MATERIALS AND METHODS
CHEMICALS

Monensin was obtained from Sigma Chemical Co., St. Louis, MO, USA. Reduced glutathione, oxidized glutathione, bovine serum albumin, adenosine triphosphate, NADPH, NADH, DTNB, NBT, CDNB, sodium pyruvate, thiamine pyrophosphate, thiobarbituric acid, glutathione reductase, DMEM, FCS, fungizone used were from Hi Media Laboratories Private Limited, Bombay. All other chemicals used were of analytical grade.

ANIMALS

Male Wistar rats having 150-200 g body weight, obtained from the central animal house, Panjab University, Chandigarh, were used in all the experiments. The animals were housed in polypropylene cages bedded with rice husk in the departmental animal house under hygienic conditions following the guidelines of the local committee for animal experimentation of the Panjab University. The animals were maintained on standard pelleted feed and water *ad-libitum* throughout the experimental period. Before initiating the experiments, the animals were well acclimatized to the laboratory conditions.

EXPERIMENTAL DESIGN

*In vivo* experiments

The animals were randomly segregated into four main groups. Group I consists of control animals, group II animals were
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administered with 2.5 mg monensin/kg b. wt., group III animals 5 mg monensin/kg b. wt. and group IV animals 10 mg monensin/kg b. wt., respectively.

Before the starting of the experiments the diet requirements of the animals in different cages were checked. The standard pelleted feed was crushed to the powder form and then monensin was mixed to the powder in the required concentration and given to the animals. The control animals were given diet free of monensin. At the completion of the treatment period i.e. 67 days, animals were killed by cervical dislocation. The organs of interest were removed and used for various analyses.

Intratesticular experiments

Monensin was administered in the following series of experiments:

In one series of experiments, the animals were divided into five groups. Monensin stock solution was prepared in 10% ethanol and administered to the animals by intratesticular injection. Group I served as control which received the vehicle only (10% ethanol) while Group II was administered with 1 μg monensin/testis, Group III 5 μg monensin/testis, Group IV 10 μg monensin/testis and Group V 20 μg monensin/testis. The animals were sacrificed after a gap of five days of the drug treatment.

In the second series, the animals were divided into two groups. Group I served as control while the group II was given
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1μg monensin by intratesticular injection. The animals were kept for 35 days and then sacrificed.

In the third series, the animals were administered with 5μg monensin / testis, and were sacrificed after a time interval of 1, 2, 3 and 4 days of the drug treatment.

In vitro experiments

The animals were fasted overnight and killed by cervical dislocation. Testes were removed and the in vitro effects of monensin at various concentrations (10μM, 20μM, 40μM, 80μM and 100μM) were observed by adding the drug in the testicular cells in culture obtained from the normal rat testis.

Testicular Cell Culture

Preparation and composition of medium

DMEM (with Glutamine, without indicator (phenol red) 9.6 g/l, HEPES 10 mM, Gentamycin 10 mg/l, Fungizone 1.25 mg/l, Sodium bicarbonate (Na2HCO3) 2.0 g/l and Fetal calf serum (FCS) 5%.

Method

The testicular cell isolation and culture was done according to the method of Steinberger, 1975. All the apparatus used in this study was either autoclaved or disposable UV-sterilized plastic ware were used.
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- Rats were sacrificed after anesthetizing with diethyl ether and testes were removed under sterile conditions into a watch glass containing phosphate buffered saline (PBS).

- The testes were decapsulated and the tissues were gently teased by pressing between the two frosted slides (Hudson and Hay, 1989) to obtain a cell suspension in the PBS.

- Cell suspension was gently aspirated repeatedly with a Pasteur pipette to further disaggregate the cell clumps.

- Cell suspension was then transferred to a polypropylene tube and allowed to stand for a minute so that the tissue pieces if present can settle down.

- The supernatant was transferred to another centrifuge tube with fresh Pasteur pipette and the cells were pelleted by centrifuging at 600 rpm for 10 minutes.

- The cell pellet obtained was washed once with PBS and recentrifuged.

- The cell pellet was then suspended in the medium (to give washing) and then centrifuged at 600 rpm for 10 minutes.

- The cell pellet obtained at this step was then suspended in fresh medium.

- Cell viability was checked by dye exclusion test using trypan blue and cell concentration was adjusted at $5 \times 10^5$ cells/ml using a hemocytometer.
• The wells of the tissue culture plate were loaded with the cell suspension. Monensin at different concentrations was added to the cell suspension in different wells.

• This was incubated at 31°C for 18 hrs in a humidified atmosphere of 5% CO₂ + 95% air.

• After the incubation, the cells were checked for their viability. Different biochemical studies were performed in the culture supernatant.

Following studies were performed after sacrificing the animals from the various treatments.

**BODY WEIGHT**

Animals were weighed both before starting the experiment and at the end of the experiment.

**ORGAN WEIGHT**

Wet weights of the testis, epididymis, seminal vesicles and prostate were recorded immediately after sacrificing the animals.

**SPERM STUDIES**

**Sperm concentration**

Sperm concentration was determined in vas deferens of each animal by the method of Hukeri (1969). Immediately after sacrificing the animals, spermatozoa from vas deferens were squeezed out onto a glass slide separately and diluted with...
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normal saline in a ratio of 1:100. The diluted samples were well mixed and charged on to the chamber of Neubauer's hemocytometer. Hemocytometer chamber contains 9 squares each having an area of 1mm². The central chamber is divided into 25 small squares. The four corner squares are divided into 16 small squares. The height of the chamber formed after placing the coverslip is 1/10mm. The spermatozoa present in all 64 small corners were counted under a low power objective in the microscope. The number was divided by four to obtain the average number in 16 small squares. One big square in the corner of the area equals to 1mm².

Calculations

Volume under 16 small squares = 1mm² x 1/10mm = 0.1mm³

Let the total number of spermatozoa counted in the above volume = N

i.e. 0.1mm³ contains N spermatozoa

or 1.0 mm³ contains N x 10 spermatozoa

or 1.0 cm³ contains N x 10 x 10³ spermatozoa

Total number of spermatozoa present per ml of sample

= N x 10⁴ x dilution factor

= N x 10⁴ x 100

= N x 10⁶ spermatozoa/ml
Sperm motility

The method of Hukeri (1969) was applied to find out the sperm motility. A known length of vas deferens was taken from each rat; spermatozoa were squeezed onto a glass slide and diluted with normal saline. The motility of spermatozoa was evaluated under a light microscope. One drop of well-mixed diluted sperm suspension was placed on a glass slide under the coverslip and spermatozoa were observed under several fields for their motility. The percentage of motile spermatozoa was evaluated according to the formula:

\[
\text{% Motility} = \frac{\text{Number of motile spermatozoa/field}}{\text{Number of total spermatozoa/field}} \times 100
\]

FERTILITY STUDIES

At the end of the treatment period of all the groups, animals were mated with the control females in the ratio of 1:2. After 10 days, the males were separated and female rats were observed for litter size for 21 days.

RECOVERY STUDIES

At the end of the differential monensin treatment, the animals were kept for other 60 days on normal diet, to observe the effects of the drug after its termination.
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BIOCHEMICAL ESTIMATIONS

Preparation of tissue homogenates

After dissecting the animals, testes were removed and the wet weight recorded. A 10% homogenate of the tissue was prepared in 0.1M Tris HCl (pH 7.4) using a Potter-Elvenhim homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 10,000g for 20 minutes to get the post mitochondrial supernatant and used for various biochemical estimations.

Superoxide dismutase (SOD)

The activity of superoxide dismutase was estimated according to the method of Kono (1978).

Principle: The method is based on the principle of the inhibitory effect of SOD on the reduction of nitroblue tetrazolium (NBT) dye by superoxide anions, which are generated by the photo-oxidation of hydroxylamine hydrochloride (NH₂OH. HCl).

Reagents:

1. Solution A: EDTA (0.1mM) containing 50mM sodium carbonate, (pH 10.0).
2. Solution B: NBT (90mM) in solution A.
3. Solution C: Triton-X-100 (0.6%) in solution A.
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**Procedure:** The reaction mixture contained 1.3ml, 0.5ml and 0.1ml of solutions A, B and C, respectively. Reaction was initiated by the addition of 0.1ml of solution D to the reaction mixture and the rate of reduction of NBT in the absence of enzyme source was recorded for about 30 seconds at 560nm in a Shimadzu 160A double beam spectrophotometer. Following this an appropriate amount of the enzyme source was added.

Percentage inhibition in the rate of NBT reduction was noted and one unit of the enzyme was expressed as inverse of the amount of protein (mg) required to inhibit the reduction rate of NBT by 50%.

**Catalase**

Catalase was estimated directly as per the method described by Luck (1963).

**Principle:** The enzyme catalyzes the decomposition of hydrogen peroxide which is measured by a decrease in the absorbance at 240nm. The concentration of H$_2$O$_2$ was determined by recording the absorbance at 240nm and taking 0.0394 mM$^{-1}$ cm$^{-1}$ as the extinction coefficient.

**Reagents:**

1. Phosphate buffer (50mM, pH 7.0)
2. Hydrogen peroxide (0.75M)
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Procedure: 1.0ml of reaction mixture in the sample cuvette contained phosphate buffer (pH 7.0), \( \text{H}_2\text{O}_2 \) and an appropriate amount of the enzyme source. The blank lacking \( \text{H}_2\text{O}_2 \) was also run simultaneously. A decrease in the absorbance at 240nm was measured on a Shimadzu 160A double beam spectrophotometer.

The enzyme activity was expressed as \( \mu \) moles \( \text{H}_2\text{O}_2 \) decomposed /min /mg protein.

Glutathione peroxidase (GSH-PX)

Glutathione peroxidase was assayed as per the method given by Paglia and Valentine (1967).

Principle: The enzyme catalyses the formation of GSSG from GSH and \( \text{H}_2\text{O}_2 \).

\[
\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG}
\]

The glutathione is continuously reduced by excess of glutathione reductase and simultaneous oxidation of NADPH is measured at 340nm.

Reagents:

1. Phosphate buffer (0.05M, pH 7.0).
2. GSH (0.15M)
3. Glutathione reductase (10 U/ml)
4. Sodium azide (0.125M)
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5. NADPH (0.08M)
6. H$_2$O$_2$ (0.002M)

Procedure: 1.0ml of the reaction mixture contained 820 μl of phosphate buffer (pH 7.0), 37.5 μl of GSH, 37.5 μl of glutathione reductase, 3.75 μl of sodium azide, 25 μl of NADPH, 37.5 μl of H$_2$O$_2$ and an appropriate amount of enzyme source. The decrease in absorbance due to NADPH oxidation was recorded at 340nm in a Shimadzu 160A double beam spectrophotometer.

The activity of the enzyme was expressed as μ moles NADPH oxidized /min/mg protein and was calculated using an extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$.

Glutathione reductase (GR)

The enzyme was assayed by the method of Massey and Williams (1965).

Principle: Glutathione reductase catalyzes the reaction

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+$$

The utilization of NADPH is directly related to the activity of GR.

Reagents:
1. Phosphate buffer (100mM, pH 7.6)
2. EDTA (60mM)
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3. BSA (20mg/ml)

4. NADPH (2mM)

5. Oxidized glutathione (60mM)

**Procedure:** 1.0ml of the reaction mixture contained 0.5ml of phosphate buffer (pH 7.6), 0.2ml of DDW, 0.05ml EDTA, 0.05ml BSA, 0.05ml NADPH, 0.05ml GSSG and an appropriate amount of the enzyme source. The appropriate blanks (lacking GSSG) were carried out simultaneously. The decrease in absorbance was monitored at 340nm in a Shimadzu 160A double beam spectrophotometer.

Glutathione reductase activity was expressed as n moles NADPH oxidized/min/mg protein, which was calculated using an extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$.

**Glutathione-S-transferase (GST)**

The assay of this enzyme was done by the method of Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate.

**Principle:** GST catalyzes the formation of glutathione conjugates with GSH.

**Reagents:**

1. Phosphate buffer (0.1M, pH 6.5)

2. CDNB (1mM)
Materials and Methods

3. GSH (1mM)

Procedure: 3.0 ml of the reaction mixture contained 2.7 ml of phosphate buffer (pH 6.5), 0.1 ml of CDNB, 0.1 ml of GSH and 0.1 ml of the enzyme source. Blank cuvette contained a similar reaction mixture but without an enzyme source. The increase in absorbance due to the formation of glutathione conjugate was recorded in a Shimadzu 160A double beam spectrophotometer at 340 nm and using an extinction coefficient of 9.6 mM⁻¹ cm⁻¹.

The enzyme activity was expressed as n moles GSH conjugates formed/min/mg protein.

Glutathione reduced (GSH)

Estimation of GSH was performed by the method of Moron et al. (1979).

Principle: GSH is a non-protein sulfhydryl compound. In this method, 5, 5'-dithiobis- (2-nitrobenzoic acid) (DTNB) is reduced by -SH groups to form one mole of 2-nitro-5-mercaptobenzoic acid per mole of –SH. The nitromercaptobenzoic acid anion released has an intense yellow color, which can be used to measure –SH groups at 412 nm.

Reagents:

1. Phosphate buffer (0.2M, pH 8.0)
2. TCA (25%)
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3. DTNB (0.6 mM)

Procedure: 0.1 ml of 25% TCA was added to 0.5 ml of the homogenate. After mixing the contents, the precipitated proteins were separated by centrifugation at 2000 rpm for 15 min. 200 μl of the supernatant so obtained was diluted in a test tube to 1.0 ml with phosphate buffer. To this was added 2.0 ml of freshly prepared DTNB.

Optical density of the yellow colored complex formed by the reaction of GSH and DTNB was measured at 412 nm against a reference which contained 5% TCA instead of the sample.

The molar extinction coefficient for GSH is 13.6 M⁻¹ cm⁻¹. The results were expressed as μ moles of GSH/mg protein.

Lipid peroxidation (LPO)

The lipid peroxidation was assayed according to the method of Buege and Aust (1978).

Principle: Lipid components in the tissue, mainly the PUFAs are highly susceptible to peroxidation by various oxidizing free radicals which are formed from various sources such as enzymatic as well as non-enzymatic oxidation reactions promoted by Fe²⁺ species etc. Cycloperoxides are formed as a result of these peroxidation reactions, which give malondialdehyde by cleavage. MDA forms pink colored complex with thiobarbiturate, which can be measured at 532 nm.

Reagents:
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1. Tris Buffer (150mM, pH 7.1)
2. FeSO₄ (1mM)
3. Ascorbic acid (1.5mM)
4. TCA (10%)
5. TBA (0.375%)

Procedure: To 0.1ml of the sample was added 0.1ml of Tris-HCl buffer, 0.1ml of FeSO₄ and 0.1ml of ascorbic acid in a reaction mixture. The volume was made to 1.0 ml. It was mixed well and incubated at 37°C for 15 minutes. After the completion of the incubation 1ml of TCA and 2 ml of TBA was added and then kept for 15 min on the boiling water bath. After that, centrifugation for 15 min at 3000 rpm was done. In the blank tube the sample was replaced by water. The absorbance was read at 532nm.

The molar extinction coefficient for MDA is 1.56 x 10⁵ M⁻¹ cm⁻¹. The results were expressed as nmoles of MDA formed /min /mg protein.

Lactate dehydrogenase (LDH)

Activity of LDH was determined spectrophotometrically by the method of Schatz and Segal (1969) by following the changes in absorbance at 340nm for 3 minutes.

Principle: The enzyme LDH catalyses the reduction of pyruvate to lactate with simultaneous oxidation of NADH to NAD⁺ causing a decrease in absorbance at 340nm.
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Pyruvate + NADH + H+ → Lactate + NAD⁺

Reagents:

(1) Tris-HCl (0.1M, pH 8)
(2) Sodium pyruvate (1mM)
(3) NADH (0.15mM)

Procedure: The reaction mixture in a total volume of 2.0 ml contained 0.1M Tris-HCl (pH 8), 1mM sodium pyruvate and 0.15mM NADH. The reaction was initiated by adding a suitable amount of the enzyme to produce a measureable decrease in absorbance at 340nm.

The enzyme activity was expressed as μ moles of NADH oxidized/min/mg protein. The extinction coefficient (6.3 x 10³ μ mol L⁻¹ min⁻¹) was used to calculate the enzyme activity.

ATPase

The enzyme activity was measured according to the modified method of Quigley and Gotterer (1969). The amount of inorganic phosphorus liberated by the action of ATPase on ATP was estimated using Fiske and Subbarow (1925) method.

Principle: Phosphate is converted into phosphomolybdic acid and reduced with 1-amino-2-naphthol-4-sulfonic acid (ANSA). The liberated P₄ forms a blue colored complex, which is measured at 595nm.
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Reagents:

(1) Tris-HCl buffer (75 mM, pH 7.1)
(2) MgCl₂ (75 mM)
(3) NaCl (120 mM)
(4) KCl (200 mM)
(5) KCl (2.5 M)
(6) ATP (50 mM)
(7) PCA (2.5 M)
(8) KH₂PO₄ (1 mg/ml)
(9) Acid ammonium molybdate (2.5%)
(10) 1 Amino -2 naphth -4 sulfonic acid (ANSA):

15g of sodium metabisulphate was dissolved in 200 ml of DDW. To 195 ml of this solution, 0.5g of ANSA and 5 ml of 20% sodium sulfate was added. This solution was decolorized with 1g of activated charcoal and kept in dark for overnight. The colorless filtrate was stored in the refrigerator for use but discarded once it turned yellowish.

Procedure: 1ml of the reaction mixture containing 0.4 ml of 75 mM Tris-HCl buffer (pH 7.1), 0.1 ml of 75 mM MgCl₂, 0.1 ml of 120 mM NaCl, 0.1ml of 200 mM KCl, 0.1 ml of 50 mM ATP, 0.15ml of distilled water and 0.05 ml of the sample was incubated
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for 20 min at 37°C. After incubation, the reaction was stopped by
the addition of 0.25 ml of 2.5 M PCA. The tubes were kept in ice
for 15 min and then 0.25 ml of 2.5 M KCl was added. Tubes were
centrifuged at 1000 rpm for 10 min. To 1 ml of the aliquot was
added 1 ml of acid ammonium molybdate, 0.5 ml of ANSA and
2 ml of DDW. Optical density of the blue-green color developed
was read at 595 nm.

Acid phosphatase

For the measurement of acid phosphatase, method of
Linhart and Walter (1965) using p-nitrophenyl phosphate as the
substrate was employed.

Principle: p-nitrophenyl phosphate is used as substrate and the p-
nitrophenol released by enzymic hydrolysis measured at 405 nm.

Reagents:

1. Acetate buffer (0.1 M, pH 4.8)
2. Para nitro phenyl phosphate (PNPP)- Dissolved 1.65 mg of
   PNPP in 1 ml of distilled water.
3. Para nitro phenol (Standard) – Dissolved 1.39 mg in
   10 ml of distilled water.
4. NaOH (0.4 N)

Procedure: 1 ml of an assay volume contained 0.9 ml of acetate
buffer (0.1 M, pH 4.8), 0.05 ml of substrate PNPP (1.65 mg/ml)
and 0.05 ml of sample. The reaction was terminated by the

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addition of 0.4 M sodium hydroxide after 30 min of incubation at 37°C. The yellow color developed due to the liberation of p-nitrophenol was read at 405 nm. Standards were run simultaneously to calculate the enzyme activity. The phosphatase activity was expressed as µ moles phenol produced /min /mg protein.

**Thiamine pyrophosphatase (TPPase)**

For determination of TPPase activity the method of Allen and Slater (1961) was followed. For the determination of inorganic phosphate released from the TPPase activity, the method of Martin and Doty (1949) was applied.

**Principle:** The phosphomolybdic acid formed in acid solution is extracted by shaking in a mixture of isobutanol and benzene. On treating with SnCl₂, phosphomolybdic acid gives a blue-green color, which can be read at 625 nm.

**Reagents:**

1. **Substrate (pH 8.0):**
   - Sodium barbital (33 mM)
   - Calcium chloride (15 mM)
   - Thiamine pyrophosphate (3.3 mM)

2. **TCA (10%)**

3. **H₂SO₄ (5 M)**
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4. 10% ammonium molybdate
5. Isobutanol-benzene mixture (equal parts).
6. 10% SnCl₂. H₂O in conc. HCl
7. 3.2% (v/v) H₂SO₄ in absolute ethanol.
8. Phosphomolybdic acid (1 mM)

Procedure: The assay was initiated by adding 0.1 ml of sample to 2.9 ml of the substrate. After the incubation of 20 min the reaction was terminated by adding 1 ml of 10% TCA. Precipitated protein was removed by centrifugation. To 3 ml of the deprotenised sample 0.5 ml of 5 M H₂SO₄, 5 ml of isobutanol-benzene mixture and 0.5 ml of 10% ammonium molybdate were added. The mixture was shaken for 15 seconds. After the separation of the two layers, a suitable amount was removed and diluted with 3.2% sulfuric acid to 5 ml. To it, 0.5 ml of 10% SnCl₂ was added and mixed immediately. A blue-green color was developed whose intensity was measured at 625 nm.

The enzyme activity was calculated by running the appropriate standards simultaneously. The enzyme activity was expressed as amount of inorganic phosphorus liberated in μ moles/min/mg protein.

Protein estimation

Protein content of various samples was estimated by the method of Lowry et al. (1951).
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**Principle:** It is based on the formation of colored cupric protein complex due to phenolic hydroxyl groups of proteins. Final color is developed by the reduction of phosphomolybdic phosphotungstic reagent by copper treated proteins.

**Reagents:**

A. Reagent – 1% CuSO₄

B. Reagent – 2% Sodium potassium tartarate

C. Reagent – 2% Anhydrous sodium carbonate in 0.1N NaOH

D. Lowry’s reagent – mixture of A: B: C in the ratio of 1:1: 98

E. Folin Ciocalteau reagent (1: 1 v/v)

F. Standard protein Solution (20mg%), bovine serum albumin (BSA).

**Procedure:** To 0.1ml of the sample, 0.9 ml distilled water and 3.0 ml of Lowry’s reagent was added, mixed well and allowed to stand for 10 min. Then 0.3 ml of Folin-Ciocalteau’s reagent was added and the contents were vigorously shaken and incubated for 30 minutes. Appropriate blanks and standards were run simultaneously and optical densities were measured at 670 nm.

**CYTOCHEMICAL ASSAY OF THIAMINE PYROPHOSPHATASE**

For cytochemical localization of the Golgi body marker enzyme thiamine pyrophosphatase, the method of Allen and Slater (1961) was applied.
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Reagents:

1. Incubating medium, (pH 9.5):
   - Sodium veronal (33 mM)
   - CaCl₂ (15 mM)
   - Thiamine pyrophosphate chloride (5 mM)
   - Cysteine hydrochloride (4 mM)

2. 2% Cobalt nitrate

3. 1% Yellow ammonium sulphide.

4. Glycerol.

5. Different grades of alcohol.

Procedure: Testes were removed and immediately processed for cryosectioning (-20°C) using 1100 CM Leica cryostat at the Department of Histopathology, Post Graduate Institute of Medical Education and Research, Chandigarh. Sections were incubated in the medium for 5 min at 37°C. After the incubation they were rinsed briefly and treated with 2% Cobalt nitrate for 3-5 min, followed by rinsing in distilled water. The sections were treated with 1% yellow ammonium sulphide for 1-2 min. In the end the sections were washed in water, dehydrated, cleared and mounted in Glycerol.

The black deposits indicated the site of thiamine pyrophosphatase activity.
DNA FRAGMENTATION STUDY

DNA fragmentation study was carried out by isolating the DNA using the standard method of CTAB/NaCl (Sambrook and Russell, 2001). The electrophoresis of DNA was carried out on 1.2% agarose gel (w/v) made in Tris-Borate-EDTA (TBE) buffer (pH 8.0). DNA was stained with ethidium bromide and then visualized on a UV transilluminator.

Reagents:

1. Lysis Buffer:
   
   10 mM Tris-Cl (pH 8.0)
   
   0.1 M EDTA (pH 8.0)
   
   0.5 % SDS

2. TE Buffer (10 mM)
   
   Tris-Cl (pH 8.0)
   
   1 mM EDTA (pH 8.0)

3. 10% SDS

4. Stock proteinase K (2 mg/ml)

5. CTAB (N-cetyl trimethyl ammonium bromide).

CTAB/NaCl solution: 4.2 g NaCl was dissolved in 80 ml of DDW. 10 g CTAB was added while stirring and the solution was
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heated at 65°C and the volume was made up to 100 ml with DDW.

6. 5M NaCl

7. Chloroform, Isopropanol, Isoamyl alcohol

8. 70% ethanol

9. Stock TBE
   
   54 g Tris base
   
   27.5 g Boric acid
   
   20 ml of 0.5 M EDTA

10. Sample buffer

   0.25% Bromophenol blue (BPB) in PBS

   40% sucrose in PBS

11. 1.2% Agarose

12. Stock ethidium bromide (2 mg/ml).

**Procedure:**

1. The testes were taken from each animal with the help of scissors, forceps and the fibrous capsule removed with a surgical blade.
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2. A 10% homogenate was prepared in lysis buffer and centrifuged at 12,000 g for 20 minutes. Supernatant was removed.

3. Pellets were suspended in 500 μl of TE buffer by gentle vortexing.

4. To the suspension 70 μl of 10% SDS and 25 μl of proteinase K was added and the mixture was kept at 65°C for 1hr.

5. After incubation, 100 μl of 5 M NaCl was added followed by 80 μl of CTAB/NaCl solution. Mixture was vortexed until the liquid content had a milky white appearance.

6. The mixture was again incubated at 65°C for 1hr.

7. An equal volume of chloroform: isoamyl alcohol (24:1) was added and the mixture vortexed for 10 seconds.

8. After vortexing, the mixture was centrifuged for 5 minutes at 12,000 g. The sample in the aqueous phase was retained.

9. Extraction was repeated with CTAB/NaCl followed by chloroform: isoamyl alcohol once more with the organic phase and added to the aqueous phase as obtained in the previous step.

10. Nucleic acids in the solution were precipitated by adding 0.6 volumes of isopropanol at -20°C for 30 min and the mixture centrifuged at 12,000g for 30 minutes.
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11. DNA pellets were given a washing with 100-200 μl of cold 70% ethanol to remove CTAB and NaCl.

12. After re-spinning, the supernatant was removed and the pellets allowed to dry at room temperature.

13. Pellets were dissolved in 40 μl of TE Buffer.

* For DNA isolation from testicular cell cultures. The cells in the culture media were suspended in the lysis buffer and processed as above.

Agarose gel electrophoresis of DNA

1. 1.2% agarose gel was prepared in a diluted TBE buffer.

2. Added 1μl of stock ethidium bromide to the gel immediately before polymerization.

3. Dissolved the DNA suspension for staining in sample buffer.

4. Added the samples to the wells in the agarose gel and the gel was run at 50V.

5. After the completion of electrophoresis, the bands obtained were visualized on a UV transilluminator.
HISTOPATHOLOGICAL STUDIES

Light microscopy

The histology of testes was performed by the standard Hematoxylin and Eosin staining (Thompson, 1966) technique. Testes from both the control and treated groups were fixed in Bouin’s fixative. After fixation, the tissues were washed properly, dehydrated in the grades of alcohol, cleared in benzene and embedded in paraffin wax. Blocks were made and 5-6 μm thick sections cut and stained. For staining, the sections were deparaffinized and hydrated down to distilled water through different grades of alcohol. Sections were stained with hematoxylin, which gives blue color to nuclei, dehydrated down to 70% alcohol and counter stained with eosin, which imparts pink color to the cytoplasm. The slides were again dehydrated up to absolute alcohol, cleared with xylene and mounted in DPX.

Electron microscopy

Testes samples were processed immediately after sacrificing the animals for histopathological examination at the electron microscopic level according to the method of David et al. (1973). Testes were fixed by immersion in the fixative having formaldehyde and glutaraldehyde made in 0.2 M sodium cacodylate buffer (pH 7.2) for 18 hrs at 4°C. The specimens were then thoroughly washed 3 times in cacodylate buffer and then post fixed for 60min in 1% Osmium tetraoxide (OsO₄) made in the same buffer. The tissues were then thoroughly washed in the
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buffer to remove the extraneous traces of OsO₄ and then dehydrated in an ascending series of acetone, allowing 20 min for each change in each of the concentration of the acetone. Specimens were infiltrated and subsequently embedded in Durcupan resin kit Supplied by Polaron Scientific Inc., U.S.A. Specimen blocks (3-5 blocks) from each animals were made out by polymerisation of the pure embedding resin at 60°C for 48-72 hrs.

Ultrathin sections of various specimen blocks were cut using a Reichert Jung Ultramicrotome. Initially, semi-thin sections of thickness about 1μm were cut using very sharp glass knives (prepared on LKB Knife maker) to locate the area of interest in different treatment groups. These semi-thin sections were stained with 0.5% toludine blue made in 1% borax solution. Ultra-thin sections of interference colors from golden to silver were cut and loaded on fine copper grids of 100-300 mesh size. Sections were double stained with Uranyl acetate and Lead citrate. These ultrathin sections were finally viewed under Transmission Electron Microscope at the Department of Anatomy, All India Institute of Medical Sciences (AIIMS), New Delhi.

STATISTICAL ANALYSIS

The difference between the means ± standard deviation of means (S.D) for the control and treated groups were examined by using Student’s ‘t’-test. Statistical differences of p-values at the level of 0.05 or less were considered significant.