MATERIALS AND METHODS

Male Balb/c mice of the weight range 20-25g were obtained from the Central Animal House of Panjab University, Chandigarh and were acclimatized for 1-2 weeks in the departmental animal house before using for experimentation. The animals were kept in polypropylene cages and water was available *ad libitum*. To create different selenium (Se) status mice, animals were divided into three groups:

Group I: Se deficient (0.02 ppm)
Group II: Se adequate (0.2 ppm)
Group III: Se excess (1 ppm)

To make different selenium status animals in different groups as stated above, the mice were kept on yeast based diet. The yeast based diet usually contains approximately 0.02 ppm selenium and hence animals fed on this diet for a minimum of 8 weeks were considered selenium deficient animals.

Animals in the group II (adequate Se) and group III (excess Se) were fed with selenium deficient diet supplemented with sodium selenite at 0.2 ppm (adequate level) and 1 ppm (excess level) selenium levels.

ANIMAL DIET PREPARATION

Selenium deficient diet with inactivated Baker's yeast, as a protein source was prepared by the method of Burk, (1987)

<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>Baker's Yeast</td>
<td>30</td>
</tr>
<tr>
<td>Corn oil</td>
<td>6.67</td>
</tr>
</tbody>
</table>

ANIMAL DIET PREPARATION

Selenium deficient diet with inactivated Baker's yeast, as a protein source was prepared by the method of Burk, (1987)
**Materials and Methods**

Mineral mixture (USP XIV) 5
Vitamin mixture 1
DL-methionine 0.3
Vitamin E 0.04

**Vitamin mixture**

The vitamins were weighed individually as given below:

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>10g</td>
</tr>
<tr>
<td>Inositol</td>
<td>1.1g</td>
</tr>
<tr>
<td>Para aminobenzoic acid</td>
<td>1.1g</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>16.5g</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>10g</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.22g</td>
</tr>
<tr>
<td>Pyridoxine 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>4.4mg</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>0.3mg</td>
</tr>
</tbody>
</table>

The above mixture was finally made to 100g with sucrose and used at 1\% level as stated above.

Oil soluble vitamins were dissolved directly in the oil to be added in the respective diets as per following scheme : Vitamin A, 20,000 IU; Vitamin K, 5mg; Vitamin E, 100mg and Vitamin D, 1000 U for 1 kg diet.
Materials and Methods

The basic diet prepared as above was supplemented with selenium as sodium selenite at the level of 0.2 ppm and 1 ppm selenium for group II and group III animals respectively.

Animals in the three groups were provided the respective diets for eight weeks.

The body weight of the animals was recorded every week. After the completion of diet feeding schedule in different groups, the mice were sacrificed under ether anesthesia followed by cervical dislocation. Tissues were removed immediately and different estimations were carried out as detailed below.

SELENIUM LEVELS

Selenium levels in testis, liver and serum were quantitated by the method of Hasunuma et al., (1982) based on the principle that selenium content in tissue on acid digestion is converted to selenous acid. The reaction between selenous acid and aromatic-O-diamines such as 2,3-diamino naphthalene (DAN), leads to the formation of 4,5 benzopia-zselenol which displays a brilliant lime green fluorescence when excited at 366nm in cyclohexane.

![4,5 benzopia-zselenol](image)

Reagents

1. 6M hydrochloric acid (HCl)
2. 0.1M perchloric acid (HClO₄)
3. 7M ammonia solution
Materials and Methods

4. 50% formic acid

5. Stabilizing solution (0.025M EDTA in 25% hydroxyl ammonium chloride)

6. 2,3-diaminonaphthalene (DAN) reagent (made fresh)

Preparation of DAN

Fifty mg DAN was dissolved fresh in 50 ml of 0.5% hydroxyl ammonium chloride in 0.1M HCl in brown bottle and kept for 1 hr. DAN solution was washed with equal volume of cyclohexane by vigorous shaking in separating funnel with stopper and kept for 2–3 min till two layers separated. Lower aqueous layer of DAN was collected. This washing procedure was repeated two more times and the final DAN solution was stored in dark bottle.

Digestion of Samples

The tissues (liver and testis) were weighed (100 mg) and transferred to digestion flask fitted with special condenser to prevent any loss of selenium as volatile selenides. For selenium estimation in serum, 100 μl of serum sample was taken.

Five ml of concentrated HNO₃ was added to the sample. The sample was then heated on the sand bath at approximately 100°C with condensers on. Heating was continued till the brown fumes ceased. Temperature of the sand bath was then lowered and 1.0 ml of HClO₄ was added to the digestion flask without removing the condensers. The flasks were then removed from sand bath after heating for 30 min at the lower temperature and allowed to cool. The condensers were removed and the volume of digest was measured for each sample.
Materials and Methods

Estimation

To one ml of digested sample in duplicate, added 1ml of 6.0M HCl, 1.0ml of 50% formic acid, 2.0ml of the stabilizing solution, 2.5ml of ammonia solution and 5.0ml of DAN solution.

After incubation at 50°C for 20min in water bath, the tubes were cooled at room temperature. 3.0ml of cyclohexane was added to each tube and mixed on vortex mixer for one minute each. The tubes were allowed to stand to separate the cyclohexane layer. The upper layer (cyclohexane) was removed and collected in a test tube. Again added 3ml of cyclohexane and mixed well for 30 sec. Cyclohexane layer was removed similarly and added to the first extract.

Fluorescence emission in the extracted cyclohexane was read on fluorescence spectrophotometer using 366nm as excitation wavelength and 520nm as the emission wavelength.

1.0ml of working standard solution (Annexure I) and 1.0ml of distilled water was used as sample in standard and blank respectively.

Calculations:

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

Tissue and serum selenium levels were expressed as μg/g and μg/l respectively.

BIOCHEMICAL ESTIMATIONS

Tissue homogenates (10% w/v) were prepared in cold 20mM Tris-HCl (pH 7.4) buffer using mechanically driven teflon fitted potter elvejham type homogenizer for one minute under ice cold conditions. Homogenates were centrifuged at 10,000g for 30 min at
4°C to get the post mitochondrial fractions (PMF). Biochemical estimations were carried out in the PMF as detailed below:

**Glutathione Peroxidase**

Activity of glutathione peroxidase (GSH-Px) was assayed in liver and testis by the coupled enzyme procedure with glutathione reductase using hydrogen peroxide as substrate (Paglia and Valentine, 1967)

Reaction mixture consisted of:
- 0.08M NADPH (7mg/ml) = 37.5μl
- Glutathione Reductase (10U/ml) = 37.5μl
- 0.15M glutathione (46mg/ml) = 37.5μl
- 0.125M sodium azide (73mg/ml) = 3.75μl
- 0.05M potassium phosphate buffer (pH 7.0) = 820 μl
- 0.002M H2O2 = 37.5μl

The reaction was started by the addition of 20μl of post mitochondrial fraction diluted with 0.05M potassium phosphate buffer (pH 7.0). Oxidation of NADPH was recorded at 340nm in spectrophotometer (UV-VIS Shimadzu UV 160A). Enzyme activity was expressed as μmoles of NADPH oxidized/min/mg protein.

**Lipid Peroxidation**

Levels of malondialdehyde (MDA), index for determining the extent of lipid peroxidation from the breakdown of polyunsaturated fatty acids, were determined in PMF of testis by following the protocol of Ohkawa *et al.*, (1979). The procedure was as follows:

In stoppered test tubes, 0.2ml of post mitochondrial fraction (PMF) from 10% testis homogenate was taken and 0.2ml of 8.1% SDS, 1.5ml of 20% acetic acid solution (pH 3.5), 1.5ml of aqueous thiobarbituric acid (TBA; 0.8%) were added. The mixture was finally
Materials and Methods

made to 4ml with distilled water and was heated in a water bath at 95°C for 60min. Further, tubes were cooled immediately under tap water and 1.0ml of distilled water was added. Then 5ml of extraction solution (15:1, v/v mixture of n-butanol and pyridine) was added to each tube and vortexed vigorously for 2min. After centrifugation at 4000 rpm for 10min, the absorbance in the organic layer (upper layer) was measured at 532nm on a spectrophotometer (UV-VIS Shimadzu UV-160A).

For standard, 2-10nmol range of 1,1',3,3'-tetraethoxypropane (TEP) in 200μl volume was used and for control distilled water was used in place of the sample. MDA levels were expressed as nmol MDA/mg protein.

Total and Oxidized Glutathione

Total and oxidized glutathione were quantitated by fluorimetric method of Hissin and Hilf (1976).

Glutathione reacts specifically with O-Phthaldehyde (OPT) at pH 8.0, resulting in the formation of a highly fluorescent product that is activated at 350nm with an emission peak at 420nm. The fluorescence intensity for the OPT-GSH reaction is directly related to GSH concentration.

Proteins were precipitated with trichloroacetic acid (TCA) at a final concentration of 5% and the supernatant was obtained by centrifugation at 10,000xg for 10min at 4°C.

Total Glutathione

The reaction procedure to quantitate total glutathione (GSH+GSSG) was as follows:


**Materials and Methods**

Supernatant (after TCA precipitation) = 100μl
0.1M phosphate-5mM EDTA buffer (pH 8.0) = 9.4 ml
O-Phthaldehyde (100μg/100μl methanol) = 500μl

Incubated at room temperature for 15min and then read at 420nm using 350nm excitation wavelength in a fluorescence spectrophotometer.

Reduced glutathione was used to make the standard curve (5-40μg). The results were expressed as nmoles of GSH per mg protein.

**Oxidized glutathione**

For the estimation of oxidized glutathione (GSSG), supernatant was mixed with N-ethylmaleimide and incubated at room temperature for 30min to inhibit reduced glutathione (GSH) at an alkaline pH(12.0). The fluorescence intensity produced by the OPT-GSSG reaction at pH 12.0 was directly related to GSSG concentration.

The supernatant (0.5ml) was incubated with 0.04M N-ethylmaleimide (0.2ml) for 30min at room temperature. After the incubation, the reaction procedure consisted of the following steps:

Sodium hydroxide (0.1N) = 9ml
Fraction from the above reaction = 500μl
O-Phthaldehyde (100μg/100μl methanol) = 500μl

Incubated for 15min at room temperature and read at 420 nm using 350nm as excitation wavelength. Oxidized glutathione in the concentration of 5-40μg was used as standard and results were expressed as nmoles of GSSG per mg protein.
Materials and Methods

Glutathione-S-Transferase

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

Reaction procedure consisted of:
0.1M potassium phosphate buffer (pH 6.5) = 1.0ml
0.001M reduced glutathione (GSH) = 0.035ml
0.001M 1-chloro-2,4-dinitrobenzene (in methanol) = 0.035ml

The reaction was started with the addition of 50μl of sample and formation of conjugated complex of CDNB was monitored at 340nm. The activity was expressed as nmol CDNB conjugated/min/mg protein using the molar extinction coefficient of CDNB (9.6mM⁻¹cm⁻¹).

Glutathione Reductase

Glutathione reductase was estimated by the method prescribed by Massey and Williams (1965).

The enzyme activity of glutathione reductase was monitored by following the oxidation of co-factor NADPH by the oxidized glutathione (GSSG), at 340nm. The utilization of the NADPH was directly related to the activity of glutathione reductase.

Reaction mixture consisted of:
3mM EDTA (100.8mg/10ml) = 0.1ml
2% BSA (200mg/10ml) = 0.1ml
0.1mM NADPH (7.4mg/10ml) = 0.1ml
3mM oxidized glutathione (184mg/10ml) = 0.1ml
0.1M potassium phosphate buffer (pH 7.5) = 0.6ml
The reaction was started by the addition of 50μl of the postmitochondrial fraction. Enzyme activity was expressed as μmoles of NADPH oxidized/min/mg protein.

**Superoxide Dismutase**

Activity of superoxide dismutase (SOD) was estimated by the method described by Kono (1978). The reaction is designed to observe the inhibition of the rate of oxidation of nitroblue tetrazolium (NBT) using hydroxylamine hydrochloride as the electron donor.

Reaction assay consisted of:

Solution A: (3.8 mg EDTA and 0.53g Na₂CO₃ in 100ml H₂O, pH 10.0) = 1.3ml
Nitroblue tetrazolium (8mg/100ml of Sol. A) = 0.5ml
0.6% Triton X-100 in solution A = 0.1ml
2mM hydroxylamine hydrochloride (pH 6) (1.4mg/10ml) = 0.1ml

After the addition of hydroxylamine hydrochloride, the change in absorbance \( A(x) \) is measured at 560nm. To this reaction mixture, enzyme source was added and again change in absorbance \( A(y) \) was recorded at 560nm. Difference in the rate is compared and expressed as percent inhibition.

\[
\text{%inhibition} = \frac{A(x) - A(y)}{A(x)} \times 100
\]

One unit of enzyme is defined as the amount of enzyme required to result in 50% inhibition of \( A(x) \). The enzyme was expressed as units/mg protein.

**Catalase**

Catalase was estimated directly as per method described by Luck (1963). Hydrogen peroxide was used as the substrate.
Materials and Methods

The reaction mixture consisted of:

- 50mM phosphate buffer (pH 7) = 2.5ml
- 0.75M Hydrogen peroxide = 50μl
- Enzyme source = 150μl

Rate of change of absorbance was noted at 240nm and the enzyme activity was calculated from the rate of decrease in the optical density (ΔA) and molar extinction coefficient of H₂O₂ (0.0394mM⁻¹cm⁻¹). Final results were expressed as μmoles of H₂O₂ decomposed/min/mg protein.

Nitric Oxide Synthase

Nitric oxide synthase activity was determined in terms of nitrite and citrulline, measured according to the standard protocols. NOS activity was determined in the post mitochondrial fraction (PMF).

Nitrite levels

Estimation was carried out by the method of Raddassi et al., (1994). Griess reagent (1:1 v/v) mixture of 0.1% N-(1-naphthyl)ethylene-diamine dihydrochloride in H₂O-1% sulfanilamide in 2.5% orthophosphoric acid) was mixed with equal volume of PMF and incubated for 30min in dark. Absorbance was measured at 545nm. The nitrite amount was calculated from sodium nitrite (NaNO₂) standard curve and expressed as nmol/mg protein.

Citrulline levels

The colorimetric estimation of citrulline is based on its reaction with diacetyl monoxime, which was performed by the method of Boyde and Rahmatullah (1980). Post mitochondrial fraction (PMF) was incubated with urease (21mM/ml) for 1hr at 37°C. Proteins were then removed by addition of TCA(7.5%) and centrifugation. 200μl of supernatant was diluted to 500μl with 0.1N HCl and 3ml of freshly prepared chromogenic solution (Annexure II) was added and
Materials and Methods

vortexed. Mixture was boiled in waterbath for 5min at 100°C in stoppered tube and absorbance was measured at 530nm.

For standard, 5-30μmole range of citrulline was used and citrulline levels were expressed as nmol/mg protein.

HORMONE ANALYSIS
LH, FSH and testosterone were estimated in the serum of mice from the three groups after 8 weeks of diet feeding by enzyme linked immunosorbent assay (ELISA).

SPERM MOTILITY STUDIES
Vas deferens were removed from each mouse. Spermatozoa were squeezed on to a glass slide in 0.9% NaCl at room temperature. The motility of spermatozoa was evaluated under light microscope. One drop of sperm suspension was placed on a glass slide, covered with a coverslip and 10 random fields of view were examined. Number of motile and nonmotile sperms were counted. Motility was then expressed as the percentage of motile sperms.

SPERM COUNT
Sperms from each epididymis were teased out in 1ml of normal saline. A drop of the sperm suspension was placed on the haemocytometer under a coverslip. The sperm number in the 64 small wells of the chamber were then counted and from the knowledge of the area and depth of the chamber, sperm counts per mm³ were calculated.

FERTILITY STUDIES
At the end of the treatment period in all the three groups, male mice were allowed to mate with normal females in the ratio of 1:2
Materials and Methods

respectively. After a week, the males were separated. Percent fertility was checked as:

\[
\%\text{Fertility} = \frac{\text{No. of females giving birth}}{\text{No. of females exposed to mating}} \times 100
\]

Female mice were observed for 21 days for giving birth to pups. In each female giving birth to pups in all the groups, the litter size was determined and averaged in each group.

HISTOLOGICAL STUDIES

The testes, immediately after removal from the animals, were washed with normal saline and were immediately transferred to zenker fixative. The testis was cut cross-sectionally after 3-4hr. The tissue was then removed from fixative after 24hr and washed in several changes of tap water to remove any traces of fixative. The tissue was then dehydrated in ascending series of alcohol. To remove any traces of mercury, the tissue was transferred to iodine solution (in 70% alcohol) during the dehydration steps. For embedding in wax, the dehydrated tissues were treated with benzene and then transferred sequentially to 1:3::wax:benzene, 2:2::wax:benzene and then to 3:1::wax:benzene. Finally, tissues were given two changes of pure wax and embedded in wax.

Eight micron thick sections of testis from different groups were cut using manual hand driven microtome and transferred to egg albumin coated glass slides. Sections were dewaxed in xylene, rehydrated in descending series of alcohol and stained with hematoxylin and eosin. After dehydration in ascending series of alcohol, stained sections were mounted in DPX mountant and viewed under a light microscope for histological examination and photographed.
TESTICULAR CELL KINETIC STUDIES

Random field were selected from the hematoxylin and eosin stained testis slides from each animal. Counts of the cell nuclei lying in transverse section of stage VII of the cycle of the seminiferous epithelium were made by the method of Leblond and Clermont (1952). Nuclei of sertoli cell, type-A spermatogonia and step 7 spermatid were scored only when nucleoli were visible. Preleptotene, pachytene and step-19 spermatid were counted on the basis of the shape of these cells. Number of germinal cells per 100 sertoli cells were also calculated.

The tubular diameter was measured using an occular micrometer in the eyepiece of the microscope and calibrating the scale with the stage micrometer.

LDH-X PURIFICATION

LDH–X was purified from mouse testes by the method of Lee et al., (1982) using AMP–affinity gel chromatography.

a. Equilibration of AMP-sepharose affinity column: 2g of 8-(6-aminohexyl)amino-AMP-sepharose was washed several times in 0.1M phosphate buffer, pH 7.0 to remove dextran and lactose preservatives. Column was slowly packed with washed AMP-sepharose and then equilibrated with 10mM potassium phosphate buffer (pH 6.5).

b. Column Regeneration: After each use, the column was regenerated with 6M urea and 2M NaCl alternatively. Before use, column was equilibrated with 10mM potassium phosphate buffer (pH 6.5).
c. *Preparation of reduced NAD'-pyruvate adduct:* The adduct was prepared by mixing 50mg of NAD\(^+\) and 50mg of pyruvate in 1ml of distilled water. pH of the above solution was adjusted and maintained at 11.5 for 20 minutes by dropwise addition of 1N NaOH. The mixture was then diluted to give an absorbance of 2 at 340 nm in 10mM phosphate buffer (pH 6.5), before being used. Absorbance ratio \(A_{260}/A_{280}\) was 2.8 in this preparation.

d. *Purification:* 20% homogenate of frozen testes was prepared in 20mM Tris-HCl (pH 7.4) buffer and centrifuged at 27,000g for 20min at 4°C. The supernatant was collected by passage through a double-layered cheese cloth, and the pellet or lipid was discarded. The supernatant was then heated at 60°C for 15min in a 65°C water bath. The precipitated protein was removed by centrifugation at 27000g for 20min at 4°C. The pH of the clear supernatant was adjusted to 6.5 by proper addition of 0.5M KH\(_2\)PO\(_4\). The enzyme solution was then loaded on an 8-(6-aminohexyl)amino-AMP-sepharose column pre-equilibrated with 10mM potassium phosphate buffer (pH 6.5). No significant activity of lactate dehydrogenase was found in the eluent. The affinity column was then washed with 100ml of 10mM potassium phosphate buffer pH 6.5, followed by another 300ml of 50mM potassium phosphate buffer of the same pH. Lactate dehydrogenase is then eluted biospecifically with NAD'-pyruvate adduct. Purified enzyme thus obtained was dialysed against normal saline.

**LDH-ASSAY**

The activity of the purified enzyme was determined at 25°C spectrophotometrically by following the change in absorbance at 340nm.
Materials and Methods

Reaction mixture consisted:

- 0.1M Tris-HCl (pH 8.0) = 800μl
- 1.0mM Sodium pyruvate = 100μl
- 0.15mM NADH = 100μl

The reaction was started by the addition of suitable amount of enzyme to produce a decrease in absorbance of 0.05 to 0.1 per minute at 340nm.

Polyacrylamide gel electrophoresis

The purity of the LDH-X enzyme was checked by 10% SDS-polyacrylamide gel electrophoresis by the method of Laemmli (1970) using minigel apparatus (BIORAD, USA). Following reagents were made:

1. Buffer A
   - 1.5M Tris.HCl (pH 8.9)
2. Buffer B
   - 0.5M Tris.HCl (pH 6.8)
3. Acrylamide stock
   - Acrylamide : 30g
   - Bis-acrylamide : 0.8g
   - Final volume was made to 100ml with double distilled water.
4. Ammonium persulphate (APS): 10% (Prepared fresh)
5. Plug gel
   - Acrylamide : 1ml
   - APS (10%) : 40μl
   - TEMED : 4μl
6. Separating gel (Prepared fresh)
   - Acrylamide stock : 1.7ml
   - Double distilled water : 1.9ml
   - Buffer A : 1.3ml
**Materials and Methods**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS (10%)</td>
<td>50μl</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>50μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2μl</td>
</tr>
</tbody>
</table>

7. Stack gel (Prepared fresh)

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

8. Sample buffer (2X) (Prepared fresh)

- SDS (40%): 100μl
- Beta-mercaptoethanol (β-Me): 40μl
- 0.5M Tris-HCl (pH 6.8): 100μl
- Glycerol: 100μl
- Bromophenol blue (BPB) (33 mg%): 160μl

9. Running Buffer (Prepared fresh)

- Tris: 3g
- Glycine: 14.4g
- SDS (10%): 1ml

Final volume was made to 1000ml with double distilled water.

10. Comassie blue stain

- Methanol: 250ml
- Glacial acetic acid: 50ml
- Comassie blue (R250): 250mg

Final volume was made to 500ml with double distilled water.

11. Destaining solution

- Methanol: 62.5ml
- Glacial acetic acid: 17.5ml
Materials and Methods

Final volume was made to 250ml with double distilled water.

Placed the spacers between the two plates. Plug gel was used to seal the bottom of the chamber so formed between the two plates. Separating gel solution was then poured and allowed the plates to stand undisturbed for one hour to polymerize the gel. After the polymerization of the gel, the butanol:water layer was removed. Dried the plates properly with the whatmann sheet and then stack gel was cast and comb was placed. The gel was allowed to stand for another half an hour at room temperature to polymerize. The comb was removed and wells were dried.

The proteins were mixed with the sample buffer (2X concentration) in 1:1 ratio and boiled for 2 min, cooled and centrifuged at 1000g for 2min. The samples containing 20μg of protein were loaded into each well and electrophoresis was carried at a constant current of 10mA through the stacker and 15mA through the separating gel. As the dye front reached the plug gel, the gel was removed and placed in the commassie blue stain for 4-5 hrs with constant shaking. The gel was then destained with destaining solution with gentle agitation. The destainer was changed as needed until the background was clear. The gel was then dried in a gel wrap.

LDH-X ANTIBODY PREPARATION

Polyclonal antibodies against purified LDH-X were raised in two female rabbits by initially injecting each (intra-dermal, multiple sites) 200μg of purified LDH-X protein emulsified in complete freunds adjuvant (CFA). After one week of initial injection, response was boosted thrice with 50μg of LDH-X protein in incomplete adjuvant with one week interval each. At the completion of booster doses, blood was collected through ear vein of rabbits and serum was
Materials and Methods

separated. Serum from the blood drawn before starting immunization of rabbits served as a control anti-sera.

WESTERN IMMUNOBLOT ANALYSIS OF LDH-X AND HSP70 CONTENT

Tissue homogenates (10% w/v) prepared at 4°C in homogenizing buffer (20mM Tris-HCl, pH 7.4) using a mechanically driven teflon fitted Potter-Elvejham type homogenizer were centrifuged at 100,000xg for 60min to get cytosol. Protein was estimated by the method of Lowry et al., (1951). Protein sample (10µg each) were separated on SDS–PAGE as explained earlier. Proteins were from PAGE electrophoretically transferred to PVDF membrane (Immobilon-P, Millipore, USA) using 48mM Tris, 39mM glycine, 20% methanol (v/v), 0.4% SDS (w/v), pH 8.3 at 200mA for 2hr using Multiphor II, electrophoresis transfer system (Pharmacia LKB Biotechnology, Sweden). Membrane was blocked with 5% non-fat milk in phosphate buffered saline (PBS; 0.01M, pH 7.2) for 1hr with constant shaking. The membrane was then incubated with either polyclonal anti-LDH-X antibody (1:500) or monoclonal anti-hsp70 antibody (Sigma–aldrich; 1:1,000) diluted with 5% skimmed milk in PBS for 90min at room temperature. The membrane was sequentially washed with PBS, 0.05% Tween–20 in PBS and PBS each for 5 min. The membrane was then incubated with peroxidase labeled anti–rabbit IgG (Sigma–aldrich; 1:20,000) for LDH–X immunoblot. For hsp70 immunoblot, membrane was incubated with biotinylated antimouse IgG (Sigma–aldrich; 1:1,000) followed by streptavidin labeled horse radish peroxidase (HRPO, Sigma–aldrich; 1:500) for 45 min each at room temperature. The membrane for both blots were
again washed sequentially with PBS, 0.05% Tween-20 in PBS and PBS for 5 min each and the bands were visualised by addition of diamino benzidine (8mg/10ml PBS + 12μl H₂O₂). The reaction was terminated by rinsing the membrane with double distilled water.

**ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR LDH-X AND HSP70**

ELISA was carried out to quantitate the LDH-X and hsp70 in testis cytosol samples. The assay was standardized by titrating the different concentrations of antigen and antibodies. Wells were coated with 5μg (for LDH-X) and 10μg (for hsp70) proteins in 100μl of 0.05M carbonate buffer (pH 9.6) and kept overnight at 4°C in a moist chamber. Flicked the plate to remove the unbound antigen solution and wells were blocked with 1% BSA in 0.1M phosphate buffered saline (pH 7.2) for 1hr at 37°C. Flicked and washed wells thrice with 200μl of PBS containing 0.05% (v/v) tween-20. Wells were then incubated with either anti-LDH-X polyclonal primary antibody (1:1,000) or monoclonal hsp70 antibody (Sigma-aldrich; 1:1,000) diluted in PBS (containing 0.05% tween-20 and 1%BSA) and kept for 2hr at 37°C. Plate was again washed and incubated with anti-rabbit secondary antibody (peroxidase labeled) (Sigma-aldrich; 1:20,000) for LDH-X and for hsp70 wells were incubated with biotinylated goat antimouse IgG (Sigma-aldrich; 1:1,000) and then with streptavidin labeled horse radish peroxidase (HRPO;1:500) for 2hr at 37°C. Wells were washed further three times as described above and color was developed by addition of 2,2'-azino-di-(3-ethyl-benzothiazolin sulfonic acid) reagent.
Materials and Methods

IMMUNOHISTOCHEMICAL LOCALIZATION OF LDH-X AND HEAT SHOCK PROTEINS

Eight micron thick testis paraffin cross-sections taken on a egg albumin coated glass slides were deparaffinized in xylene and rehydrated in descending series of alcohol. Endogenous peroxidase activity was blocked by incubating sections with 3% H₂O₂ for 30min at room temperature. Nonspecific staining was blocked by incubation with 2% BSA in phosphate buffered saline (PBS; 10mM, pH7.2). Sections were then incubated with monoclonal antibodies (Sigma-aldrich) against either hsp60(1:100), hsp70(1:2,000), hsp90(1:300) or with polyclonal antibody against LDH-X (1:500) diluted in PBS containing 1% BSA for 90min in moist chamber at room temperature. For negative control, only diluent (PBS with 1% BSA) was added. Further, after proper washing with PBS-Tween(PBS with 0.05% Tween-20), sections were incubated with peroxidase labeled anti rabbit IgG for LDH–X (1:10,000). For heat shock proteins, sections were incubated with mouse IgG (1:15) followed by streptavidin labeled horse radish peroxidase (1:15). The sections were washed with PBS and then with PBS–Tween and reaction product was developed using DAB plus H₂O₂ as described earlier. Reaction was terminated by washing with distilled water, sections were mounted in glycerol jelly (Annexure III) and viewed / photographed under microscope.

DNA FRAGMENTATION ANALYSIS

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
**Materials and Methods**

consisted of 10mM Tris-Cl (pH 8.0), 0.1M EDTA (pH 8.0), 20µg/ml RNase and 1% SDS. The solution was incubated for 1hr at 37°C. Added proteinasek to the 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 3hr 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. Mixed the two phases slowly by inversion for 10min. Two phases were separated by centrifugation at 10,000g for 15min 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 added two volumes of ice-cold isopropanol and kept at -70°C for 1hr. 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 20µl of TE buffer (pH 8.0). The absorbance of DNA was measured at 260nm and 280nm. The ratio of A$_{260}$ and A$_{280}$ should be greater than 1.75.

**Agarose Gel Electrophoresis**

1.8% agarose was prepared in TAE buffer in a flask under boiling water bath. 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. The gel was allowed to stand at room temperature for 30-45min. Separating buffer (TAE) was added in the chamber. The DNA solution (10µg) mixed with the sample buffer was loaded into slots of the submerged gel and electrophoresis was carried at constant current of 50mA, the bands were viewed using ultraviolet transilluminator (BIO–RAD Systems, USA) and photographed.