Male infertility and abnormal progeny outcome are some of the consequences resulting from exposure of germ cells to various stressors. Of these oxidative stress emerges as one of the most important mediator of testicular dysfunctions. Numerous epidemiological observations and in vitro studies in mammalian spermatozoa indicate the involvement of oxidative stress in abnormal spermatogenesis and hence infertility in the subjects.

Selenium is an essential micronutrient and is of fundamental importance to human health. It is essential for normal functioning of the male reproductive system and its deficiency has been linked with testicular dysfunction and reduced sperm motility. High concentration of selenium is also toxic and linked to decrease in fertility.

Selenium, a well known antioxidant, gets incorporated into proteins in the form of amino acid selenocysteine (Sec). Most of the selenoproteins viz. glutathione peroxidase, thioredoxin reductase, selenoproteins P etc. have ROS scavenging activities. On the other hand selenium is an effective anticancer agent and inhibits cell proliferation in various tumor cells. Several studies indicate that selenium affects cellular activities presumably by acting on proteins important for signal transduction. Two transcription factors-activator protein 1(AP1) and nuclear factor $\kappa$B (NF$\kappa$B) are sensitive to the cellular redox status and regulate the activity of several genes associated with cell growth, proliferation, differentiation, inflammation and apoptosis. Both these transcription factors are known to be expressed in the testis, and previous studies have indicated their involvement in the process of spermatogenesis.

Considering the above, the present study aimed to investigate the involvement of the upstream kinases for the transcription factors along with other parameters in the progression of spermatogenesis and apoptosis under selenium stress conditions. Moreover, the study was further extended to correlate the alterations in the expression of these kinases with the apoptotic activity of germ cells in terms of the apoptotic factors involved.

Different selenium status was generated viz. selenium deficient (group I), selenium adequate (group II) and selenium excess (group III) in mice by feeding the respective diets for a period of 4 (group Ia, Ila, IIIa) and 8 weeks (group Ib, IIb, IIIb).
The animals in selenium deficient groups (Ia and Ib) received 0.02ppm selenium which is well below the adequate levels of 0.2ppm given to animals in the adequate group (IIa and IIb). Animals in the excess groups (IIIa and IIIb) received 1ppm selenium which is considered in the supranutritional range but is well below the toxic level (3-4ppm).

No significant change in body weight of the animals was observed. Significant decrease in selenium levels was observed in the testis in selenium deficient groups. Reduction in glutathione peroxidase (GSH-Px) activity and mRNA expression, observed presently during selenium deficiency further confirms the establishment of selenium deficient state in the body. Low selenium levels are associated with reduced sperm function, reduced sperm motility and loss of fertility. Selenium levels increased in excess group. Glutathione peroxidase activity and mRNA expression increased in the selenium excess group at 4 weeks but no further increase was observed at 8 weeks suggesting that incorporation of selenium in this enzyme is under a homeostatic control.

Lipid peroxidation was found to increase in selenium deficient groups which substantiates the fact that glutathione peroxidase plays an important role in free radical scavenging and indicates increased oxidative stress in selenium deficient animals.

It was observed that selenium deficiency and excess conditions progressively increased Reactive Oxygen Species (ROS) generation. Numerous studies have linked increased production of ROS with increased oxidative stress, increased testicular dysfunction, decreased sperm motility and infertility.

Decrease in reduced glutathione (GSH) levels and increase in oxidized glutathione (GSSG) levels was observed in both selenium deficient and selenium excess groups. Glutathione redox ratio (GSH/GSSG) decreased in animals from both selenium deficient and selenium excess groups. The rate limiting enzyme in glutathione synthesis is γ-Glutamyl cysteine synthase (γGCS). mRNA expression studies indicated decrease in γGCS after 8 weeks in selenium deficient and excess animals. This indicates that selenium is a potential thiol perturbing agent. It is the most common non-protein thiol antioxidant and forms important constituent of several antioxidant enzymes such as glutathione peroxidase, glutathione reductase and glutathione-S-transferase.
In the present study, glutathione reductase (GR) activity and mRNA expression decreased in selenium deficient animals at 4 weeks and progressive decrease was observed after 8 weeks. In the selenium excess diet fed group decrease was noticed only after 8 weeks. Glutathione reductase is responsible for converting GSSG back to GSH. This suggests the accumulation of oxidized glutathione in these groups. Glutathione-S-transferase (GST) activity increased in selenium deficient and selenium excess groups after 4 and 8 weeks. mRNA expression of GST also found to increase in both selenium deficient and selenium excess groups after 8 weeks. GST is responsible for detoxification of ROS. Increase in the activity implies an adaptive mechanism to cope up oxidative stress.

Catalase and Superoxide dismutase (SOD) activity also decreased in selenium deficient groups at both 4 and 8 weeks. Increase in catalase activity was observed in selenium excess group at 8 weeks. However, SOD activity decreased at 4 weeks while no change was observed after 8 weeks in selenium excess groups. This further suggests that selenium deficiency leads to increased accumulation of $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ that builds up oxidative stress.

The direct effect of different selenium status on male reproductive activity was determined in terms of sperm motility, sperm concentration and fertility status of the treated male mice. Sperm motility and concentration were found to be significantly reduced in selenium deficient groups at both 4 and 8 weeks. Supplementation of 1 ppm selenium also affected sperm concentration and motility but this was less as compared to selenium deficient animals. The fertilizing ability (percent fertility) and litter size were not affected at 4 weeks. However, significant reduction in both these parameters was observed at 8 weeks in both selenium deficient and excess groups when treated males were mated with normal females. These results demonstrate the functional relevance of oxidative stress generated during selenium deficiency and excess and the development of male infertility.

Histopathological changes observed in the selenium deficient animals included shrinkage of seminiferous tubules, reduced lumen size and displacement of germ cells. Also, appreciable decrease in the number of pachytene spermatocytes, spermatids and mature spermatozoa was observed, suggesting a disruption during meiosis. This suggests that selenium deficiency generates oxidative stress which interferes with the progression of spermatogenesis. Animals fed selenium excess diet for 4 weeks did not reveal any change but prolonged supplementation for 8 weeks
resulted in shrinkage of seminiferous tubules and reduced germ cell height. Significant change in the number of various spermatogenic cells was observed at 4 and 8 weeks.

To understand the mechanism by which selenium exerts its effects on transcription factors AP1 and NFκB, the various kinases which regulate their function were studied.

AP1 is supposed to have predominant role in spermatogonial proliferation and in the physiology of mammalian and non mammalian germ cells. The various kinases that regulate AP1 are collectively termed as the Mitogen Activated Protein Kinases (MAPKs). These include c-Jun N terminal Kinase (JNK), p38 kinase and Extracellular signal regulated kinase (ERK). Expression of these kinases was studied in the present study at both transcriptional (RT-PCR) and translational (western immunoblot) level.

mRNA expression analyses for JNK revealed increase in expression of the kinase in selenium deficient and excess groups. This was further confirmed by Western immunoblot analyses where also increase in protein expression was observed. This increase in expression is suggestive of the involvement of this kinase in the progression of spermatogenesis and also in apoptosis under selenium stress conditions.

Immunohistochemical analysis also indicated increase in the number of JNK positive cells in selenium deficient and excess groups as compared to the selenium adequate group. It is likely that under selenium deficient and excess conditions, an increase in the generation of ROS as a consequence of lipid peroxidation, and a decrease in the cellular GSH pool, might lead to phosphorylation and activation of this kinase. Once activated, JNK might further activate a series signal transduction events, involving the caspases, ultimately culminating in apoptosis. During selenium deficiency and excess conditions, this activation of JNK kinase might account for the reduced number of post-meiotic cells-spermatids and spermatozoa observed in these groups.

mRNA expression analyses for p38 revealed increase in expression of the kinase in selenium deficient and excess groups, which was further confirmed by Western immunoblot analyses where similar results were obtained. This increase in expression is also suggestive of the involvement of this kinase in the progression of spermatogenesis and also in apoptosis under selenium stress conditions.
Immunohistochemical analysis also indicated increase in the number of p38 positive cells in selenium deficient and excess groups as compared to the selenium adequate group. Similar explanation as above could explain the observations. Selenium deficient and excess conditions lead to an increase in the generation of ROS as a consequence of lipid peroxidation, and a decrease in the cellular GSH pool. This might lead to phosphorylation and activation of this kinase. Once activated, p38 kinase might further activate a series signal transduction events, involving the caspases, ultimately culminating in apoptosis. During selenium deficiency and excess conditions, this activation of p38 kinase might also account for the reduced number of post-meiotic cells-spermatids and spermatozoa observed in these groups.

mRNA expression and protein expression analyses for ERK revealed no change in expression of the kinase in selenium deficient and excess groups. This observation is suggestive of its non involvement in the progression of spermatogenesis and also in apoptosis under selenium stress conditions. These observations were further strengthened by immunohistochemical analyses, where similar results were obtained.

NFκB is another redox sensitive transcription factor that also plays an important role in the development of spermatozoa. The kinases responsible for regulation of NFκB include IκB kinase alpha (IKKα) and NFκB Inducing Kinase (NIK). Activation of NFκB requires degradation of IκB and translocation of the active dimer (p65-p50) into the nucleus. This is achieved by its two upstream kinases. Presently, mRNA expression of IKKα and NIK increased in selenium deficient animals after 4 and 8 weeks. The protein expression of the kinases also revealed increase in expression after 4 and 8 weeks of selenium deficient diet feeding schedule. Increase was also evident in selenium excess groups, but was substantially less compared to the former. Immunohistochemical localization studies also confirmed the above observations. Previous studies have reported an increase in the mRNA expression of p65 and p50 in selenium deficient animals after 8 weeks. The protein expression of p65 in the nuclear fraction also increased after 8 weeks of selenium deficient diet feeding schedule. Increase was also evident in selenium excess group, but was substantially less compared to the former. Simultaneously decrease in IκBα mRNA expression as well as protein levels was recorded in selenium deficient and excess group after 8 weeks in previous studies. This indicates that the increased levels of ROS generated, particularly H$_2$O$_2$ leads to phosphorylation and activation of NIK.
The activated NIK then phosphorylates and activates IKKa, which then acts upon the NFkB-IkB complex, finally degrading the IkB protein and rendering the NFkB molecule free to be translocated to the nucleus. Previous studies have also revealed reduction in the levels of the inhibitory protein IkB, up regulation of p65 gene and its translocation into the nucleus especially during selenium deficiency.

All these observations indicate the activation and translocation of this transcription factor into the nucleus. Activation of NFkB is reported to inhibit steroidogenesis and it also exerts proapoptotic function in testis. Since the entire process of spermatogenesis is based on the delicate balance mediated by hormones, NFkB activation might be a possible mechanism for significant decrease in percentage fertility and reduced reproductive performance as observed presently.

DNA damage is known to be the most important outcome of the oxidative stress. Interplay of a variety of molecules and pathways have been found to execute the process of DNA damage induced apoptosis. So, it was relevant to study the molecular mechanism/s of oxidative stress mediated DNA damage and hence apoptosis.

Currently an enhanced expression of caspase3 and caspase8 was detected in selenium deficient and selenium excess group animals in comparison to animals that were fed on diet supplemented with adequate levels of selenium at both the treatment intervals. These modulations in the expression of the apoptotic factors are mainly attributable to the induction of free radical mediated oxidative stress in selenium deficient and selenium excess group of animals. These findings point to the existence of a positive relationship between sperm damage by ROS and higher levels of caspases, which indicates the presence of apoptosis in germ cells following variations in dietary selenium.

Irregularities in germ cell apoptosis are associated with infertility and also the sperm DNA integrity is a more objective marker of sperm function. DNA fragmentation was observed in testis from selenium deficient and selenium excess group of animals. This may be attributed to the enhanced oxidative stress in these groups. This can be related to the poor sperm motility and morphology as observed in selenium deficient and excess group of animals at both the treatment intervals.

In the present study, TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) assay was used to identify and characterize apoptotic germ cells. The frequency of TUNEL positive cells increased in both selenium deficient
and excess group of animals. Spermatogonial cell populations were found to be maximally apoptotic in both the groups at both the treatment intervals. However, in selenium deficient animals, there was also a simultaneous increase in the TUNEL positive spermatocytes than the adequate group where no such DNA damage was observed.

Glutathione, the common cellular non-protein thiol is closely involved in selenium metabolism. In order to study and clearly ascertain the observed in vivo effects associated with different selenium status in the present study, testicular germ cells were maintained in the presence of Butthionine Sulphoximine (BSO) (glutathione depletor) and selenium (sodium selenite) was added at 0.5µM and 1.5µM concentration which were largely non-cytotoxic. BSO and selenium alone and in combination, lead to increased generation of ROS. BSO and selenium alone and when co administered caused decrease in GSH levels in a dose dependent manner and an increase in the levels of GSSG.

Coadministration of selenite and BSO induced the expression of JNK and p38 kinase while no change was observed in the expression of ERK kinase as studied by RT-PCR. Thus, the increased expression seen in the present study demonstrates that glutathione depletion affects the apoptotic response of a cell since JNK and p38 kinase are involved in the apoptotic response of a cell.

In lieu of strengthening the above observation, apoptotic factors like caspase3 and caspase8 were studied which showed highly induced expression when BSO and selenium were administered alone and in combination. On the other hand, anti apoptotic Bcl2 was highly suppressed in these conditions, suggestive of the presence of apoptosis. Also, the integrity of DNA was damaged in case BSO and selenium were administered alone and in combination.

In conclusion present study indicates that selenium is essential for the progression of spermatogenesis. Stress responsive kinases that regulate the activity of transcription factors AP1 and NFκB appear as the key signaling molecules involved in the process by which selenium regulates spermatogenesis. JNK and p38 kinase that regulate the expression of AP1 showed a pro apoptotic response under selenium deficient and selenium excess conditions, whereas ERK was demonstrated to be non responsive. The kinases for NFκB; IKKα and NIK also showed a pro apoptotic response under selenium deficient and selenium excess conditions. This pro apoptotic
Summary

response demonstrated by the kinases that regulate redox sensitive transcription factors AP1 and NFkB might be the reason for reduced number of spermatids and spermatozoa in selenium deficient animals and reduced germ cell height in selenium excess group. Based on these observations the mechanism behind regulation of spermatogenesis under selenium status has been proposed.