MATERIALS AND METHODS

CHEMICALS PROCUREMENT:
Sodium selenite, 2,3-diamino napthalene (DAN), Bovine serum albumin (BSA), Buthionine sulfoximine (BSO), Diethyl pyrocarbonate (DEPC), Agarose, Ethidium bromide, EDTA, Sodium acetate, NADPH, Tetraethoxypropane (TEP), Glutathione reductase, o-pthaldehyde (OPT), N-ethylmaliemide (NEM), Oxidised Glutathione (GSSG), Reduced Glutathione (GSH), Proteinase K, Dichlorofluorescin diacetate (DCFH-DA), Dithiothreitol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco’s modified eagle’s medium, DMEM (with L-glutamine and 15mM HEPES), Fungizone, Fetal Calf Serum (FCS) and Acridine orange were purchased from Sigma-Aldrich, St Louis (USA). Molecular biology grade chemicals for RNA isolation e.g. chloroform, isopropanol, ethanol and formaldehyde were purchased from Amresco, Ohio (USA). Formamide, Methanol, Hydroxyl ammonium chloride, Cyclohexane (HPLC grade) and sucrose were purchased from E. Merck (India) Ltd., India.

Antibodies for JNK, p38 and mouse β-actin were from Sigma-Aldrich, St Louis (USA). Antibodies for IKKa, NIK and ERK were from Santa Cruz Biotechnology, Santa Cruz, CA (USA). All other chemicals and reagents used in the present study were of analytical grade and were procured from Indian manufacturers, viz. SRL, CDH, SD-Fine etc.

TREATMENT PROTOCOL:
Male Balb/c mice in the weight range of 20-25g procured from the Central Animal House, Panjab University, Chandigarh (India) were used in the present study. They were housed in the departmental animal room in polypropylene cages at 12/12 hr light/dark cycle. Food and water were provided ad libitum.

To create different selenium (Se) status in the animals viz. 0.02ppm, 0.2ppm and 1ppm, in different groups (Group I, II and III respectively), the mice were kept on yeast-based diet. The yeast-based diet usually contains 0.02ppm selenium and hence animals fed on this diet were considered selenium deficient animals (Group I). For the selenium supplemented groups, selenium was added at 0.2ppm (Group II; adequate level) and 1ppm (Group III; excess level) as sodium selenite to the selenium deficient diet. 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).
DIET PREPARATION:

Selenium deficient diet: Selenium deficient diet was prepared using inactivated Baker’s yeast as a protein source in the laboratory itself according to the composition given by Burk (1987) shown in Table B.

Table B: Composition of the diet

<table>
<thead>
<tr>
<th>Components</th>
<th>Percentage by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>56.99</td>
</tr>
<tr>
<td>Bakers yeast (inactivated)</td>
<td>30.00</td>
</tr>
<tr>
<td>Corn oil</td>
<td>6.67</td>
</tr>
<tr>
<td>Mineral mixture (USP XIV)</td>
<td>5.00</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1.00</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.30</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Water soluble vitamins: Water soluble vitamins were weighed individually to make a vitamin mixture as given in Table C which was then made 100g with sucrose. This was used at a final concentration of 1% in the diet.

Table C: Water soluble vitamin mixture

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>10.00g</td>
</tr>
<tr>
<td>Inositol</td>
<td>1.10g</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>1.10g</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1.650g</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>1.00g</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.22g</td>
</tr>
<tr>
<td>Pyridoxin hydrochloride</td>
<td>0.22g</td>
</tr>
<tr>
<td>Calcium pentothenate</td>
<td>0.66g</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>0.22g</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.02g</td>
</tr>
<tr>
<td>d-biotin</td>
<td>4.40mg</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.3mg</td>
</tr>
</tbody>
</table>
Materials and Methods

Oil soluble vitamins: Oil soluble vitamins were dissolved directly in corn oil and added in the diet as shown below (for 1kg diet):

Table D: Oil soluble vitamin mixture

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>20,000 IU</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>5 mg</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>100 mg</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>1000 U</td>
</tr>
</tbody>
</table>

Selenium supplemented diet: For the selenium supplemented groups, selenium was added at 0.2 ppm (Group II; adequate level) and 1 ppm (Group III; excess level) as sodium selenite in the above diet. This 1 ppm selenium dose was so chosen that the level is in excess to the adequate level but is well below the toxic limits (3-4 ppm).

All the animals were fed with the respective diets for 4 weeks (Group Ia, Ila and IIIa) and 8 weeks (Group Ib, IIb and IIlb). The body weight of the animals was recorded every week. After completion of the diet-feeding schedule, animals were sacrificed using ether anesthesia followed by cervical dislocation. Tissues were removed immediately to analyze the following parameters:

1. Selenium estimation in testis of mice from different treatment groups after four and eight weeks of diet feeding schedule.
2. Effect of different levels of selenium status on the oxidation-reduction system by carrying out biochemical estimations for the following: glutathione peroxidase (GSH-Px), lipid peroxidation (LPO), redox status (reduced glutathione/oxidized glutathione; GSH/GSSG), catalase, superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST).
3. Determination of ROS generation in testis.
4. Evaluation of the reproductive potential of mice after completion of the diet feeding schedule by measuring sperm concentration in epididymis, sperm motility in vas deferens and percentage fertility.
5. To examine the changes in testis at microscopic level using light microscopy and testicular germ cell kinetics.
Materials and Methods

6. mRNA expression for the following genes was studied in testis by RT-PCR (reverse transcriptase-polymerase chain reaction): GSH-Px, γ-glutamylcysteine synthetase (γGCS), GST, MnSOD, catalase and GR. mRNA expression of stress responsive protein kinases, viz; JNK (c-jun N-terminal kinase), p38, ERK (Extracellular signal regulated kinase), IKKα (Inhibitory kappaB kinase alpha) and NIK (NF-κB inducing kinase) were also analyzed.

7. Protein expression of JNK, p38 and ERK by Western immunoblot analysis.

8. Protein expression of IKKα and NIK by ELISA.

9. Immunohistochemical localization of these kinases in testis sections by immunohistochemistry.

10. Evaluation of apoptotic factors in all the treatment groups by analyzing the mRNA expression of caspase 3, caspase 8 and Bcl-2.

11. Evaluation of apoptosis in testis in all the treatment groups using TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) and DNA ladder studies.

12. Total testicular cell maintenance was used to study the effect of selenium in modulating the mRNA expression of the above mentioned kinases under conditions of experimental inhibition of glutathione synthesis using buthionine sulfoximine (BSO), a well-known inhibitor of γGCS.

13. Evaluation of apoptosis in the above maintained testicular cells by ethidium bromide/acridine orange staining, by analyzing the mRNA expression of caspase 3, caspase 8 and Bcl-2, and by DNA ladder studies.

1) SELENIUM ESTIMATION:

Selenium levels were estimated by following the method of Hasunuma et al., (1982) based on the principle that selenium content on acid digestion is converted into selenous acid. Further, the reaction between selenous acid and aromatic-o-diamine such as 2,3-diamino naphthalene (DAN) leads to the formation of 4,5-benzopiaz-selenol, which displays brilliant lime green fluorescence when excited at 366nm in cyclohexane.

All the reagents were made in double distilled water.
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Preparation of DAN: 50mg of DAN was dissolved in 50ml of 0.5% hydroxyl ammonium chloride in 0.1M HCl in brown bottle and kept for 1hr on magnetic stirrer for proper dissolution. DAN was washed with equal volume of cyclohexane by vigorous shaking in a separating funnel with stopper and kept for 2-3 minutes till the two layers got separated. The lower aqueous layer of DAN was collected. This washing procedure was repeated thrice to clear DAN of all the impurities. The DAN solution was finally stored in a dark coloured bottle.

Digestion of samples: For digestion of tissues, modified kjeldahl type flasks with long air condensers were used to prevent any loss of selenium as volatile selenides. 5ml of concentrated nitric acid (HNO₃) was added to 100mg of tissue (testis) in the digestion flasks and heated continuously on a hot sand bath till the brown fumes ceased. After this, temperature of sand bath was lowered and 1ml of perchloric acid (HClO₄) was added without removing the condensers. Heating was resumed for another 30min, following which the flasks were removed from the sand bath and cooled. The resultant solution was referred as digest. Volume of each digest was measured.

Estimation: To 1ml of the digest, 1ml each of 6N HCl and 50% formic acid were added. Then 2ml stabilizing solution (0.025M EDTA in 2.5% hydroxyl ammonium hydrochloride), 2.5ml of ammonia solution and 5ml of DAN solution were added. This mixture was incubated at 50°C for 20min in water bath. The tubes were cooled and 3ml cyclohexane was added to each tube. The samples were thoroughly mixed on a vortex mixer for 1min and the tubes were allowed to stand to separate the two layers. The upper layer (cyclohexane) was carefully removed and collected in a separate test tube. This process was repeated twice and the cyclohexane layers pooled. Fluorescence of 4,5-benzopia-zselenol formed was quantitated on spectrophlorometer (Perkin Elmer, LS55, USA) using 366nm as excitation and 520nm as emission wavelength.

One ml of working standard and 1ml of distilled water was added to respective standard and blank tubes.

Working standard solution: Stock solution (1mg Se/ml) was prepared by adding 2.3 mg of dried sodium selenite in 0.1ml perchloric acid and final volume was
made 1ml with distilled water. This solution was diluted to 0.1μg Se/ml and used as the working standard solution.

**Calculations:**

\[
\frac{\text{Test reading}}{\text{Standard reading}} \times \frac{\text{Concentration of standard}}{\text{Total volume of digest}} \times \frac{\text{Total volume of digest}}{\text{Tissue weight (g)}}
\]

Selenium levels were expressed as μg selenium/g testis.

2. **BIOCHEMICAL ESTIMATIONS:**

To study the oxidation-reduction system of the tissue, homogenates were prepared (10%w/v) in 20mM Tris-HCl (pH 7.4) using mechanically driven teflon fitted potter elvejham type homogenizer under ice-cold conditions. Homogenates were centrifuged at 10,000g for 30min at 4°C to get post mitochondrial fraction (PMF). The following parameters were analyzed:

i) **Glutathione peroxidase:**

Total glutathione peroxidase (GSH-Px) activity was estimated in testis by the method of Lawrence and Burk, (1976) using H$_2$O$_2$ as substrate. The reaction mixture consisted of:

- 50mM Potassium phosphate buffer, pH 7.0: 566μl
- 1mM EDTA: 100μl
- 1mM Sodium azide: 10μl
- 0.2 mM NADPH: 100μl
- Glutathione reductase (1EU/ml): 4μl
- 1mM GSH: 100μl
- 0.25 mM H$_2$O$_2$: 100μl

The reaction was started by adding 20μl of PMF to 880μl of the above mixture (except H$_2$O$_2$) followed by incubation for 5min at room temperature. Then 100μl of H$_2$O$_2$ was added. Absorbance was recorded at 340nm (UV-160A, Shimadzu) for 3min. Enzyme activity was calculated from the slope of these lines and expressed as μmoles NADPH oxidized /min/mg protein.

ii) **Lipid peroxidation:**

The levels of malondialdehyde (MDA) were used as an index for measuring the extent of lipid peroxidation according to the method of Wills, (1966).
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**Procedure:** To 250µl of PMF, 500µl of ice-cold TCA (10%) was added and centrifuged at 800g for 10min. The supernatant was collected and to 500µl of supernatant an equal volume of thiobarbituric acid, TBA (0.67% in distilled water) was added. The tubes were kept in boiling water bath for 10min. Since malondialdehyde is a degradation product of peroxidized lipids, the development of pink colour (with the absorption characteristics: maxima 532nm) of TBA-MDA chromophore was taken as an index of lipid peroxidation. For standard, 2-10nM range of 1,1',3,3'-tetraethoxypropane (TEP) in 250µl volume was used and for control, distilled water was used instead of the sample. Results were expressed as nmoles MDA/mg protein.

**iii) Estimation of Glutathione levels:**

The levels of total and oxidized glutathione were measured by the fluorimetric method of Hissin and Hilf, (1976). This method is based on the principle that glutathione reacts specifically with o-pthaldehyde (OPT) at pH 8 and with GSSG at pH 12 resulting in the formation of a highly fluorescent product which is activated at 350nm with an emission at 420nm.

**Procedure:** To the PMF, trichloroacetic acid (TCA; final concentration 5%) was added to precipitate the protein and supernatant was obtained after centrifugation at 10,000g for 10min at 4°C. The samples were then processed differently for measuring total and oxidized glutathione.

**Total glutathione:**
The reaction mixture consisted of:

- 0.1 M sodium phosphate-5mM EDTA buffer (pH 8) 9.4 ml
- Tissue supernatant (after TCA precipitation) 100µl
- OPT (100mg% in methanol) 500µl

The samples were mixed by vortex and incubated at room temperature for 15min. Reduced glutathione (GSH) in the range of 5-40µg was used as standard.

**Oxidized glutathione (GSSG):** For the estimation of GSSG, 0.5ml of the supernatant after TCA precipitation was mixed with 200µl of 0.04M N-ethylmaleimide (NEM) at room temperature for 30min. This causes inhibition of GSH at an alkaline pH 12. The reaction mixture contained:
Materials and Methods

0.1N sodium hydroxide 9.4ml
Fraction from above reaction 100μl
OPT (100mg% in methanol) 500μl

The samples were mixed by vortex and incubated at room temperature for 15min. Oxidized glutathione (GSSG) in the range of 5-40μg was used as standard. The fluorescence intensity was recorded in a fluorescence spectrophotometer (Perkin Elmer, USA) with an excitation filter of 350nm and an emission filter of 420nm. Results were expressed as nmoles of GSSG/mg protein.

Reduced glutathione (GSH): GSH levels were obtained by subtracting the values of GSSG levels from the total glutathione levels. Results were expressed as nmoles of GSH/mg protein.

iv) Catalase:
Catalase activity was estimated according to the method of Luck, (1963) using hydrogen peroxide as the substrate.
The reaction mixture consisted of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM phosphate buffer (pH 7)</td>
<td>2.5ml</td>
</tr>
<tr>
<td>0.75 M H₂O₂</td>
<td>50μl</td>
</tr>
</tbody>
</table>

The reaction was started by adding 100μl of enzyme (PMF) to the above reaction mixture and the rate of change in absorbance was recorded at 240nm for 2min. The enzyme activity was expressed as the rate of decrease in absorbance using the molar extinction coefficient of H₂O₂ (0.0394 mM⁻¹cm⁻¹). Results were expressed as μmoles of H₂O₂ decomposed/min/mg protein.

v) Superoxide dismutase:
Activity of superoxide dismutase (SOD) was estimated by the method of Kono, (1978). The reaction is designed to observe the inhibition of the rate of oxidation of nitroblue tetrazolium (NBT), which was recorded using hydroxylamine hydrochloride as the electron donor.
The reaction mixture consisted of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A (3.8mg EDTA and 0.53 g Na₂CO₃ in 100ml distilled water, pH 10)</td>
<td>1.3ml</td>
</tr>
<tr>
<td>NBT (8 mg/100ml of Sol A)</td>
<td>0.5ml</td>
</tr>
<tr>
<td>0.6% Triton X in Solution A</td>
<td>0.1ml</td>
</tr>
<tr>
<td>2mM hydroxylamine hydrochloride (pH 6)</td>
<td>0.1ml</td>
</tr>
</tbody>
</table>
Materials and Methods

After the addition of hydroxylamine hydrochloride, the change in absorbance $A_x$ was measured at 560nm. To this reaction mixture, enzyme source (PMF) was added and again change in absorbance $A_y$ was recorded at 560nm. Difference in the rate was compared and expressed as percent inhibition.

$$\% \text{ inhibition} = \frac{A_x - A_y}{A_x} \times 100$$

One enzyme unit is defined, as the enzyme required resulting in 50% inhibition of $A_x$. The enzyme activity was expressed as units/mg protein.

vi) Glutathione reductase:

Glutathione reductase activity was estimated according to the method of Massey and Williams, (1965). The activity was monitored by following the oxidation of co-factor NADPH by oxidized glutathione (GSSG) at 340nm. The utilization of NADPH was directly related to the activity of glutathione reductase.

The reaction mixture consisted of:

- 3mM EDTA 0.1ml
- 2%BSA 0.1ml
- 0.1mM NADPH 0.1ml
- 3mM GSSG 0.1ml
- 0.1M potassium phosphate buffer (pH 7.5) 0.6ml

The reaction was started by the addition of 50μl of the PMF and the oxidation of NADPH was recorded at 340nm for 3min. Enzyme activity was expressed as μmoles of NADPH oxidized/min/mg protein.

vii) Glutathione-S-transferase:

The activity of Glutathione-S-transferase (GST) was measured by using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to the method of Habig et al., (1974).

The reaction mixture consisted of:

- 0.1M potassium phosphate buffer (pH 6.5) 1ml
- 1mM reduced glutathione (GSH) 35μl
- 1mM CDNB (in absolute alcohol) 35μl

To the above mixture, 50μl of the tissue sample (PMF) was added and the formation of conjugated complex of CDNB was monitored at 340nm. The activity
was expressed as μmoles CDNB conjugated/min/mg protein using the molar extinction coefficient of CDNB (9.6mM⁻¹cm⁻¹).

viii) **Protein estimation:**

Protein estimation was done by the method of Lowry et al., (1951). To 10μl of sample from each treatment group, 3ml of 50:1 mixture of 2% sodium carbonate in 0.1N NaOH and 0.5% CuSO₄ /1% Na-K tartrate in distilled water was added. The tubes were incubated for 10min at room temperature. Then 300μl of 1N Folin's phenol reagent was added to each tube, mixed and again incubated for 30min at room temperature. The optical density was then measured at 620nm on spectrophotometer (UV-160A, Shimadzu). Bovine serum albumin (BSA) was used as standard (10-100μg).

3. **DETERMINATION OF FREE RADICALS IN TESTIS:**

Determination of free radicals was based on the modified method of Driver et al., (2000). This method is based on the principle that the acetate group of Dichlorofluorescein diacetate (DCFH-DA) will be cleaved by esterases activated by various free radicals to form a fluorescent product, dichlorofluorescein (DCF) whose intensity can be measured at 530nm.

**Solutions:**

1. Locke’s Buffer:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>154mM</td>
</tr>
<tr>
<td>KCl</td>
<td>5.6mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>3.6mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.0mM</td>
</tr>
<tr>
<td>D-glucose</td>
<td>10mM</td>
</tr>
<tr>
<td>HEPES (pH 7.4)</td>
<td>5mM</td>
</tr>
</tbody>
</table>

**Procedure:** Testicular homogenates were prepared in ice-cold Locke’s buffer. The homogenates were warmed at 21°C for 5 minutes. The reaction mixture containing 10μM Dichlorofluorescein diacetate (DCFH-DA) and 5mg tissue/ml was incubated for 15 min at room temperature (21°C) to allow the probe to be incorporated into any membrane-bound vesicles and the diacetate groups cleaved by esterases. After another 30 min. of incubation, the conversion of DCFH to the fluorescent
Materials and Methods

product Dichlorofluorescein (DCF) was measured using fluorescence spectrophotometer with excitation at 485nm and emission at 530nm. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by inclusion of parallel blanks. The relative difference in fluorescence intensity was taken as the measure of amount of ROS in different treatment groups.

4. EVALUATION OF REPRODUCTIVE POTENTIAL:
   i) Sperm Concentration: Epididymal spermatozoa were suspended in 1ml of normal saline. One drop of the sperm suspension was placed on the haemocytometer and covered with a coverslip. The number of spermatozoa were counted in all the 64 small wells of the chamber and then from the knowledge of the area (1mm²) and depth (0.1mm) of the chamber, sperm number per mm³ were calculated.
   ii) Sperm Motility Studies: Spermatozoa were teased out from the vas deferens in a watch glass containing 1ml normal saline (0.9% sodium chloride) at room temperature. Clumps were removed by gently mixing through a micropipette. 100µl of this suspension was placed on a slide and covered with a coverslip. The number of motile and non-motile spermatozoa was counted in several random fields. Motility was then expressed as the percentage of motile sperms.
   iii) Fertility Studies: At the end of the treatment period, male mice were allowed to mate with normal females in the ratio of either 1:3 or 1:2 for 7 days, after which the males were separated. The females were observed for 21 days for signs of pregnancy or for giving birth to pups. Percentage fertility was calculated as:

   \[
   \text{Percentage fertility} = \frac{\text{Number of females giving birth}}{\text{Number of females exposed to mating}} \times 100
   \]

   For each female giving birth, the litter size was recorded and averaged for each group.

5. HISTOLOGICAL STUDIES:
   i) Light Microscopy: On completion of the diet feeding schedule, testis from the animals were used for histological studies using light microscopy. Testis were removed and immediately transferred to Zenker’s fixative. After 3-4hr of fixation,
transverse cut was given to allow easier penetration of the fixative and the fixation process was continued for 24hr. The tissues were then thoroughly washed in running water overnight to remove all traces of the fixative. Next, the tissue was dehydrated in ascending series of alcohol (30%, 50%, 70%, 90% and 100%) for 1hr each. Traces of mercury were removed by placing the tissue pieces in iodine solution (prepared in 70% alcohol) during dehydration at 70% alcohol level. For embedding, the dehydrated samples were placed in benzene (30min), then sequentially in 1:1 benzene: wax (1hr) and then given two changes of 1hr each in pure wax before finally embedding in wax. 5μm thick sections were obtained using a manual hand driven microtome and transferred to the glass slides. These were dewaxed in xylene, rehydrated in descending series of alcohol and stained with haematoxylin and eosin (H and E). Stained sections were mounted in DPX after dehydration and viewed under a light microscope.

ii) Testicular Cell Kinetic Studies: Twenty five random fields were selected from the haematoxylin and eosin stained testis slides. Cells at stage VII were counted in the seminiferous tubules according to the method of Leblond and Clermont, (1952). Sertoli cell, typeA spermatogonia, preleptotene (PL), pachytene (P) and step 19 spermatids were counted on the basis of their shape. Number of germinal cells per 100 sertoli cells was also calculated.

5. RNA EXPRESSION STUDIES:

mRNA expression analysis by RT-PCR was performed using QIAGEN one step RT-PCR kit (Germany). Various antioxidant enzymes: GSH-Px, γGCS, MnSOD, GST, catalase, GR; kinases involved in the regulation of AP-1 (JNK, p38, ERK) and NF-κB (IKK, NIK); caspase 3, caspase 8 and Bcl-2 associated with apoptosis were analyzed. Mouse β-actin was used as an internal control. For these studies, total RNA isolation was carried out from the mice testis.

i) Total RNA Isolation: Total RNA was isolated from mice testis using TRI-REAGENT (Mol Res Centre, Inc, Ohio, USA). TRI-REAGENT combines phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate and most effective inhibition of RNase activity. It helps in the isolation of complete spectrum of RNA molecules. To obtain RNA, following procedure was performed:
**Materials and Methods**

**Homogenization:** 50mg testis from different treatment groups was homogenized in 0.5ml TRI-REAGENT in 1.5ml polystyrene microfuge tubes using hand homogenizer.

**Phase Separation:** The samples were kept at room temperature for 5min to permit the complete dissociation of nucleoprotein complexes. Then 0.1ml chloroform was added and mixed vigorously for about 15 seconds. The homogenates were then kept at room temperature for 10min followed by centrifugation at 12,000g for 15min at 4°C. Following centrifugation three distinct layers, a lower phenol chloroform phase, interphase and an upper colorless aqueous phase were seen. The upper phase that is roughly 60% the volume of TRI-REAGENT contains RNA.

**RNA Precipitation:** The aqueous phase was transferred to fresh tubes and then 250μl isopropanol was added to precipitate the RNA. The samples were kept at room temperature for 10min and then centrifuged at 12,000g for 10 min at 4°C. RNA precipitate was seen as a small white pellet on the side of the tube.

**RNA Wash:** The pellet was washed with 0.5ml 75% ice-cold ethanol by centrifugation at 7500g at 5min at 4°C.

**RNA Solubilisation:** After removing the ethanol, the RNA pellet was briefly air-dried (not completely), and then dissolved the pellet in 10μl of diethyl pyrocarbonate (DEPC) treated water.

**ii) Estimation of Purity and Concentration of RNA**

**Purity:** This was checked by determining the ratio of absorbance values at 260nm and 280nm. The final ratio for all the RNA preparations was approximately 1.6, which indicates a pure preparation.

**Concentration:** Concentration of RNA was estimated by measuring the absorbance at 260nm in spectrophotometer (UV-160A, Shimazdu) using $A_{260}=1\equiv40μg/ml$.

**iii) Agarose Gel Electrophoresis of RNA:** Integrity and size distribution (quality) of isolated total RNA was checked by denaturing agarose-gel electrophoresis according to the method of Lehrach *et al.*, (1977).

**Solutions:**

1. Preparation of 10X MOPS: 10.46g of 3-(N-morpholino) propanesulfonic acid (MOPS) was dissolved in 225ml of diethyl pyrocarbonate (DEPC) treated
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water and to this 4.17ml of 3M sodium acetate was added. The pH was adjusted to 7 with 10M NaOH and then the volume was made up to 250ml. After autoclaving, 5ml of EDTA (pH 8) was added and the solution was stored in a dark bottle.

2. Composition of 10X RNA formaldehyde gel loading buffer:

- 50% glycerol
- 1mM EDTA (pH 8)
- 0.25% bromophenol blue
- 0.28% xylene cyanol

Procedure: 1.2 % agarose gel (40ml) was prepared in DEPC treated water in an Erlenmeyer flask using microwave oven, allowed to cool to 60°C and then 5ml of 10X MOPS and 1.5ml of formaldehyde were added and mixed well. The gel was poured into horizontal gel electrophoresis chamber with comb and allowed to stand at room temperature for 30min to polymerize. Samples were prepared by mixing the following in microfuge tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>4.5μl (5μg)</td>
</tr>
<tr>
<td>10X MOPS</td>
<td>1.0μl</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>3.5μl</td>
</tr>
<tr>
<td>Formamide</td>
<td>8.0μl</td>
</tr>
<tr>
<td>Ethidium bromide (1mg/ml)</td>
<td>1.0μl</td>
</tr>
</tbody>
</table>

The samples were incubated at 65°C for 15min and then chilled on ice. To all the samples, 1.5μl of 10X RNA loading buffer was added and mixed well. 1X MOPS was used as running buffer and electrophoresis was carried out at 70V. Finally, the bands were viewed and photographed using Gel Doc (BioRad, UK).

iv) Primer designing and synthesis: Optimal primer pairs were designed using the software “Gene Runner” or their sequence were obtained from literature and were got synthesized from Sigma-Aldrich (USA). Lengths of the primers chosen were ~20bp. Primer sequences for different genes along with the estimated product size are listed in the table E.
### Materials and Methods

**Table E: Primer pairs used**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Reference</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH-Px</td>
<td>Sense 5'-CCT CAA GTA CGT CGC ACC TG-3'</td>
<td>Chambers et al., 1986, 197bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-CAA TGT CTT GGC ACA ACC CC-3'</td>
<td></td>
</tr>
<tr>
<td>γGCS</td>
<td>Sense 5'-CTT TCT GGC ACA GCA GTG TG-3'</td>
<td>Yan and Meister, 1990, 346bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-TAA GAC GGC ATC TCG CTC CT-3'</td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>Sense 5'-CCA AAG AGA AGC CAA GAC TGC-3'</td>
<td>NM_008181, 228bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-AGG CTG CTG ATT GTG CTC TGG-3'</td>
<td></td>
</tr>
<tr>
<td>MnSOD</td>
<td>Sense 5'-GCA CAT TAA GGC GCA GAT CA-3'</td>
<td>Ho and Crapo, 1987, 241bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-AGC CTC CAG CAA TCC TCC TT-3'</td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>Sense 5'-TGA GCC GCC TGA ACA CCA CT-3'</td>
<td>NM_010344, 316bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-TTG GGGATCC GTG GCA ATG G-3'</td>
<td></td>
</tr>
<tr>
<td>JNK</td>
<td>Sense 5'-TCG ACC ACC CAT ACA TCA AGG-3'</td>
<td>BC_053027, 324bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-ACC CAA GGC CCA AGT CAT C-3'</td>
<td></td>
</tr>
<tr>
<td>p38</td>
<td>Sense 5'-TAT CCA CTC GGC GGG CAT CA-3'</td>
<td>NM_011161, 342bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GTT CCC ACC ACC TCC ATG AT-3'</td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>Sense 5'-TGT TCT CCT TCC TCG TTC C-3'</td>
<td>NM_011949, 258bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-ATG GGT GGC CCG ATG ATG-3'</td>
<td></td>
</tr>
<tr>
<td>IKKα</td>
<td>Sense 5'-ACC ATT TGC ATC CAG AAG TTT ATC-3'</td>
<td>McKenzie et al., 2000, 264bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GAT AAC CAA TGA CAC CAA CCT C-3'</td>
<td></td>
</tr>
<tr>
<td>NIK</td>
<td>Sense 5'-CTG GCC TGT GTA GAC AGC CAG A-3'</td>
<td>Russo et al., 2002, 314bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-ACC CTT TGA GTG CTC TCC AAT-3'</td>
<td></td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Sense 5'-AGT CAG TGG ACT CTG GGA TC-3'</td>
<td>Grimm et al., 2000, 346bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GTA CAG TTC TTT GTG GAG CA-3'</td>
<td></td>
</tr>
<tr>
<td>Caspase 8</td>
<td>Sense 5'-TGC CCT CAA GTT CCT GTG CCT GGA-3'</td>
<td>Zender et al., 2003, 228bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-GGA TGC TAA GAA TGT CAT CTC C-3'</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Sense 5'-AGA GGG GCT AGC AGT GGG AT-3'</td>
<td>Levine et al., 1993, 450bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-CTC AGT CAT CCA CAG GGC GA-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense 5'-ATC CGT AAA GAC CTC TAT GC-3'</td>
<td>Yamada et al., 1996, 287bp</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-AAC GCA GCT CAG TAA CAG TC-3'</td>
<td></td>
</tr>
</tbody>
</table>
v) Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): One step-RT-PCR kit was used (QIAGEN Inc, Germany), in which cDNA synthesis and PCR are carried out sequentially in the same tube (Labware Scientific Inc., USA).

Procedure: RT-PCR was performed according to the manufactures instructions. 3μg RNA was used for each reaction. Firstly, a master mix was prepared as follows:

<table>
<thead>
<tr>
<th>Master mix</th>
<th>Vol/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X QIAGEN RT-PCR buffer</td>
<td>10μl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2μl</td>
</tr>
<tr>
<td>QIAGEN one step RT-PCR enzyme mix</td>
<td>2μl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1μl</td>
</tr>
</tbody>
</table>

The master mix was mixed thoroughly and 15μl of it was added to each PCR tube. To this 5μl of each sense and antisense gene specific primers (from 10μM stock) were added. Then 3μg template RNA was added and final volume was adjusted to 50μl with RNase free water provided in the kit. All the reactions were carried out on ice. The PCR tubes were gently vortexed and centrifuged in order to settle all the components at the bottom. The PCR tubes were placed in a thermal cycler (Techmne Inc., UK), which was programmed as follows:

**Table F: Thermal cycler conditions**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>50°C</td>
<td>50 min</td>
</tr>
<tr>
<td>Initial PCR activation</td>
<td>95°C</td>
<td>15 min</td>
</tr>
<tr>
<td>3 step cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>variable*</td>
<td>45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>No of cycles 35**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min</td>
</tr>
<tr>
<td>Final Hold</td>
<td>10°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

*Annealing temperatures were:

53°C: IKKα

Materials and Methods
53.5°C: Caspase 3, Caspase 8
56°C: GST, γGCS, MnSOD, GSH-Px, catalase, JNK, p38, ERK, β-actin
56.6°C: NIK
58°C: Bcl2

** In initial experiments, RT-PCR was performed by using different number of cycles-20, 25, 30 and 35. It was found that the PCR products were progressively amplified till 35 cycles and hence 35 amplification cycles were performed.

The PCR products were analyzed on agarose gel electrophoresis.

v) **Agarose Gel Electrophoresis for PCR Products:**

**Solutions:**

1. Preparation of 50X TAE buffer (100ml):
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>24.2 g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5.71ml</td>
</tr>
<tr>
<td>0.5 M EDTA (pH 8)</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

2. DNA gel loading buffer:
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol blue</td>
<td>0.25%</td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td>0.25%</td>
</tr>
<tr>
<td>Glycerol in water</td>
<td>30%</td>
</tr>
</tbody>
</table>

**Procedure:** 30ml of 1.5% agarose was prepared in 1X TAE buffer in an Erlenmeyer flask in a microwave oven. After cooling to approximately 60°C, ethidium bromide was added to a final concentration of 0.5μg/ml. The gel was poured in to horizontal plastic tray with comb and left undisturbed for 30min to allow it to polymerize.

3μl of the PCR product was mixed with 1μl DNA gel loading buffer and the samples were loaded in separate wells. 1X TAE was used as the running buffer. Electrophoresis was carried out at 70V. The bands were visualized and photographed on Gel Doc (BioRad, UK).

vi) **Densitometric analysis of bands:** Densitometric analysis of bands was done by using the Image J software (NIH).
6. PROTEIN EXPRESSION STUDIES:

A) Western Immunoblot Analysis:

Solutions:

1. PBS (0.01M, pH 7.4)
2. Lysis Buffer
   - Tris (pH 8.0) 10mM
   - EDTA 150mM
   - Triton X-100 1.0%
   - PMSF 1mM
   - DTT 1mM

Protease inhibitor: A stock solution of 100mM phenyl methyl sulphonyl fluoride (PMSF) in isopropanol was prepared. A final working concentration of 1mM was added to lysis buffer just before use.

Procedure: Cytoplasmic extract were prepared according to the method of Kim et al., (2004) with some modifications. Briefly, 100mg testis was gently homogenized in 0.5ml of ice cold PBS. After centrifugation at 5000g (2min) at 4°C, the supernatant was removed and the pellet was lysed in 500µl lysis buffer and kept on ice for 30 min, followed by centrifugation at 10,000g for 10min at 4°C. The supernatant was collected as cytoplasmic extract. Protein concentration was determined by the method of Lowry et al., (1951).

ii) Polyacrylamide Gel Electrophoresis (SDS-PAGE): The protein samples were separated on 12% SDS-PAGE by the method of Laemmli, (1970) using minigel apparatus (BioRad, UK).

Solutions:

1. Buffer A 1.5 M Tris HCl (pH 8.9)
2. Buffer B 0.5 M Tris HCl (pH 6.7)
3. Stock acrylamide solution (for 100ml)
   - Acrylamide 30g
   - Bis-acrylamide 0.8g

Final solution was made to 100ml with double distilled water. The solution was filtered through whatmann filter paper and stored in a dark colored bottle.

4. 10% Ammonium persulphate solution (APS; prepared fresh)
5. 10 % Sodium dodecyl sulphate (SDS)

6. Plug gel
   - Acrylamide stock 500μl
   - APS (10%) 20μl
   - TEMED 2μl

7. Separating gel (10%)
   - Acrylamide stock 1.7ml
   - Double distilled water 1.9ml
   - Buffer A 1.3ml
   - APS (10%) 50μl
   - SDS (10%) 50μl
   - TEMED 2μl

8. Stack gel
   - Acrylamide stock 330μl
   - Double distilled water 1.4ml
   - Buffer B 250μl
   - APS (10%) 20μl
   - SDS (10%) 20μl
   - TEMED 2μl

9. Sample buffer (2X)
   - 40% SDS 100μl
   - β-mercaptoethanol 40μl
   - Buffer B 100μl
   - Glycerol 100μl
   - Bromophenol blue (33mg%) 160μl

10. Running buffer
    - Tris base 3g
    - Glycine 14.4g
    - SDS 1g

    Final volume was made to 1000ml with distilled water.
11. Coomassie blue stain
   Methanol  250ml
   Glacial acetic acid  50ml
   Coomassie blue (R250)  250 mg
   Final volume was made to 500ml with distilled water.

12. Destaining solution
   Methanol  62.5ml
   Glacial acetic acid  17.5ml
   Final volume was made to 250ml with distilled water.

Procedure: In order to seal the bottom of the chamber formed between the plates by using 1mm spacers; the plug gel was poured first. Then the separating gel was poured and it was immediately overlaid with water saturated with butanol. It was left undisturbed to polymerize for 30min. After this, the top of the separating gel was rinsed with distilled water and dried using a whatmann sheet. Then the stack gel was poured with the comb properly placed in it. This was allowed to stand for another 30min at room temperature to polymerize.

Cytoplasmic extract (60µg protein) was mixed in equal ratio with 2X sample buffer and heated in a boiling water bath for 1min. Samples were loaded in the respective wells and electrophoresis was carried out at a constant current of 5mA through stack gel and 10mA through separating gel. One of the gels was stained in Coomassie blue (R250) for 3-4hr with constant shaking. The gel was then destained in destaining solution with gentle agitation. It was then dried in a gel wrap.

iii) Western Immunoblot:

Solutions:
1. Transfer buffer
   Tris buffer  4.5g
   Glycine  21.6g
   SDS  3g
   Methanol  150ml
   Final volume was made to 1200 ml with distilled water.

2. Phosphate buffer saline, PBS (0.01M, pH 7.4)
   Na$_2$HPO$_4$  1.452g
   NaH$_2$PO$_4$  0.132g
   NaCl  9g
   Final volume was made to 1L.
**Materials and Methods**

**Procedure:** First placed the gel, whatmann sheets (cut approx equal to the size of the gel) and two foam pads in transfer buffer. The PVDF membrane was first soaked in methanol briefly and then kept in transfer buffer. The sandwich of gel and membrane was prepared and filter sheets and foam pads were kept on each side. The gel was kept at the cathode side and membrane at the anode side of the unit. The sandwich was properly tightened with rubber bands and placed in the transfer chamber, filled with chilled transfer buffer. Transfer was done at 200mA for 2.5hr.

After transfer, the membrane was washed with PBS for 10min and then placed in blocking solution (5% non-fat milk in PBS) for 1hr with constant shaking. Then the membrane was incubated with polyclonal primary antibodies (JNK, 1:8000), (p38, 1:5000), (ERK, 1:500) and mouse β-actin (1:4000). Dilutions were made with blocking solution and incubation time was 2hr at room temperature with constant shaking. The membrane was sequentially washed with PBS, 0.05% Tween-20 in PBS and PBS, each for 5min. Then the membrane was incubated with peroxidase labeled secondary antibody (1:500) anti-rabbit IgG (Sigma-Aldrich, USA) for 2hr. Thereafter, the membrane was washed again as described above. The blot was developed by adding diaminobenzidine (DAB; 8mg/10ml PBS containing 12μl H₂O₂). Reaction was terminated by rinsing the membrane with double distilled water and the membrane was air dried.

iv) **Densitometric analysis of bands:** Densitometric analysis of bands was done by using the Image J software (NIH).

**B) Immunohistochemical localization studies:**

5μm thick paraffin sections of mice testis were incubated at 60°C for 30min and deparaffinized in xylene for 2hr. The sections were then gradually hydrated in descending series of alcohol (100%, 90%, 70%, 50% and 30%). Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 30min at 37°C. The non specific staining was blocked by incubating sections with 2% BSA in phosphate buffer saline (PBS 10mM, pH 7.2). The sections were then incubated with various antibodies seperately (anti-JNK, 1:8000), (anti-p38, 1:5000), (anti-ERK, 1:500), (anti-NIK, 1:2000), and (anti-IKKα, 1:500) in a moist chamber for 2hr at 37°C. For negative control, only diluent was added (PBS with 1% BSA). After incubation with the respective primary antibodies, washings were given with PBS, PBS tween (PBS with 0.05% tween-20) and PBS successively for 5min each. The sections were then
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incubated with peroxidase labeled anti-rabbit IgG (1:500) for 2hr. Sections were washed again in the same manner as described above and the reaction product was developed using diaminobenzidine (DAB) and $\text{H}_2\text{O}_2$. Reaction was terminated by washing with distilled water and sections were mounted in glycerol jelly.

C) Enzyme Linked Immunosorbent Assay (ELISA):

**Solutions:**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCL</td>
<td>50mM, pH 7.4</td>
</tr>
<tr>
<td>PBS</td>
<td>0.1M, pH 7.2</td>
</tr>
<tr>
<td>Carbonate Buffer</td>
<td>0.06M</td>
</tr>
<tr>
<td>Citrate Buffer</td>
<td>0.1M, pH 5.0</td>
</tr>
</tbody>
</table>

**Substrate:**

2,2-azino-di-(3-ethylbenzothiozolinsulphonic acid) ABTS 0.5mg/ml of Citrate Buffer Hydrogen Peroxide 5μl/10ml of Citrate Buffer

**Procedure:** ELISA was carried out to quantitate the levels of NIK and Iκκa in various treatment groups under study. The assay was standardized by titrating the different concentration of antigens and antibodies. 10% (w/v) tissue homogenates were prepared in 50mM Tris-HCl (pH 7.4) under ice-cold conditions. The homogenates were then centrifuged at 10,000rpm for 30 minutes. The supernatant (post mitochondrial fraction, PMF) thus obtained was quantitated for protein by Lowry method and collected for ELISA. 2.5μg protein was loaded onto ELISA strip wells in 100μl carbonate buffer and kept overnight at 4°C, in a moist chamber. Flicked the plate to remove the unbound antigen and the wells were blocked with 1% BSA in 0.1M PBS for 1hr at 37°C. The plate was then flicked and washed thrice with PBS containing 0.05% (v/v) Tween-20. Wells were then incubated with primary antibody diluted in PBS containing 0.05% Tween-20 and 1% BSA [ NIK (1:2000), Iκκa (1:500)] and kept for 2hr at 37°C. Plate was again washed thrice in the same manner and incubated with peroxidase labeled anti-rabbit IgG diluted in PBS containing 0.05% Tween-20 and 1% BSA (1:500) and kept for 2hr at 37°C. The plate was again washed thrice as above and one last washing was given with distilled water since Tween acts as an inhibitor of the substrate; ABTS [2,2-azino-di-(3-ethylbenzothiozolinsulphonic acid)]. The substrate was then added to each well and the plate was kept in dark for 30min after which the color developed was read at 405nm.
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7. APOPTOTIC STUDIES

A) DNA Fragmentation Analysis:

i) DNA Isolation: Freshly excised tissue was grounded to powder form under liquid nitrogen using pestle and mortar. The powdered tissue was added to approximately 10 volumes of extraction buffer, which consisted of

- Tris-Cl 10mM (pH 8.0)
- EDTA 0.1M (pH 8.0)
- RNase 20µg/ml
- SDS 1%

The solution was incubated for 1 hr at 37°C. Proteinase K was added to a final concentration of 100µg/ml. The enzyme was gently mixed with the viscous lysate. The suspension of lysed cells was placed in a waterbath for 3 hr at 50°C. Viscous solution was swirled periodically. The solution was allowed to cool to room temperature and an equal volume of phenol: chloroform: 1:1 was added. The two phases were mixed slowly by inversion for 10 min. Two phases were separated by centrifugation at 10,000g for 15 min at room temperature. Viscous aqueous phase was transferred to a clean centrifuge tube and repeated the extraction twice with phenol: chloroform. To the aqueous phase added equal volume of 0.06M sodium acetate. To this were added two volumes of ice-cold isopropanol and kept at -70°C for 1 hr. The above solution was centrifuged at 10,000g for 20 min to isolate DNA. The pellet obtained was washed with 70% ethanol and dissolved in 20 µl of TE buffer (pH 8.0).

ii) Estimation of Purity and Concentration of DNA:

Purity: This was checked by determining the ratio of absorbance values at 260nm and 280nm. The final ratio for all the DNA preparations was greater than 1.75, which indicates a pure preparation.

Concentration: Concentration of DNA was estimated by measuring the absorbance at 260nm in spectrophotometer (UV-160A, Shimazdu) using A_{260}=1=50µg/ml.

iii) Agarose Gel Electrophoresis:

30 ml of 1.8% agarose was prepared in 1X TAE buffer in an Erlenmeyer flask in a microwave oven. The gel was cooled to 60°C and ethidium bromide was added to a final concentration of 0.5µg/ml and mixed thoroughly. The gel was poured into the horizontal gel electrophoresis chamber with comb and was allowed to stand at room temperature for 30 min.
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5μg of the DNA was mixed with 1μl DNA gel loading buffer and the samples were loaded in separate wells. 1X TAE was used as the running buffer. Electrophoresis was carried out at 70V. The bands were visualized using ultraviolet transilluminator (BioRad systems, USA) and photographed on Gel Doc (BioRad, UK).

B) Involvement of Apoptosis using TUNEL (Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling):

Study of the apoptotic cell death was performed by the In Situ Cell Death Detection Kit (Roche Diagnostics, Germany).

Solutions:

1. Buffer A
   - Tris chloride (pH 7.5) 50mM
   - MgCl₂ 10mM
   - BSA 1mg/ml

2. Proteinase K (nuclease free) solution
   - Stock solution: 2mg/ml in 10mM Tris chloride (pH 7.4-7.8)
   - Working solution: 20μg nuclease free proteinase K in 10mM Tris chloride buffer (pH 7.4-7.8)

3. TUNEL reaction mixture
   - Label solution 450μl
   - Enzyme solution 50μl
   (Prepared immediately before use)

4. 0.1M PBS (pH 7.5) for washing

5. DNase I: 1500 U/ml in buffer A

Procedure: The selected sections were dewaxed in xylene and rehydrated through graded series of alcohol. The tissue sections were incubated with proteinase K for 20min at 25°C. The sections were rinsed in PBS twice. TUNEL reaction mixture was prepared by mixing 450μl of label solution and 50μl of enzyme solution provided in the kit. 50μl of this TUNEL reaction mixture was added to each sample. To the negative control, only 50μl of label solution was added. For the positive control, DNase I (50μl) was added for 10min at 25°C, and then TUNEL reaction mixture was added. The samples were incubated for one hour at 37°C in a humid chamber in dark. The slides were then thoroughly rinsed in PBS. 50μl of
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Convertor peroxidase (POD) was added and the slides were further incubated for 30 min at 37°C. Next, the slides were rinsed thrice with PBS and then 100 µl of DAB substrate was added (1 mg/ml in PBS). The reaction was terminated with double distilled water and the slides were mounted in glycerol jelly and viewed under a microscope.

9. In Vitro STUDIES:

To investigate the effect of altered redox status on the mRNA expression of JNK, p38, ERK, Bcl2, Caspase 3 and Caspase 8 and the role played by selenium in regulating this process, in vitro experiments were carried out. The cellular glutathione levels were depleted by using buthionine sulfoximine (BSO), which is a well known inhibitor of the enzyme γ-glutamylcysteine synthetase (γGCS).

Testicular cell maintenance:

Solutions:

1. Composition of Media

   Dulbecco’s Modified Eagle’s Medium
   DMEM (with L-glutamine and 15 mM HEPES) 9.6 g/l
   Sodium bicarbonate 2.0 g/l
   Fetal Calf Serum (FCS) 5%
   Gentamycin 10 mg/l
   Fungizone 1.25 mg/l
   (Prepared in autoclaved water)

2. 0.1 M Phosphate buffer saline, PBS (pH 7.4) in autoclaved water.
3. Sodium selenite stock solution (4 mM) in water
4. 100 mM BSO stock solution in media

   Dilutions for different selenium concentrations and BSO were prepared in media

Procedure: Male Balb/c mice were sacrificed by cervical dislocation under ether anesthesia and testicular cells were isolated by the method of Steinberger, (1975) with minor modifications. The testis were removed and decapsulated. Then, the seminiferous tubules were teased gently by pressing between two frosted slides to get a cell suspension in PBS. The cell suspension was gently aspirated using a pasteur pipette to disaggregate cell clumps. The suspension so obtained was then filtered
through a stainless steel mesh to remove cell aggregates and tissue debris and the filtrate was centrifuged at 1000g for 10min at 4°C to obtain a cell pellet. The cells were then suspended in media supplemented with 5% FCS. Cells were counted and plated at a concentration of 0.4×10^6 cells/well. Selenium was added as sodium selenite at a final concentration of 1.5μM or 0.5μM either alone or together with buthionine sulfoximine, BSO (10nM). All stock solutions and dilutions of sodium selenite, BSO were prepared in media only. The cells were maintained at 32°C in a humidified atmosphere of 5% CO_2 for 4hr. After completion of incubation, the cells were collected from the wells and centrifuged at 10,000g at 4°C to obtain pellet.

Following parameters were analyzed:

i) **Assessment of cell viability by MTT assay:**

Cytotoxic effects of different concentrations of selenium and BSO on testicular cells were determined as a function of cellular mitochondrial activity by MTT assay (Alley *et al.*, 1988). This is based on the principle that MTT produces a purple formazon product when incubated with live cells. In principle, the number per well is directly proportional to the production of the formazon, which following solubilization with DMSO can be measured spectrophotometrically.

**Procedure:** To 100μl of the supernatant added 10μl of MTT solution (5mg/ml in 0.1M PBS; pH 7.4) and incubated in a humidified atmosphere of 5% CO_2 at 32°C for 3hr. After discarding the supernatant, the formazon crystals so obtained were dissolved in 200μl of DMSO. The absorbance was read at a wavelength of 580nm using ELISA reader (Stat fax 325). Blanks contained DMSO only.

ii) **Estimation of glutathione levels:**

Cells were harvested in ice-cold PBS (0.1M, pH 7.4) and centrifuged at 10,000g for 10 min. The pellet so obtained was lysed in 0.1M phosphate EDTA buffer (pH 8) containing 0.1% Triton X100 (approx. for 5×10^5 cells, 100μl lysis buffer was used). Protein was precipitated with 5% TCA. The lysates were mixed well and then protein was pelleted by centrifugation at 10,000g for 10min at 4°C. Total and oxidized glutathione were estimated separately in the supernatant, in a similar manner as described earlier.

iii) **ROS determination:**

ROS accumulation in the testicular cells was detected with a carboxy-H_2-DCFDA (Molecular Probes) staining method (Wang *et al.*, 2001). This assay is based
on the principle that the nonpolar, nonionic dichlorofluorescein diacetate dehydrate (H$_2$-DCFDA) crosses cell membranes and is enzymatically hydrolyzed into nonfluorescent dichlorofluorescein dehydrate (H$_2$-DCF) by intracellular esterases. In the presence of ROS, H$_2$-DCF is rapidly oxidized to become highly fluorescent dichlorofluorescein (DCF). At the end of the incubation, cells were incubated at 37°C for 1 hour with 5 μM carboxy- H$_2$-DCFDA dissolved in the medium. The resulting fluorescence intensity as a measure of ROS generation was recorded on a spectofluotometer using excitation and emission filters of 488nm and 530nm respectively.

iv) RNA Isolation and RT-PCR analysis:

RNA Isolation: Total RNA was isolated from mice testicular cells using TRI-REAGENT. Cells from different treatment groups were harvested in media and centrifuged at 10,000g for 10min to obtain a pellet. TRI-REAGENT was added (at a concentration of 1ml per 5×10$^6$ cells) and cells were homogenized in 0.4ml TRI-REAGENT in 1.5ml polystyrene microfuge tubes using hand homogenizer. The samples were kept at room temperature (20-25°C) for 5min, and then 80μl chloroform was added and mixed vigorously for about 15sec. The homogenates were then kept at room temperature for 10min followed by centrifugation at 12,000g for 15min at 4°C. Following centrifugation, three distinct layers were formed. The upper colorless aqueous phase, which contains RNA, was collected. To this was added 200μl isopropanol in each tube in order to precipitate RNA. The samples were mixed well and kept at room temperature for 10min. Then samples were centrifuged at 12,000g for 10min at 4°C. RNA precipitate was seen as a small white pellet on the side of the tube that was washed with 0.5ml 75% ice-cold ethanol by centrifugation at 7500g at 5min at 4°C. After removing the ethanol, the RNA pellet was briefly air-dried (not completely) and then dissolved the pellet in 10μl of DEPC treated water. The purity, concentration and integrity of RNA were checked as described previously.

RT-PCR was carried out using gene specific primers for JNK, p38, ERK, Bcl2, Caspase 3 and Caspase 8 in similar way as described previously.

v) DNA isolation: DNA was isolated using the standard phenol/chloroform extraction method. Briefly, control and treated cells were pelleted by centrifugation and were then suspended in lysis buffer. The suspension was incubated at 37° C for 1 hr. Proteinase K was then added at a concentration of 200μg/ml and the suspension
was incubated for another 3 hrs at 50° C. Equal volume of phenol was then added and the suspension was centrifuged at 10,000 rpm for 30 min. The aqueous layer obtained was collected and 0.2 volumes of ammonium acetate and 2 volumes of 70% ethanol were added to it. This was centrifuged at 5000 rpm for 5 min. The precipitate so obtained was washed with 70% ethanol and was finally dissolved in TE buffer. The DNA obtained was quantitated by noting the ratio of absorbances obtained at 260nm and 280 nm. To 10μg of DNA sample from each treatment, gel loading dye was added and horizontal electrophoresis was carried out in 1X TAE buffer on 1.8% agarose gels.

vi) Acridine orange /Ethidium bromide staining for Apoptosis detection:

For Apoptosis detection, cells were subjected to Acridine Orange/ethidium bromide staining according to the method of Heftsi et al., 2004.

After completion of incubation, the cells were centrifuged at 1500g for 5 min at room temperature and then resuspended in a 25μl of medium, to which 2μl of a solution containing 100μg/ml Acridine Orange and 100μg/ml ethidium bromide in PBS was added. The cells were then visualized by fluorescence microscopy (Olympus BX60, equipped with a CCD camera), using a blue filter.

11. STATISTICAL ANALYSIS OF DATA:

The difference between Means ± Standard Deviations (SD) for control and treated groups were examined by using the Student’s t-test for unpaired values. Statistical difference of p-value at the level of 0.05 or less were considered significant.