	ACCESSION U36277	403 bp
	Antisense	5′-GGC CTC CAA ACA CAC AGT CA-3′		
iNOS	Sense	5′-TCA CGC TTG GGT CTT GTT CA-3′	Melby *et al.* 1998	773 bp
	Antisense	5′-TTG TCT CTG GGT CCT CTG GT-3′		
p65	Sense	5′-TGGCGAGAGAAGCACAGATA-3′	ACCESSION M61909	272 bp
	Antisense	5′-TGTTGGTCTGGATTCGCTG-3′		
p250	Sense	5′-GAGTCACGAAATCCAACGCA-3′	ACCESSION NM_008689	318 bp
	Antisense	5′-CCAGCAACATCTTCACATCC-3′		
β actin	Sense	5′-ATC CGT AAA GAC CTC TAT GC-′3,	Yamada *et al.* 1996	287 bp
	Antisense	5′-AAC GCA GCT CAG TAA CAG TC-3′		

Results

Selenium estimation

Se was estimated in the testis and liver of mice from all the three treatment groups after 4 and 8 weeks of diet feeding. The data are shown in Table 2. In testis, a significant decrease in Se level was observed in group Ia which became highly significant at 8 weeks (group Ib). Significant increase was observed in the Se excess group IIIa and IIIb. In liver also similar changes in selenium levels were observed in all the groups of animals.

Glutathione peroxidase

GSH-Px activity was measured in the testis and liver. In testis, a significant decrease was observed at 4 weeks in Se deficient (group Ia) and significant increase was observed in Se excess (group IIIa) as compared to group IIa. At 8 weeks, the GSH-Px levels declined further in group Ib. However no change was observed in the Se excess feeding in group IIIb. Similar changes were observed in liver (Table 2).

Lipid peroxidation (LPO)

Significant increase in LPO was observed in Se deficient and Se excess group at 4 weeks of diet feeding. This increase became more significant at longer duration of 8 weeks in both the groups Ib and IIIb as compared to the Se adequate group IIb (Table 2).

NO levels

The data are represented in Table 2. *S*ignificant increase in NO levels was observed in Se deficient group Ia which became highly significant at 8 weeks (group1b). Similarly, increase was observed in the Se excess mice. However, it was comparatively less than the Se deficient animals.

Fertility studies

Data are shown in Table 3. The percentage fertility decreased in Se deficient Group Ia. It decreased further at 8 weeks in group Ib. Reduced fertility was also observed in Se excess animals (group IIIb). No significant change in litter size was observed at 4 weeks in groups Ia and IIIa. However, it significantly decreased to 3.5 in Group Ib and 4.6 in group IIIb as compared to a litter size of 7 in the Se adequate Group II.

RT-PCR analysis

The levels of p65 decreased at 4 weeks, but a significant increase was observed at 8 weeks under conditions of Se deficiency (Figure 1). In Se excess group while no change was observed at 4 weeks interval, increase in expression was evident at

Table 2. Selenium, glutathione peroxidase, lipid peroxidation and nitric oxide levels in testis after 4 and 8 weeks of diet feeding schedule.

	4 weeks			8 weeks		
	Deficient Ia	Adequate IIa	Excess IIIa	Deficient Ib	Adequate Iib	Excess IIIb
Selenium levels (μg Se/g tissue)						
Testes	0.612 ± 0.063^{b}	0.735 ± 0.068	0.792 ± 0.063^{a}	$0.405 \pm 0.046^{c,f}$	0.733 ± 0.061	$0.888 \pm 0.056^{c,f}$
Liver	0.505 ± 0.061^{c}	0.663 ± 0.043	0.761 ± 0.051^{c}	$0.313 \pm 0.031^{c,f}$	0.676 ± 0.068	$0.941 \pm 0.061^{c,f}$
Glutathione peroxidase (μmol NADPH oxid/min/mg protein)						
Testes	201.90 ± 2.60^{c}	259.53 ± 33.90	295.52 ± 22.6^{b}	$120.65 \pm 15.6^{c,f}$	257.91 ± 8.14	286.09 ± 25.9^{b}
Liver	634.28 ± 39.04^{c}	870.73 ± 91.84	921.59 ± 64.92^{a}	$405.51 \pm 58.56^{c,f}$	885.10 ± 76.68	964.18 ± 92.42^{a}
Lipid peroxidation (nmoles MDA/mg protein)	0.65 ± 0.09^{c}	0.48 ± 0.07	0.58 ± 0.17^{b}	$0.98 \pm 0.14^{c,f}$	0.48 ± 0.09	$0.81 \pm 0.14^{c,f}$
NO levels (nmoles NO/mg protein)	5.83 ± 0.64^{a}	4.93 ± 0.94	4.24 ± 0.26^{a}	$9.58 \pm 0.64^{c,f}$	4.66 ± 0.51	$6.06 \pm 0.30^{c,f}$

Values are Mean ± SD of six observations. $^{a}p < 0.05$, $^{b}p < 0.01$ and $^{c}p < 0.001$ represent comparison between Se deficient, Se excess animals with respect to Se adequate animals at 4 and 8 weeks. $^{d}p < 0.05$, $^{e}p < 0.01$ and $^{f}p < 0.001$ represent comparison between Se deficient, Se excess animals and Se adequate animalsat 4 and 8 weeks.

Table 3. Changes in the fertility status in mice after 4 and 8 weeks of diet feeding schedule.

	4 weeks			8 weeks		
	Deficient Ia	Adequate Iia	Excess IIIa	Deficient Ib	Adequate Iib	Excess IIIb
Fertility (%)	72.7 ± 22.9^{a} (6)	88.86 ± 18.8 (6)	88.86 ± 18.8 (6)	$41.65 \pm 8.54^{c,e}$ (6)	88.86 ± 18.8 (6)	$44.43 \pm 8.54^{c,f}$ (6)
Litter size	7.2 ± 1.81 (5)	7.0 ± 1.27 (6)	6.6 ± 1.76 (6)	3.5 ± 0.67^{cf} (2)	7.0 ± 1.27 (6)	$4.6 \pm 0.34^{a,d}$ (3)

Values are Mean ± SD. $^{a}p < 0.05$, $^{b}p < 0.01$ and $^{c}p < 0.001$ represent comparison between Se deficient, Se excess animals with respect to Se adequate animals at 4 and 8 weeks. $^{d}p < 0.05$, $^{e}p < 0.01$ and $^{f}p < 0.001$ represent comparison between Se deficient, Se excess animals and Se adequate animals at 4 and 8 weeks. Values in parentheses represent number of animals.

8 weeks diet feeding schedule. The p50 expression however remained unaffected at the excess level even at 8 weeks but in Se deficient animals a time dependent increase in p50 expression was observed. RT-PCR analysis for IκBα (Figure 2) showed highly significant time dependent decrease of around 26% in the Se deficient animals compared to 13% in the Se excess animals at 8 weeks. iNOS expression (Figure 3) also increased at 8 weeks in Se deficient group. Bands of equal intensity were observed for β actin in all the samples.

Western blot analysis

Distinct bands for both NFκB (p65) and IκBα were observed in the testicular nuclear extract and cytoplasmic extract respectively. The densitometric analysis of these bands was done and the data is represented in Figure 4a,b, which indicates the activation pattern of this transcription factor. For IκBα, a highly significant decrease was observed at 8 weeks (lane 4) in Se deficient mice. In Se excess group decrease was evident at 8 weeks (lane 6). A time dependant decrease was observed at both Se deficient and excess groups.

In case of p65, highly significant increase in expression was recorded at both 4 (lane 1) and 8 weeks (lane 4) in Se deficient mice. Change was indicated at excess level but was less pronounced compared to the deficient group. However, a time dependent increase in the expression was indicated in both deficient and excess groups.

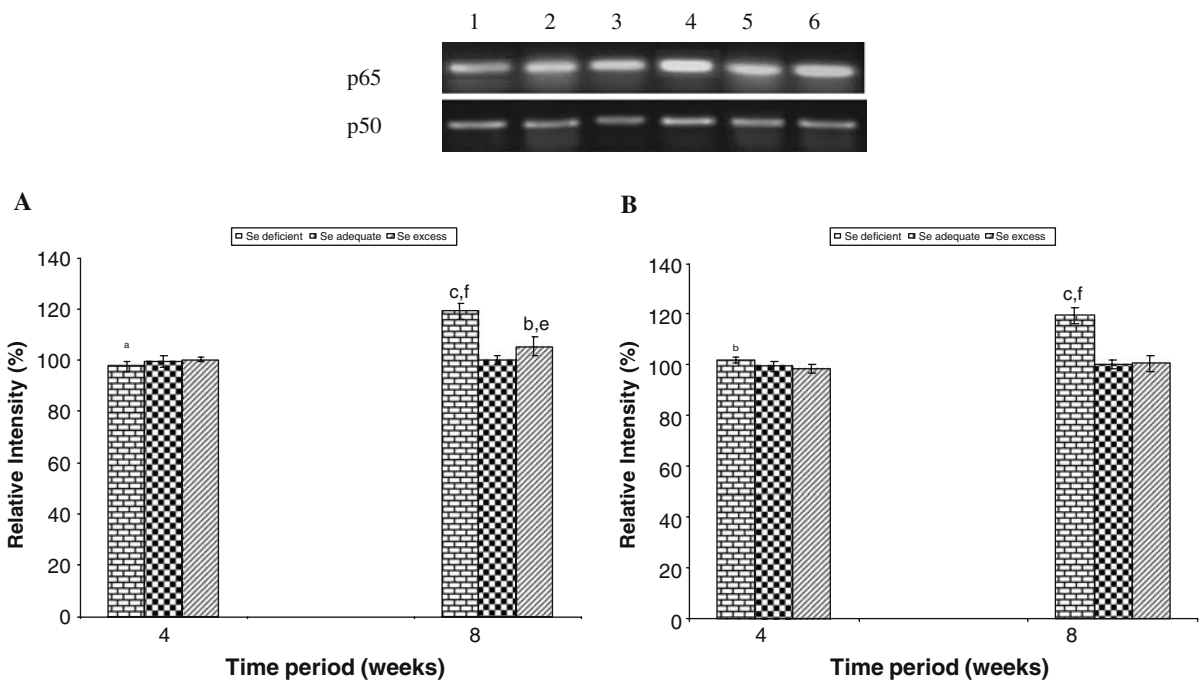

Figure 1. RT-PCR analysis for NFκB and densitometric analysis of the PCR products A:p65 and B:p50 in different groups. Values are Mean ± SD of four observations from different animals. $^{a}p < 0.05$, $^{b}p < 0.01$ and $^{c}p < 0.001$ represent comparison between Se deficient, Se excess animals with respect to Se adequate animals at 4 and 8 weeks. $^{d}p < 0.05$, $^{e}p < 0.01$ and $^{f}p < 0.001$ represent comparison between Se deficient, Se excess and Se adequate animals at 4 and 8 weeks.

Discussion

Selenium, a potent antioxidant, constitutes an essential component of GSH-Px which catalyses the degradation of peroxides specially lipid peroxides to control the level of cellular H_2O_2 and hydroperoxides and helps in the maintenance of redox status of cells (Spallholz 2001). Presently, decreased Se levels and GSH-Px activity in the liver and testis of Se deficient animals was observed, which might contribute to the building up of oxidative stress. In the Se excess group (IIIa,b), significant increase in the GSH-Px activity and Se levels was observed initially at 4 weeks, but no further increase was observed at 8 weeks. Various researchers (Hafeman et al. 1974, Lei *et al.* 1995) suggested that a homeostatic process controls the level of glutathione peroxidase and higher intake beyond the nutritionally adequate level does not elicit any further increase in enzyme activity.

Significant increase in lipid peroxidation (LPO) was recorded in both the Se deficient and Se excess supplemented groups. The drastic increase in LPO in Se deficient group can be explained on the basis of decreased levels of GSH-Px. Surai *et al.* (1998) demonstrated that this enzyme represents more than 75% of the total enzyme activity in spermatozoa. As a result of the loss of GSH-Px activity, peroxides accumulate and disturb the redox environment of the cells. In Se excess group, increased LPO could be attributed to the ability of selenite to form a highly reactive species, GSSe-, a selenopersulfide (Shen *et al.* 2001), which generates free radical, superoxide as well as other ROS. Evidence to this also comes from the work of other researchers (Lane & Medina 1985) who showed increased lipid peroxidation in mouse mammary glands as the selenite concentration increased to supranutritional levels.

Reduced percentage fertility was observed in mice fed with Se deficient diet (group I) and excess (group III) as compared to its Se adequate counterpart. Because of the high concentration of polyunsaturated fatty acids and low concentration of cytoplasmic antioxidants (Jones *et al.* 1979), spermatozoa are highly susceptible to oxidative

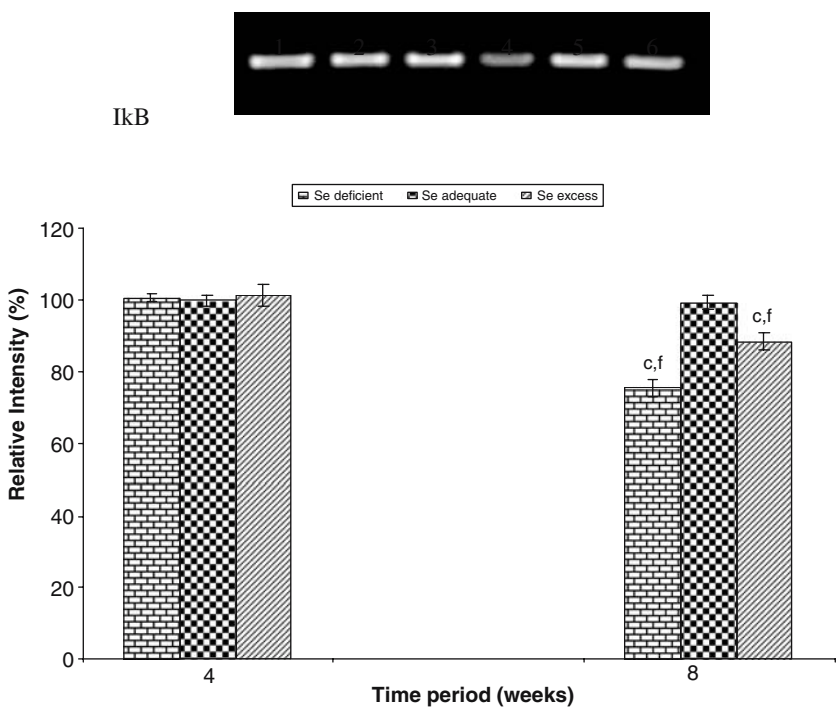

Figure 2. RT-PCR analysis for IκB and densitometric analysis of the PCR product of IκB in different groups. Values are Mean ± SD of four observations from different animals. [a]$p < 0.05$, [b]$p < 0.01$ and [c]$p < 0.001$ represent comparison between Se deficient, Se excess animals with respect to Se adequate animals at 4 and 8 weeks. [d]$p < 0.05$, [e]$p < 0.01$ and [f]$p < 0.001$ represent comparison between Se deficient, Se excess and Se adequate animals at 4 and 8 weeks.

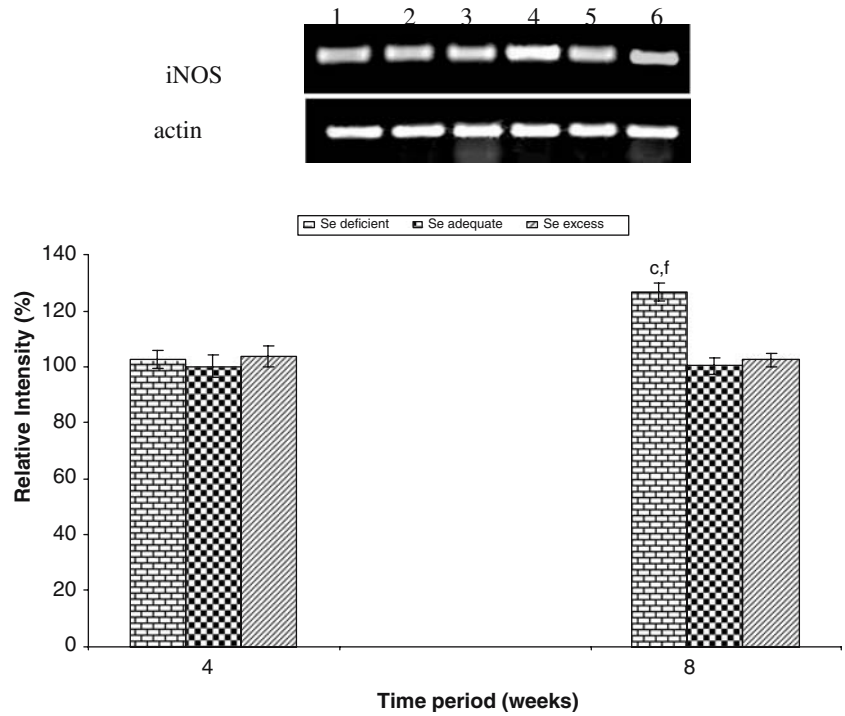

Figure 3. RT-PCR analysis for iNOS and densitometric analysis of the PCR product of iNOS in different groups. Values are Mean ± SD of four observations from different animals. $^{a}p < 0.05$, $^{b}p < 0.01$ and $^{c}p < 0.001$ represent comparison between Se deficient, Se excess animals with respect to Se adequate animals at 4 and 8 weeks. $^{d}p < 0.05$, $^{e}p < 0.01$ and $^{f}p < 0.001$ represent comparison between Se deficient, Se excess and Se adequate animals at 4 and 8 weeks.

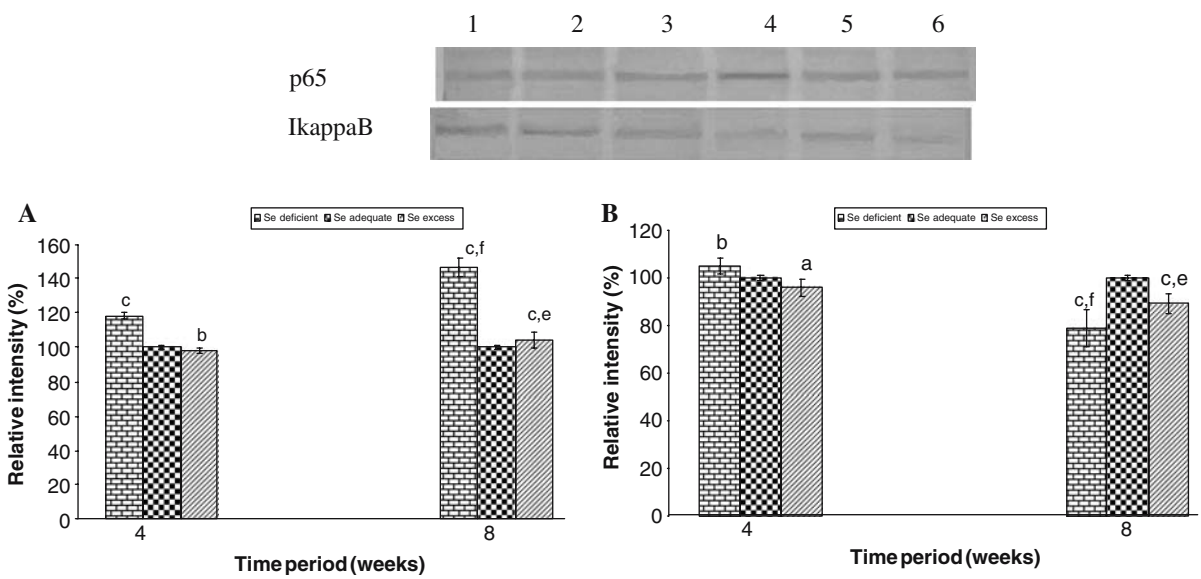

Figure. 4 Western Blot analysis for (a) NFκB (p65) and (b) IκBα and densitometric analysis of the respective proteins in different groups. Lane 1: 4 weeks Se deficient, Lane 2: 4 weeks Se adequate, Lane 3: 4 weeks Se excess, Lane 4: 8 weeks Se deficient, Lane 5: 8 weeks Se adequate, Lane 6: 8 weeks Se excess. Values are Mean ± SD of four observations from different animals. $^{a}p < 0.05$, $^{b}p < 0.01$ and $^{c}p < 0.001$ represent comparison between Se deficient, Se excess animals with respect to Se adequate animals at 4 and 8 weeks. $^{d}p < 0.05$, $^{e}p < 0.01$ and $^{f}p < 0.001$ represent comparison between Se deficient, Se excess and Se adequate animals at 4 and 8 weeks.

damage. During maturation, in the epididymis, they get exposed to this oxidative environment for a long time because of their slow transit time and prolonged storage (Cummins *et al*. 1994). Thus, decreased GSH-Px activity during Se deficiency and increased lipid peroxidation in mice fed excess Se, indicate increased levels of free radicals which disrupts the normal spermatogenic process and might be responsible for reduced sperm motility and viability leading to reduced fertility and fewer litters.

NFκB is a well-known redox regulated transcription factor, which plays an important role during the development of sperm cells (Lilienbaum *et al*. 2000). The expression of both p65 and p50 increased significantly in the Se deficient group I after 8 weeks. p65 (*Rel A*) showed a significant increase in the Se excess group but no change was evident in the p50 gene. The protein expression profile also indicated highly significant increase in p65 levels after 8 weeks of the Se deficient diet feeding schedule. Increase was also evident in Se excess group, but substantially less compared to the former. This indicated that the increased levels of ROS generated, particularly H_2O_2 leads to the up-regulation of p65 gene. Simultaneously decrease in IκB mRNA expression as well as protein levels was recorded. This again indicates that the activation of NFκB is probably due to both reduced levels of the inhibitory protein IκB and increased *p65* levels. Earlier studies have indicated activation of NFκB as a consequence of loss of GSH-Px, increased H_2O_2 levels, and increased oxidative stress (Taylor *et al*. 2004; Crack & Taylor 2005). Increase in the *in vivo* expression of the *p50* and *p65* under conditions of increased oxidative stress has been reported (Pardo *et al*. 1998). This demonstrates that Se deficiency disturbs the redox environment of the cells and these effects are mediated at the transcriptional level. Increase in the mRNA expression of p50 gene was observed. The p50 promoter is under a positive auto regulatory loop and can be activated by either of the two subunits of NFκB (p50 and p65), and more strongly by the combination of both (Ten *et al*. 1992). Enhanced binding of NFκB to its consensus sites has been shown under Se deficient conditions (Christensen & Pusey 1994). This phenomenon probably causes its continuous expression by oxidants. p50 gene expression was not affected in Se excess mice. In the excess Se supplemented group IκB levels decreased marginally indicating activation of NFκB, but this was less pronounced compared to the Se deficient group. iNOS expression was not affected by Se supplementation. This is perhaps due to over expression of GSH-Px and generation of only a mild form of oxidative stress. It has been reported (Kretz-Remy *et al*. 1996) that GSH-Px overexpression alters the isoform composition of IκB-α, leading to the accumulation of the more basic isoform of this protein which prevents proteolysis of IκB-α, a phenomenon that precedes and controls the degradation and activation of NF-kappa B.

Substantially elevated NO levels and iNOS expression were observed in both the groups but was significantly enhanced in the Se deficient group. Increased levels of NO builds up oxidative stress, which if continued for long results in testis and sperm dysfunction (Romeo *et al*. 2001). In micromolar concentrations NO reduces sperm motility (Tomlinson *et al*. 1992) but at higher micromolar concentrations it severely affects both motility and sperm viability (Hellstrom *et al*. 1994). This is detrimental to male fertility. NO is shown to be toxic to human sperm (Rosselli *et al*. 1998) and elevated levels of NO are reported in the seminal plasma of infertile men (Zini *et al*. 1996).

A number of genes expressed in the testis, including the androgen receptor, urokinase, proenkephalin are regulated by NFκB. Modulation of this transcription factor by Se indicates its possible implications in spermatogenesis and reproductive potential. Also, TNF-α, a known inducer of NFκB, has been demonstrated to inhibit steroidogenesis in Leydig cells at the transcriptional level of steroidogenic enzymes (Hong *et al*. 2004). During our previous studies, the levels of testosterone, FSH and LH were significantly reduced during Se deficiency, which has been implicated in testicular dysfunction (Kaur & Bansal 2004). Since the entire process of spermatogenesis is based on the delicate balance of these endocrine factors, NFκB activation might be a possible mechanism for significant decrease in percentage fertility and reduced reproductive performance. Reports also suggest that during testicular stress NFκB proteins exert proapoptotic effects on germ cells, which raises the possibility that increased expression of NF-κB could lead to situations involving excessive germ cell death and low fertility. (Pentikainen *et al*. 2002).

In conclusion, a certain basal level of NFκB activation is required for the normal progression of spermatogenesis. However, Se deficiency disturbs the intracellular redox balance and increases oxidative stress. This causes activation of transcription factor NFκB that in return initiates a cascade of events inducing several target genes such as iNOS and reduced androgen levels that severely affects the male reproductive ability.

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