1. Semen characteristics.

Seminal content of epididymis was obtained by cutting the cauda epididymis using surgical blades and squeezing onto a sterile clean watch glass.

1.1 Sperm motility

Reagents

Normal saline – 0.89% Sodium chloride.

Procedure

A small drop of diluted semen was taken in a microscope slide. Covered this with a cover slip by the wet mounting technique. Rimmed the cover slip with vaseline to avoid drying. Examined this under microscope and the percentage of motility and mode of movement, were noted.

1.2 Sperm Count

The concentration of spermatozoa was determined using the haemocytometer method described in WHO manual 2010.

Reagent

Diluent: 5 g Sodium carbonate 1 mL of 35% (v/v) formalin and 99 mL water.

Procedure

Semen collected from the cauda epididymis was taken in a tube. Diluted 50 mL of the semen with 950 mL of diluent, made a 1:20 dilution. From that diluted semen transferred 10-20 mL to the chamber of an improved Neubauer haemocytometer and covered with a cover glass. The haemocytometer was allowed
to stand for about 5 mins. After the cells sedimented, this was counted and then counts / mL was calculated.

1.3 Sperm morphology


Reagents

1. Eosin Y (10 g/ L in distilled H₂O)
2. Nigrosin (100 g/ L in distilled H₂O)

Procedure

Mixed one drop of semen with two drops of 1% Eosin Y. After 30 sec, added three drops of 10% Nigrosin solution and mixed. Placed a drop of the semen-Eosin-Nigrosin mixture on a clear microscopic slide and made a smear. Allowed to air dry. Then examined the morphological changes of sperm. Counted at least 100 to 200 spermatozoa. From this percentage of morphological change was calculated.

1.4 Vital staining

Reagents

1. Sperm cells suspended in 0.9% physiological saline
2. Eosin V
3. Nigrosin

Procedure

100 µL of 1% aqueous solution of Eosin V is mixed with one drop of Nigrosin in an eppendorf. One drop of this stain is mixed with one drop of semen and the mixture is placed on a glass slide. Observed under microscope using 40X
objective. Dead sperms with damaged plasma membrane will be stained pinkish violet and the live ones will remain unchanged.

2. Assay of Aspartate amino transaminase (AST) [E.C.2.6.1.1.] and Alanine amino transaminase (ALT) [E.C.2.6.1.2]

AST and ALT activities were determined by the method of Reitman and Frankel (1957).

Reagents

1. Phosphate buffer, pH 7.4: Dissolved 2.26 g anhydrous Na$_2$HPO$_4$ and 0.54 g anhydrous KH$_2$PO$_4$ in 100 mL distilled water and pH adjusted to 7.4. The final volume was adjusted to 200 mL with distilled water.

2. AST substrate: (200 mM of DL-aspartic acid and 2 mM α-ketoglutaric acid). Dissolved 13.3 g of DL-Aspartic acid in minimum amount of sodium hydroxide which would dissolve it and produced a solution with pH 7.4. About 90 mL was required. Added 0.146 g α-ketoglutaric acid, dissolved it by adding a little more sodium hydroxide. Adjusted the pH to 7.4 and made up to 500 mL with phosphate buffer. Stored at -15°C.

3. ALT substrate: 0.9 g alanine is dissolved in 9 mL distilled water and pH adjusted to 7.4 by 1 N NaOH and added 0.0146 g α-ketoglutaric acid and dissolved by adding little more NaOH, pH adjusted to 7.4 and made up to 50 mL with phosphate buffer pH 7.4. Stored at -15°C.

4. Color reagent: 2, 4 – dinitrophenyl hydrazine (19.8 mg DNPH is dissolved in 90 mL distilled water) and added 10 mL con. HCl.

5. Pyruvate standard: 22 mg sodium pyruvate dissolved in 10 mL phosphate buffer. Stored at -5°C
6. Pyruvate working standard: 1 mL stock is diluted with 5mL phosphate buffer.

7. 0.4 N NaOH: 16 g of sodium hydroxide per litre of water.

**Procedure**

The reaction mixture contained 0.5 mL of the substrates (0.2 µM of DL-aspartic acid and 0.002 M α-ketoglutaric acid), 0.1 mL serum and incubated for 60 mins at 37°C. The reaction was stopped by the addition of 2, 4 dinitro phenyl hydrazine. Then 0.4 N NaOH was added and the oxaloacetic acid liberated was quantitatively measured by the colorimetric method. The activity of AST is expressed as µM of oxaloacetic acid liberated/min/mL of serum. In the case of ALT, the incubation medium contained 0.5 mL of the substrate (1.78 g alanine and 30 mL α-ketoglutaric acid in 0.5 mL of NaOH and made upto 100 mL), 0.1 mL serum/tissue homogenate was incubated for 60 mins at 37°C. The reaction was stopped by the addition of color reagent. The 0.4 N NaOH was added and the pyruvate liberated was measured colorimetrically. The activity of ALT is expressed as µM of pyruvate liberated /min/ litre serum.

3. **Assay of Gamma glutamyl transferase (GGT) (EC.2.3.2.2)**

Activity of GGT was estimated by the method of Szasz (1969)

**Reagents**

1. Buffer substrate: 6.25 mM gamma glutamyl-p-nitroanilide/L in 100 mM Tris buffer containing 50 mM glycylglycine. pH adjusted to 9.0 at 25°C. Dissolved freshly before use.

2. Acetic acid: 100 mL glacial acetic acid diluted to 1 L with water.

3. p-nitroanilide solution: 100 mM/L.
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**Procedure**

Added 50 µL serum to 450 µL pre-warmed buffer substrate, incubated at 37°C for 30 mins, then added 2.5 mL acetic acid to stop the reaction and the optical density was read at 450 nm against a blank prepared by adding 50 mL serum to 450 µL buffer substrate and 2.5 mL acetic acid. Unit is defined as µM of p-nitroaniline liberated per min per mg protein.

4. **Estimation of Malondialdehyde (MDA)**

Malondialdehyde was estimated by the method of Ohkawa (1979)

**Reagents**

1. KCl (1.15%)
2. Sodium dodecyl sulphate (SDS) (8.1%)
3. Acetic acid (20%): pH of the 20% acetic acid solution was adjusted to 3.5 with NaOH
4. Aqueous solution of 0.8% thiobarbituric acid (TBA)
5. N- butanol pyridine (15:1 v/v)
6. 1, 1, 3,3- tetra methoxy propane (TMP as standard)

**Procedure**

1 g tissue was homogenized in 9 volumes of 1.15% KCl, centrifuged and took the supernatant. 0.2 mL 8.1% SDS, 1.5 mL 20 % acetic acid (pH 3.5) and 1.5 mL of aqueous solution of TBA were added to 0.2 mL of tissue homogenate/ serum. The mixture was made up to 4 mL with distilled water and then heated in a water bath at 95°C for 60 mins in a condenser. After cooling under tap water, 1 mL of distilled water and 5 mL of n-butanol: pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 mins, the organic layer was
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taken and its absorbance at 532 nm was measured against a butanol blank. TMP was used as the internal standard. Lipid peroxide levels were expressed as mM of malondialdehyde produced.

5. Estimation of Hydroperoxide

Hydroperoxides were estimated by the iodometric method of Mair and Hall (1977).

Reagents

1. Tris-HCl buffer (0.1 M), pH 7.5
2. Chloroform: methanol (2:1 v/v mixture)
3. Acetic acid: chloroform (3:2 v/v mixture)
4. Potassium iodide (KI): 6 g KI in 5 mL ice cold distilled water.
5. Cadmium acetate: 0.5% (0.5g in 100 mL water)

Procedure

Weighed tissues were homogenized in 0.1 M Tris-HCl buffer, pH 7.5 and allowed to stand for 5 mins. The supernatant was used for the estimation of hydroperoxides. 1 mL of the tissue homogenate was mixed thoroughly with 5 mL of chloroform: methanol (2:1) followed by centrifugation at 1000 g for 5 mins to separate the two phases. 3 mL of the lower chloroform layer was recovered using a syringe and placed in a water bath at 45°C under a stream of nitrogen. 1 mL of acetic acid: chloroform (3:2) mixture followed by 0.05 mL of KI was quickly added and the test tubes were stoppered and mixed. The tubes were kept in the dark at room temperature for exactly 5 mins, followed by the addition of 3 mL of cadmium acetate. The solution was mixed and centrifuged at 1000 g for 10 mins. The absorbance of the upper phase was read at 353 nm against blank containing the
complete assay mixture except the tissue homogenate. Molar extinction coefficient of hydroperoxide is $1.73 \times 10^{-4} \text{ m}^{-1} \text{ cm}^{-1}$.

6. Estimation of Conjugated dienes

Conjugated dienes were estimated according to the method of Recknagel and Ghoshal (1966).

Reagents

1. Tris-HCl buffer (0.025 M) pH 7.5  
2. Chloroform: methanol (2:1)  
3. Cyclohexane

Procedure

Tissue was homogenized in Tris-HCl buffer. Centrifuged and 1 mL of the supernatant was mixed with 5 mL of chloroform: methanol (2:1). Centrifuged for 5 mins at 1000 g. The upper layer was removed by aspiration and 3 mL of the lower layer was taken in a test tube and evaporated to dryness by placing in a water bath at $45^\circ \text{C}$. Dissolved the residue left after evaporation in 3 mL cyclohexane and absorbance was read at 233 nm against cyclohexane blank. The conjugated dienes have a molar extinction coefficient of $2.52 \times 10^{-4} \text{ m}^{-1} \text{ cm}^{-1}$.

7. Estimation of protein carbonyls

Protein carbonyls were estimated using the method of Abraham and Packer (1993).

Reagents

1. 2.5 M HCl  
2. 10 mM DNPH in 2.5 M HCl
3. 20% TCA
4. 10% TCA
5. Ethanol: ethyl acetate (1:1 v/v)
6. 6 M guanidine HCl containing 20 mM potassium phosphate. Adjusted pH to 2.3 with con. HCl

Procedure

Proteins were precipitated with 20% TCA and centrifuged. The precipitate was resuspended in 2, 4 DNPH and vortexed for 10 mins intervals for 2 h at room temperature. The pellets were washed thrice with ethanol-ethyl acetate to remove the free reagent before centrifugation. The pellets were then resuspended in 0.6 mL of guanidine hydrochloride, incubated at 37°C for 15 mins and centrifuged at 5000 g for 3 mins. The absorbance of the supernatant was measured photometrically at 366 nm for carbonyl content and calculations were performed with an extinction coefficient value of 22,000 M⁻¹cm⁻¹.

8. Assay of Superoxide dismutase (SOD) [E.C.1.15.1.1]

SOD activity was determined by the method described by Kakkar et al. (1984).

Reagents
1. Sucrose (0.25 M)
2. Ammonium sulphate (90%)
3. Tris-HCl buffer (0.0025M, pH 7.4)
4. Sodium pyro phosphate buffer (pH 8.3, 0.052M)
5. Phenazine methosulphate (1.2 µM)
6. Nitro blue tetrazolium (NBT) (300 µM)
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7. NADH (780 µM)

8. n-butanol

Procedure

Tissues were homogenized in 0.25 M sucrose and differentially centrifuged under cold conditions to get the cytosol fraction. Before estimation of SOD, the protein was precipitated from the supernatant with 90% ammonium sulphate. The precipitated proteins were removed by centrifugation. The SOD in the supernatant fraction was dialysed overnight in Tris-HCl buffer (0.0025 M, pH 7.4) and freed from (NH₄)₂SO₄. The supernatant was used as the enzyme source.

The assay mixture contained 1.2 mL sodium pyrophosphate buffer, 0.1 mL of 1.2 µM phenazine methosulphate, 0.3 mL of NBT, 0.2 mL NADH approximately diluted enzyme preparations and water in a total volume of 3 mL. Reaction was started by the addition of NADH. After incubation at 30°C for 90 sec, the reaction was stopped by the addition of 1 mL glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4 mL of n-butanol. The mixture was allowed to stand for 10 mins, centrifuged and butanol layer was taken out. Colour intensity of the chromogen in the butanol fraction was measured at 560 nm against butanol. A system devoid of the enzyme served as control.

One unit of the enzyme activity is defined as the enzyme concentration required to inhibit the chromogen production by 50% in 1 min under the assay conditions.

9. Assay of Catalase [EC.1.11.1.6]

The enzyme activity was assayed by the method of Maehly and Chance (1954).
Reagents

1. M/15 Phosphate buffer pH 7.00: Dissolved 3.52 g of potassium dihydrogen phosphate and 7.27 g of disodium hydrogen phosphate in double distilled water and made up to 100 mL.

2. \( \text{H}_2\text{O}_2\)-phosphate buffer (M/15 phosphate buffer \(1.25 \times 10^{-2}\) \(\text{H}_2\text{O}_2\) pH 7.00): Diluted 0.16 mL \(\text{H}_2\text{O}_2\) (30 % w/v) to 100 mL with buffer. The OD of the solution was about 0.5 at 240 nm with 1 cm light path.

Procedure

The enzyme extract was prepared by homogenizing the tissue in phosphate buffer and centrifugation at 5000 g. The reaction mixture contained phosphate buffer, 2 mM \(\text{H}_2\text{O}_2\) and the enzyme extract. The decrease in the absorbance at 230 nm was measured spectrophotometrically. Unit is defined as the velocity constant /second.

10. Glucose 6- phosphate dehydrogenase [EC 1.1.1.49]

The enzyme was assayed by the method of Kornberg and Horecker (1955)

Reagents

1. 0.02 M Glucose-6-phosphate
2. \(1.5 \times 10^{-3}\) M NADP
3. 0.1 M \(\text{MgCl}_2\)
4. 0.04 M Glycylglycine buffer (pH 7.5)

The chilled tissues were homogenized with three volumes of 0.04 M glycylglycine buffer pH 7.5. The homogenate was centrifuged at 2000 g at 0°C for 10 mins. The supernatant was used as enzyme source.
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Procedure

To 1 mL of the substrate in a quartz cell, added 0.1 mL of NADP, 0.25 mL buffer and 0.2 mL MgCl₂. To this, 0.05 mL of enzyme was added and the absorbance was read at 340 nm at 1 min interval. One unit of the enzyme was defined as that amount which causes an initial change of optical density of 0.1/min under the above conditions of assay.

11. Malic enzyme [EC.1.1.1.40]

The activity was assayed by the method of Ochoa (1955).

Reagents

1. 0.25 M Glycylglycine buffer, pH-7.4
2. 0.05 M Manganese chloride
3. 0.00675 M NADP
4. 0.03 M L-Malate, pH-7.5

The chilled tissues were homogenized with three volumes of 0.25 M glycylglycine buffer, pH 7.4 at 0°C and supernatant obtained by centrifuging at 2000 rpm at 0°C for 10 mins was used as the enzyme.

Procedure

The reaction mixture in a quartz cell consisted of 0.3 mL buffer, 0.06 mL of manganese chloride, 0.2 mL NADP, 0.05 mL of L-malate, enzyme and water to a final volume of 3.0 mL. The assay was carried out at room temperature (23-25°C). The reaction was started by the addition of either malate or enzyme and the optical density (OD) at 340 nm was read against a blank containing all the components except NADP, at intervals of 15 sec, for 1-2 mins. One unit of the enzyme was
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defined as that amount which causes an initial change of optical density of 0.01/ min under the above conditions of assay.

12. Acid and Alkaline phosphatase

The activity of acid phosphatase and alkaline phosphatase was assayed by the method described by Kornberg and Horecker (1955).

Reagents

1. Alkaline Buffer: Mixed 7.5 g glycine, 0.095 g magnesium chloride, 750 mL water and 85 mL 1 N NaOH, diluted with 1 L water.

2. Stock substrate: Dissolved 0.4 g p-nitrophenyl disodium phosphate in 100 mL water.

3. Alkaline buffered substrate: Mixed equal volumes of solution 1 and 2, adjusted pH to 10.3 to 10.4, dispensed 1 mL into test tubes, stoppered and stored in freezer.

4. 0.02 N sodium hydroxide: Dissolved 0.8 g NaOH pellets in 1L water.

5. Conc HCl

6. Acid Buffer: Mixed 18.90 g citric acid, 180 mL 1N NaOH and 100 mL 0.1 N HCl diluted to 1 L with water.

7. Stock substrate same as reagent 2.

8. Acid buffered substrate: Mixed equal volumes of 6 and 7, adjusted the pH to 4.8-4.9, dispensed and stored as reagent.

9. 0.1 N NaOH: Dissolved 4 g NaOH in 1 L water.
12.1. Alkaline phosphatase [EC.3.1.3.1]

Procedure

1 mL alkaline substrate was incubated at 37°C in a water bath for 5 mins. Then added 0.1 mL serum/tissue homogenate. For the blank, 0.1 mL water was added. Then incubated in the water bath for exactly 30 mins. Added 10 mL, 0.02 N NaOH and mixed by inversion. Read the optical density against the blank at 415 nm. Added concentrated HCl to each tube and mixed by inversion. Read samples against blank at 415 nm. The difference between the first and second optical densities was recorded and the activity was expressed as μmoles of p-nitrophenol liberated/min/100 mg protein.

12.2. Acid Phosphatase [EC.3.1.3.2]

Procedure

0.1 mL acid substrate was incubated at 37°C in a water bath for 5 mins to bring the solution to incubation temperature. Then added 0.1 mL serum/seminal plasma/tissue homogenate to each tube for samples and 0.1 mL water to the tube for blank. Then incubated in the water bath for exactly 30 mins. During the incubation period prepared the serum blanks for each sample by mixing 5 mL of 0.1 N NaOH and 0.2 mL serum/seminal plasma/tissue homogenate. After incubation added 4 mL 0.1 N NaOH to each tube and mixed by inversion. Read OD against blank at 415 nm. Read blanks against 0.1 N NaOH. Subtracted blank reading from sample readings. A standard curve was prepared by following the whole procedure for various dilutions of the working standard solution. Activity was expressed in terms of μmoles of p-nitrophenol liberated/min/mg protein.
13. Assay of Glutathione peroxidase [EC1.11.1.9]

The activity of glutathione peroxidase was determined by the method of Lawrence and Burk (1976) as modified by Agergaard and Jensen (1982).

Reagents

1. Sucrose (0.25 M)
2. Sodium azide (1 mM)
3. Reduced glutathione (1 mM)
4. Phosphate buffer (50 mM)
5. EDTA (1.5 mM)
6. 1 mM NADPH: 8 mg in 1 mL 1% NaHCO₃

Procedure

Tissue homogenate was prepared in 0.25 M sucrose, centrifuged at 1000 g for 30 mins and the supernatant fraction was used for assay. Assay system contained 1.5 mL phosphate buffer, 0.2 mL EDTA, 0.3 mL sodium azide, 0.1 mL reduced glutathione, 0.1 mL NADPH and the enzyme homogenate (1.0 µM/ mL). Optical density was measured at 340 nm at 20 sec intervals. Enzyme activity is defined as µ moles of NADPH oxidized/min/mg protein using 0.25 mM H₂O₂ as substrate.

14. Assay of Glutathione reductase [EC1.6.4.2]

Glutathione reductase was assayed by the method of David and Richard (1983).

Reagents

1. Sucrose (0.25 M)
2. Phosphate buffer (0.12 M)
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3. EDTA (1.5 mM)
4. Oxidized glutathione (65.3 mM)
5. NADPH (9.6 mM)

Procedure

Tissue homogenate was prepared in sucrose, centrifuged at 10,000 g for 30 mins and the supernatant was used for assay. The reaction mixture contained phosphate buffer (92.6 µL), EDTA (0.1 mL), oxidized glutathione (0.1 mL) and enzyme extract (0.1 mL). To this, 0.05 mL of NADPH was added and the absorbance was read at 340 nm at 20 sec intervals for 3 mins. The activity is expressed as µmoles of NADPH oxidized/min/mg protein.

15. Assay of Glutathione S-transferase (GST) [EC 2.5.1.18]

Glutathione –S-transferase was assayed by the method of Habig et al. (1974).

Reagents

1. 0.3 M phosphate buffer (pH 6.5)
2. 30 mM reduced glutathione-Dissolved 100 mg of GSH in 1 mL of distilled water.
3. 30 mM 1-chloro-2, 4 dinitrobenzene (CDNB) - Dissolved 60.6 mg of CDNB prepared in 1 mL of 95% alcohol.

Procedure

Tissue was homogenized in phosphate buffer (pH-6.5) and centrifuged at 1, 05,000 g and the supernatant after appropriate dilution was used as the enzyme source. 1 mL of phosphate buffer, 0.1 mL CDNB and 0.1 mL enzyme were taken in a quartz cuvette. The volume was adjusted to 2.9 mL with distilled water. The
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reaction mixture was preincubated at 37°C for 5 mins. The reaction was started by the addition of 0.1 mL of GSH. The absorbance was followed for 5 mins at 340 nm. The reaction mixture without enzyme was used as blank. One unit of the enzyme is the amount required to conjugate 1 µmol of the substrate with glutathione in 1 min.

16. Assay of β-Hydroxy methyl glutaryl CoA reductase (HMG CoA reductase) [EC.1.1.1.34]

HMG CoA reductase activity of testis was estimated as described by Rao and Ramakrishnan (1975), by determining the ratio of HMG CoA to mevalonic acid.

Reagents

1. Saline arsenate: 1g of sodium arsenate per litre of physiological saline
2. Dilute Perchloric acid: 50 mL/L
3. Hydroxylamine hydrochloride reagent: 138.98 g/L
4. Hydroxylamine hydrochloride reagent for mevlonate: Equal volumes of Hydroxylamine hydrochloride reagent and water were mixed. Prepared freshly before use.
5. Hydroxylamine hydrochloride reagent for HMG CoA: Equal volumes of Hydroxylamine hydrochloride reagent and NaOH solution (180 g/L) were mixed. Prepared freshly before use.
6. Ferric chloride reagent: 5.2 g of trichloroacetic acid (TCA) and 10 g of FeCl₃ were dissolved in 50 mL 0.65 N HCl and diluted to 100 mL with water.
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Procedure

Equal volumes of fresh 10% tissue homogenate and dilute perchloric acid were mixed, kept for 5 mins and centrifuged at 2000 g for 10 mins. To 1 mL of the supernatant, 0.5 mL of freshly prepared hydroxylamine hydrochloride reagent was added and mixed. After 5 mins, 1.5 mL of FeCl₃ was added and mixed well. The optical density was read after 10 mins at 540 nm against a similarly treated saline arsenate blank. The ratio between HMG CoA and mevalonate is taken as index of activity of enzyme which catalyses the conversion of β-hydroxy methyl glutaryl CoA to mevalonate. Lower the ratio, higher the enzyme activity.

17. Extraction of tissue for lipid estimation

The tissue was extracted according to the procedure of Folch et al. (1957).

The tissue was homogenized and extracted with chloroform: methanol (2:1). The mixture was incubated at 50°C for 15 mins. It was filtered and the residue washed with chloroform: methanol (2:1) at least 3 times. The filtrates were combined. To the filtrate, 0.7% KCl (20% of the total volume of the extract) was added and mixed. The aqueous upper phase was removed with a pasteur pipette and the lower layer was washed three times with chloroform: methanol: KCl (2:48:47) solution. The washed lower layer of chloroform was evaporated to dryness and the residue was redissolved in a known volume of chloroform. Aliquots of the extract were used for the estimation of various lipids.

17.1. Estimation of cholesterol

The cholesterol was estimated by the method of Abell et al. (1952).
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Reagents

1. Potassium hydroxide (33%)
2. Absolute alcohol
3. Ethanolic KOH: 6 mL of 33% KOH in water was added to 94 mL of absolute ethanol.
4. Petroleum ether (60-80°C)
5. Color reagent: 20 mL of acetic anhydride was cooled in ice; 1 mL of conc. H₂SO₄ was added to this with shaking. It was again cooled for 10 mins and 10 mL of glacial acetic acid was added and allowed to attain room temperature.

Procedure

An aliquot of the two lipid extract was pipetted out in a glass stoppered centrifuge tube and was evaporated to dryness. 5 mL of ethanolic KOH was added, stoppered and was shaken well. It was then warmed in a water bath at 37-40°C for 55 mins. After cooling to room temperature, 10 mL of petroleum ether was added and mixed. 5 mL of water was then added and shaken vigorously for 1 min. It was then centrifuged at a low speed for 5 mins. 4 mL of petroleum ether layer was pipetted into a test tube and evaporated to dryness at 60°C. A standard was also treated in the same manner. 6 mL of color reagent was added to each tube and kept at 25°C after thorough shaking. 6 mL of color reagent was taken as a blank. After 30 mins, the optical density was read at 620 nm.
17.2. Estimation of Triglycerides

Triglycerides were estimated by the method of Van Handel and Zilversmith (1957) with the modification that flurosil was used to remove phospholipids.

Reagents

1. Chloroform
2. Flurosil
3. 0.4% Ethanolic KOH
4. 0.2 N H$_2$SO$_4$
5. 0.05 M Sodium metaperiodate
6. 0.5 M Sodium arsenite
7. Chromotropic acid: 2 g of chromotropic acid was dissolved in 200 mL distilled water. 600 mL of concentrated sulphuric acid was added slowly to 300 mL of chilled distilled water. This chilled and diluted acid was then added to the chromotropic acid solution.
8. Glycerol: 9mg/mL

Procedure

2 g of flurosil was taken in a glass stoppered tube. 3 mL of chloroform was added. An aliquot of the extract was layered on the top of flurosil and mixed. Chloroform was then added to make total volume to 10 mL. It was then stoppered, shaken intermittently for about 10 mins and then filtered through a filter paper. After filtration, 1mL of the filtrate was pipetted into each of the three tubes. 1 mL working standard of glycerol was pipetted into each of the three tubes. The solvent was evaporated at 60-70°C. 0.5 mL ethanol was added to the third tube (unsaponified
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sample). The tubes were then closed and kept at 60-70°C for 15 mins. 1 mL of glycerol standard and 1 mL water was taken as the blank. 0.5 mL of 0.2 N H₂SO₄ was added to each of the tubes and placed gently in a boiling water bath for 15 mins to remove alcohol. They were then cooled to room temperature. 0.1 mL of sodium metaperiodate was added to each tube and kept for 10 mins. 0.1 mL of sodium arsenite was then added to each tube and mixed. The tubes were closed and then heated in a boiling water bath for 30 mins. They were then cooled and absorbance was read at 570 nm.

17.3. Estimation of Free fatty acids

Free fatty acids were estimated according to the procedure of Falholt et al. (1973).

Reagents

1. Extraction solvent: chloroform: heptane: methanol (5:5:1)
2. 33 mM Phosphate buffer (pH 6.4): 2 volumes of KH₂PO₄ (4.53 g/L) and 1 volume Na₂HPO₄ (5.93 g/L) were mixed.
3. 500 mM stock copper solution: 12.07 g Cu(NO₃)₂·3H₂O made upto 100 mL
4. 1 M Triethanolamine
5. 1 M NaOH
6. Copper Reagent: 10 mL of copper solution, 10 mL of triethanolamine and 6 mL of 1N NaOH were mixed and diluted to 100 mL. 33 g NaCl was added and the pH was adjusted to 8.1.
7. Diphenyl carbazide solution: 4 g/L in ethanol. Prepared immediately before use by adding 40 mg in 10 mL ethanol with 0.1 N triethanolamine solution.

8. 2 mM Standard palmitic acid: 51.2 mg palmitic acid in extraction solvent and made upto 100 mL. Stored in a tightly stoppered container.

9. Working standard: 5 mL stock solution made upto 20 mL with extraction solvent to give solution containing 500 µM.

**Procedure**

0.1 mL sample was evaporated to dryness at 60-65°C in a water bath. Then 1 mL phosphate buffer, 6 mL extraction solvent and 2.5 mL copper reagent were added. Blank contained 1 mL phosphate buffer, 6 mL extraction solvent and 2.5 mL copper reagent. 0.2 mL of the working standard was pipetted out into a test tube and added 1 mL phosphate buffer, 5.8 mL extraction solvent and 2.5 mL copper reagent. Then the solution was transferred to a stoppered tube and shaken vigorously for 90 sec. It was allowed to stand for 15 mins, centrifuged at 4000 g for 5 mins and the supernatant was transferred (0.1 mL) was transferred to a tube containing 0.5 mL diphenyl carbazide solution. The contents were mixed carefully, diluted with 5 mL of the extraction solvent. The optical density was read after 5 mins at 550 nm.

**17.4. Estimation of phospholipids**

Phospholipids were estimated according to the method of Zilversmith and Davis (1950).

**Reagents**

1. 2 N HNO₃
2. 5 N H₂SO₄
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3. 2.5% Ammonium molybdate

4. ANSA reagent: a mixture of 1.2 g sodium metabisulphite, 1.2 g sodium sulphite and 0.2 g 1-amino-2-naphtha-4-sulphonic acid were powdered and dissolved in 100 mL distilled water.

5. KH$_2$PO$_4$ standard solution

Procedure

An aliquot of the extract was taken in a digestion tube and evaporated to dryness in a warm water bath. Added 1 mL of 5 N H$_2$SO$_4$ and digested for 3 hr. When charring occurred, one drop of 2 N HNO$_3$ was added and digested till it became colourless. Cooled and added 1 mL of water and kept in boiling water bath for 5 mins. Pipetted out different volumes of standard and made up to 1 mL with water. Blank containing 1 mL water was also taken. Then added 1 mL of 5 N H$_2$SO$_4$ and 1 mL of 2.5% ammonium molybdate solution followed by 0.1 mL ANSA reagent to all tubes. Mixed well and added 6.9 mL of water and read the optical density within 10 mins at 660 nm against the blank.

18.1. Estimation of Hepatic bile acids

The bile acids were extracted from liver according to the procedure of Okishio et al. (1967).

Reagents

1. 95% (v/v) ethanol containing 0.1% (v/v) of ammonium hydroxide.

2. HCl

3. Chloroform : methanol (1:1)

4. Ethyl acetate: Conc. Sulphuric acid (15:1)
5. 2% vanillin in ethanol

6. 77% phosphoric acid

**Procedure**

The tissue was homogenized with 95% (v/v) ethanol containing 0.1% (v/v) of ammonium hydroxide and was refluxed for 30 mins. After filtration, the residue was reextracted twice with the same volume of solvent and filtrate. The combined filtrate was then concentrated in vacuum, made alkaline (pH 10) by the addition of sodium hydroxide and equal volume of water was added and extracted with petroleum ether to remove neutral sterols. The aqueous solution was acidified with HCl to pH 2, and extracted with chloroform: methanol (1:1) three times. The chloroform layer after washing once with a little water was dried over anhydrous sodium sulphate. After filtration and evaporation, the bile acids were dissolved in a known volume of chloroform and aliquots were taken for the estimation of bile acids. The bile acids were estimated according to the procedure described by Palmer (1967).

**18.1.1. Estimation of Cholic acid**

Evaporated an aliquot (0.5 or 1 mL) of the sample containing 0.02-0.2 g of bile acid to dryness. Then added 0.2 mL of 2% solution of vanillin in ethanol to all tubes except the blank and again evaporated to dryness. Added 5 mL of 77% phosphoric acid (Sp gr. 1.625) and heated for 10 mins at 50°C. 5 mL phosphoric acid was taken as blank. Read the colour against the reagent blank at 465 nm.
18.1.2. Estimation Chenodeoxy cholic acid

Evaporated 0.5 mL aliquot containing 0.2-1.5 mg chenodeoxy cholic acid to dryness at 100°C. Cooled to room temperature and added 3 mL of ethyl acetate: con. H₂SO₄ (50:1) to blank, standard and test. Mixed well. Kept for 2-5 mins and read at 600 nm.

19. Extraction and estimation of 17-ketosteroids

By the method of Gibson and Robbie (1943).

Reagents

1. Carbon tetrachloride
2. Con HCl
3. Absolute ethanol
4. Androsterone as standard- 50mg/mL
5. Dinitrobenzene-2%
6. 2.5 N KOH

Procedure

Extraction of ketosteroids from urine: A 24 hr urine sample was collected from the control and experimental rats. 10 mL of the urine in a 100 mL conical flask were treated with 1 mL of con HCl and boiled for 10 mins under a reflux condenser. The flask was cooled and 3 mL of CCl₄ were added to the condenser. The mixture was again boiled for 10 mins. It was cooled and transferred to a separating funnel and CCl₄ was allowed to run off. 2 mL of additional CCl₄ were added and the mixture well swirled, not shaken. The CCl₄ was allowed to separate and was drained. 1 mL of fresh CCl₄ was added, the mixture well swirled again, separated
and the CCl₄ layer removed. The first and second extractions were combined. 6 mL of combined CCl₄ extract was washed in a clean separating funnel with 2 mL of water, then with NaOH to remove acid and phenols and finally with 2 mL of water.

The washed CCl₄ extract was carefully evaporated to dryness in 100 mL flask on a water bath. The residue was dissolved in 2 mL of absolute alcohol added to the warm flask which was stoppered and shaken by swirling to dissolve the residue.

**Colorimetric estimation**

Took 0.2 mL of alcoholic extract as test, 0.2 mL of standards and 0.2 mL of alcohol were taken as blank. To this, added 0.2 mL of alcoholic solution of 2% dinitrobenzene and 0.2 mL of alcoholic 2.5 N of KOH. The tubes were stoppered and placed in a beaker of water at 25°C for 1 hr in a dark room. 5 mL of absolute alcohol was added to each of the tubes and the contents were mixed. OD was read at 620 nm.

20. **Assay of 3β-Hydroxysteroid dehydrogenase (3β-HSD) (EC.1.1.1.145)**

By the colorimetric assay of Shivanandappa and Venkatesh (1997)

**Reagents**

1. Phthalate buffer (50 mM, pH 3): 2.55 g potassium hydrogen phthalate dissolved in a mixture of 51 mL N/10 HCl and 2.5 mL Tween 20. pH was adjusted and then made up to 250 mL with HCl.

2. Tris HCl buffer: 0.1 M, pH 7.8

3. NAD: 5 mM
4. Color reagent: 40 mg INT (Iodonitro tetrazolium chloride) 10 mg PMS and 0.5 mL Tween 20 were dissolved in 50 mL water (used for the standard curve).

5. Substrate: The pregnenolone or DHEA (Dehydroepiandrosterone) was first dissolved in 0.3 to 0.5 mL dimethyl formamide (DMF) and was prepared in 50 or 100 mL Tris HCl buffer (0.1 M, pH 7.8).

Procedure

**Standard curve:** 1 M solution of NADH was dissolved in water. Different concentration (0 to 150 mL) was reacted with 0.5 mL color reagent and after the color was formed, 2 mL phthalate buffer was added and absorbance was read at 490 nm.

**Enzyme assay:** Tissue was homogenized in 0.1M Tris HCl and centrifuged at 12,000 g at 40°C. To 0.05 mL homogenate added 1 mL NAD, 1 mL DHEA and 0.9 mL water and incubated for 1 hr at 37°C. The reaction was stopped by the addition of 2 mL phthalate buffer. The turbidity was removed by centrifugation at 3000 rpm for 20 mins. Supernatant was read at 490 nm. 1 unit of enzyme activity was equivalent to the absorbance/mg protein.

21. Assay of 17β-Hydroxysteroid dehydrogenase (17β-HSD) (EC.1.1.1.51)

17β-HSD activity was determined by the method of Jarabak et al. (1969).

**Reagent**

1. Sodium pyrophosphate buffer, pH 10.2 (440 µL)
2. Crystalline BSA (25 mg)
3. Testosterone (0.3 µL)
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4. 1.1 μmol NADP

Procedure

The testicular supernatant (1 mL) was mixed with 440 μL of sodium pyrophosphate buffer (pH 10.2), 25 mg crystalline BSA and 0.3 μL of testosterone. The total volume was made up to 3 mL. Enzyme activity was measured after the addition of 1.1 μmole NADP to the incubation mixture in a spectrophotometer at 340 nm. Blank contained all the reagents except NADP. One unit of enzyme activity was equivalent to a change in absorbance of 0.001/min at 340 nm.

22. Estimation of Glycogen

The glycogen was estimated by the method of Carroll et al. (1956)

Reagents

1. 5% trichloro acetic acid
2. 95% ethanol
3. Anthrone reagent: 10.5 % anthrone and 1% thiourea in 72 % by volume of H₂SO₄.

Procedure

The tissue was homogenized at 0°C with appropriate volume of 5% TCA (3 mL). The homogenate was centrifuged at 0°C at 2000 g for 15 mins and the supernatant was filtered through an acid washed filter paper. Two more extractions were made in the same manner. The filtrate was made up to a known volume (10 mL) with 5% TCA. The final volume was adjusted in such way so as to contain 10 to 200 μg of glycogen/mL.
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To an aliquot of the TCA filtrate (1 mL), added 5 volumes of 95% ethanol. The tubes were capped with clean rubber stoppers and were allowed to stand overnight at room temperature. After precipitation was completed the tubes were centrifuged at 3000 g for 15 mins. The supernatant was decanted off and the tubes were allowed to drain in an inverted position for 10 mins. The glycogen was dissolved in water (2 mL). The reagent blank, 2 mL water and a standard 2 mL of glucose containing 100 µg of glucose were also prepared. 10 mL of anthrone reagent was added into each tube with constant mixing. When the tubes reached the temperature of the cold water, they were immersed in a boiling water bath for 15 mins and then removed to a cold water bath. Cooled to room temperature and read the OD at 620 nm.

23. Estimation of Sialic acid

Sialic acid was estimated by the TBA methods of Warren (1959)

Reagents

1. 0.2 M Sodium meta periodate in 9 M phosphoric acid.
2. 10% sodium arsenite in 0.5 M sodium sulphate solution
3. 0.6 % 2-thiobarbituric acid (TBA) in 0.5 M sodium sulphate (slightly heated to dissolve TBA in Na₂SO₄)
4. Cyclohexane

Procedure

Sample (0.1 mL) hydrolyzed in 0.2 N Na₂SO₄ at 80°C for 1 h. 2 mL water was taken as blank. 1 mL of periodate solution was added to each tube and carefully mixed. It was allowed to stand at room temperature for 20 mins and 1mL of sodium arsenite was then added to this. The tubes were then shaken till the yellow colour
disappeared. 3 mL of thiobarbituric acid solution was added to each tube and the contents were mixed. The tubes were closed and heated vigorously in a boiling waterbath for 15 mins and then cooled in waterbath for 5 mins. 5mL cyclohexanone was added to each tube and shaken vigorously in order to extract the red colour into organic phase. The tubes were centrifuged for about 3 mins at 1000 rpm. The clear upper layer was transferred and the absorbance was read at 549 nm.

24. Determination of fructose in seminal plasma

Seminal fructose content was determined by the method of Karvonen and Malm (1955).

Reagents

1. 1.8% Zinc sulphate (ZnSO₄·7H₂O)
2. 0.1 M Sodium hydroxide
3. Indole reagent: 200 mg of benzoic acid was added to 100 mL of distilled water and dissolved. Repeated shaking in a hot water bath (about 60°C). When all benzoic acid was dissolved, 25 mg of indole was added. The solution was filtered and stored at 4°C.
4. Stock fructose standard (2.8 mM): 50.4 mg of fructose dissolved in 100 mL of distilled water.
5. Working standard: On the day of analysis, one portion of the stock standard was diluted to 0.28 mM and 0.14 mM respectively.

Seminal plasma preparation: Diluted seminal plasma 1:50 by adding 0.1 mL of seminal plasma to 4.9 mL of distilled water. Added 1 mL of diluted seminal plasma to centrifuge tube. Added 0.3 mL of 1.8% zinc sulphate. Mixed and added
0.2 mL of NaOH, allowed the tubes to stand for 15 mins and centrifuged at 2000 g for 20 mins. 0.5 mL of the clear supernatant was used for analysis.

**Procedure**

Took four glass tubes. Into the first, added 0.5 mL supernatant from the diluted and deprotenized seminal plasma, into the second, 0.5 mL of 0.28 mM working fructose standard, into the third, 0.5 mL of distilled water (blank). Added to each tube, 0.5 mL of indole reagent and 5 mL of conc HCl. Stoppered the glass tubes and incubated for 20 mins at 50°C. Cooled in ice water to room temperature and read the absorbance at 470 nm.

25. Measurement of Neutral α-Glucosidase in seminal plasma

Neutral α-glicosidase in seminal plasma was measured by the method of Cooper et al. (1990).

**Reagents**

1. Buffer 1 (0.2 mol/L phosphate, pH 6.8): Dissolved 4.56 g K$_2$HPO$_4.3H_2O$ in 100 mL of purified water. Dissolved 2.72 g of K$_2$PO$_4$ in a separate 100 mL aliquot of purified water. Mixed approximately equal volumes of each until the pH is 6.8.

2. Buffer 2: Dissolved 1 g of SDS in 100 mL of buffer 1. SDS will precipitate on storage at 4°C, but redissolves on gentle warming.

3. Colour reagent 1 (for stopping the reaction, 0.1 mol/L sodium carbonate): Dissolved 6.20 g of Na$_2$CO$_3.H_2O$ in 500 mL of water.

4. Colour reagent 2: Dissolved 0.1 g of SDS in 100 mL of colour reagent 1.

5. Substrate p-nitrophenol glucopyranoside (PNPG) (5 mg/mL): Dissolved 0.1 g of PNPG in 20 mL of buffer 2 and warmed the solution on a
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hotplate at about $50^\circ C$ with stirring for about 10 mins. A few crystals may remain undissolved. The solution kept at $37^\circ C$ during use. Prepared fresh solution for each assay.

6. Standard curve of product p-nitrophenol (PNP) (5 mmol/L): Dissolved 69.5 mg of PNP in 100 mL of purified water, warmed the solution if necessary. Stored at $4^\circ C$ in the dark in aluminium foil-covered or brown glass bottle.

Procedure

Centrifuged the semen sample for 10 mins at 1000 g. Decanted and stored the sperm-free seminal plasma at $-20^\circ C$ until analysis. Thawed the sperm-free seminal plasma and mixed well on a vortex mixer. After thawing the sample, seminal plasma was diluted 1:5 with 100 mM phosphate buffer, pH 6.8, and centrifuged for 15 mins at 10 000 g. Then 10 µL diluted seminal plasma were placed into a microtiter plate well and 100 µL of 0.5% (w/v) 4-nitrophenyl-α-D-glucopyranoside (PNPG) and 1% (w/v) SDS in 100 mM phosphate buffer, pH 6.8, were added, mixed well, and incubated at $37^\circ C$ for 4 hours. Finally, 100 µL of 0.2 M Na$_2$CO$_3$ were added to each well, including the blanks, and the samples were measured in duplicate against standards of p-nitrophenol at 405 nm using an EAR microtiter plate reader.

One international unit (IU) of glucosidase activity is defined as the production of 1 µmol of product (PNP) per min at $37^\circ C$. 
26. **Assay of Sorbitol dehydrogenase [EC.1.1.1.14]**

Activity of sorbitol dehydrogenase was determined by the method described by Wootten (1964).

**Reagents**

1. 0.05 M Tris buffer, pH 8.8: 1.21 g tris hydroxymethyl aminomethane dissolved in water, 16 mL 0.1 N HCl and made upto 200 mL with H$_2$O.
2. 0.1 M D-sorbitol per mL of buffer.
3. 5 mg NAD per mL of buffer.
4. 10% aqueous trichloro acetic acid.
5. Resorcinol-thiourea reagent: Dissolved 0.1 g resorcinol and 0.25 g thiourea in 100 mL glacial acetic acid and stored in an amber colored bottle and discarded when discolored.
6. 30% HCl: Mixed well 850 mL HCl of specific gravity 1.18 and 1.5 mL of H$_2$O.
7. Colour reagent: Mixed well in the proportions of 7:1, 30% HCl, resorcinol-thiourea reagent and H$_2$O, prepared fresh daily.
8. Standard fructose: Dissolved 16.2 g fructose in 1L of 0.25% benzoic acid.

**Procedure**

Into the two tubes labeled test and control, pipetted 0.3 mL of testicular homogenate and 0.2 mL coenzyme solution and allowed to attain $37^\circ$C in the water bath. Added 0.1 mL of substrate to the test only and incubated for exactly 30 mins. Added 0.1 mL of substrate to control tube, 0.4 mL TCA to both, mixed well and
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centrifuged. To 0.5 mL of supernatant, added 4.5 mL of colour reagent and heated at 75°C for exactly 15 mins. Treated tubes containing 0.5 mL of standard and 0.5 mL of water were also run simultaneously in the same manner as the assay of supernatant aliquots. The enzyme activity was expressed as mg of fructose liberated/mL.

27. Assay of Lactate dehydrogenase [EC 1.1.1.27]

Lactate dehydrogenase activity in the testis was measured by the method of Bergmeyer (1974). Enzyme was extracted by homogenization, in isotonic saline until no solid macroscopic fragments remained. The resultant suspension was centrifuged for 5 mins at 2000 g and the supernatant was used for assay.

Reagents

1. Phosphopyruvate
2. 11.3 mM NADH

Procedure

To 3.5 mL of phosphopyruvate, 0.05 mL coenzyme (11.3 mM NADH) and 0.10 mL of supernatant were added. Blank tubes were run simultaneously without addition of NADH. The tubes were vortexed and optical density read at 60 sec interval for 3 to 5 mins at 340 nm and 360 nm. The enzyme activity was expressed as μmoles of NAD oxidised/ min/mg protein.

28. Assay of Alcohol dehydrogenase (ADH) [EC 1.1.1.1]

ADH activity was assayed in testis and liver by the method of Koivisto and Salaspuro (1996).
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Reagents

1. 100 mM glycine buffer, pH 9.6
2. 1 mM NAD
3. 25 mM ethanol

Procedure

The testicular and liver supernatant was mixed with 100 mM glycine buffer, pH 9.6, 1 mM NAD, and 25 mM of ethanol. NADH formation was measured spectrophotometrically at 340 nm at 25°C. One unit of the enzyme activity is defined as the enzyme concentration required to convert 1.0 µmole of ethanol to acetaldehyde per min at pH 8.8 at 25°C.

29. Assay of Acetaldehyde dehydrogenase (ALDH) [EC 1.2.1.3]

ALDH was determined by the activity of Canuto et al. (1983).

Reagents

1. Sodium phosphate buffer (60 mM-1 mM EDTA, pH-7.8)
2. 1 mM NAD⁺
3. 1 mM Propionaldehyde

Procedure

The cytosolic/mitochondrial fraction of testis was mixed with sodium phosphate buffer (60 mM-1mM EDTA, pH-7.8), 1 mM NAD⁺ and 1 mM propionaldehyde. The increase in absorbance was measured at 340 nm. The enzyme activity was expressed as nanomoles of NAD oxidised/ min/mg protein.

30. Estimation of protein

Protein was estimated by the method of Lowry et al. (1951).
Reagents

1. Reagent A: 2 g sodium carbonate was dissolved in 100 mL 0.1 N NaOH solution.

2. Reagent B: solution 1- 500 mg copper sulphate was dissolved in 50 mL water; Solution 2- 1 g sodium potassium tartarate was dissolved in 50 mL water

3. Mixed 0.5 mL solution 1 and 0.5 mL solution 2

4. Reagent C (alkaline copper reagent): 50 mL of reagent A and 1 mL of reagent B were mixed fresh at the time of use.

5. Folin –Ciocalteau reagent: Commercially produced reagent was diluted in the 1:2 ratios with distilled water.

6. Protein standard: 4 g BSA was dissolved in 10 mL of distilled water to make protein standard solution of 400 µg /L.

Procedure

An aliquot of 0.1 mL extract was taken and made up to 0.5 mL with distilled water. 5 mL alkaline copper reagent was added and mixed well. After allowing to stand for 10 mins at room temperature, 0.5 mL of Folins reagent was added, mixed and optical density was read at 510 nm after 20 mins in dark. A standard curve was prepared by using different concentrations of BSA.

31.1. Determination of ascorbic acid by High performance liquid chromatography (HPLC)

One part serum/testicular homogenate/mitochondrial fraction/seminal plasma with four parts of 6% metaphosphoric acid were mixed in a polypropylene storage vial. The vial contents were vortexed and centrifuged at 10,000 g for 15 mins at 4°C
and the supernatant was used for analysis. HPLC analysis was done by the Shimadzu Prominsence SCL-20AHTb (Shimadzu) system and the separation of ascorbic acid was done by isocratic gradient elution using a Luna 5S NH2 100A column (Phenomenex). The length of the column was 250 mm x 4.6 mm and the particle size was 5 µm. The mobile phase was HPLC-grade water (eluent A) and methanol (eluent B) in a 1:1 ratio, the total flow rate was 1.0 mL per min and the time of analysis was 15 mins. The detector's wavelength was set at 268 nm. The injection volume was 20 µL and the temperature of the column was thermostated at 40°C.

31.2. Determination of alpha-tocopherol by High performance liquid chromatography (HPLC)

Testicular homogenate/seminal plasma was homogenized in 1.15% KCl and tocopherols were extracted in 1 mL of hexane. Samples were centrifuged at 1500 g for 5 mins at 4°C. The hexane layer was pooled and dried under stream of nitrogen and reconstituted in 0.1 mL spectroscopic grade methanol. HPLC analysis was done by the Shimadzu Prominsence SCL-20AHTb (Shimadzu) system and the separation of alpha-tocopherol was done by isocratic gradient elution using a Luna 5S NH2 100A column (Phenomenex). The length of the column was 250 mm x 4.6 mm and the particle size was 5 µm. The mobile phase was HPLC-grade acetonitrile (eluent A) and methanol (eluent B) in a 1:1 ratio, the total flow rate was 1.0 mL per min and the time of analysis was 15 mins. The detector's wavelength was set at 268 nm. The injection volume was 20 µL and the temperature of the column was thermostated at 40°C.
32. DNA Fragmentation Assays for Apoptosis

Apoptotic changes in testis were evaluated colorimetrically by DNA fragmentation and by agarose gel electrophoresis according to the procedure of Perandones et al. (1993).

Reagents

1. Absolute isopropyl alcohol
2. 0.5 M NaCl
3. Tris-EDTA buffer
4. 0.5 M Perchloric acid
5. Ethidium bromide
6. Diphenyl amine

Procedure

Testis samples were homogenized in 700 µL hypotonic lysis buffer and centrifuged for 15 mins at 11,000 rpm. The supernatant containing small DNA fragments were separated; one-half the volume was used for gel electrophoresis and the other half, together with the pellet containing large pieces of DNA were used for quantification of fragmented DNA by the Diphenyl amine (DPA) assay. The samples were treated with equal volumes of absolute isopropyl alcohol and 0.5 M NaCl to precipitate DNA. The samples were then kept at -20°C overnight and centrifuged at 11,000 rpm for 15 mins. The pellets were then washed with 500 µL of 70% ethanol and allowed to dry at room temperature. Extracted DNA was reconstituted in 12 µL of Tris-EDTA buffer and 3 µL loading buffer. The samples were incubated at 37°C for 20 mins, and then electrophoresed on 1% agarose gels containing 0.71 µg/mL ethidium bromide. At the end of the runs, gels were
examined using UV transilluminator. The DPA assay reaction was modified by Perandones et al. (1993) from Burton (1956). Briefly, perchloric acid (0.5 M) was added to the pellets containing native DNA (reconstituted in 400 µL of the hypotonic lysis buffer) and to the supernatants containing fragmented DNA followed by the addition of 2 volumes of DPA solution. The samples were kept at 4°C for 48 h. The colorimetric reaction was then measured spectrophotometrically at 575 nm. The percentage of DNA fragmentation was calculated.

33. Caspase-3 assay

Caspase-3 activity were measured through cleavage of a colorless substrate specific for caspase-3 (Ac-DEVD-pNA) releasing the chromophore, p-nitroaniline (pNA). An increase in absorbance at 405 nm was used to quantify the activation of caspases activities. Testicular homogenate were collected and lysed in lysis buffer containing 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.4), 1 mM CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and 1 mM DTT (Dithiothreitol) for 15 mins on ice. Cell lysates were centrifuged at 18,000 g for 15 mins at 4°C. Caspase-3 activity in the supernatant was assayed using the colorimetric caspase 3 assay kit procured from Sigma–Aldrich, USA (Kit No: CASP3C-1KT058K4147) according to the manufacturer’s instructions.

34. Determination of Intra-cellular Reactive Oxygen Species (ROS)

The extent of intra-cellular ROS production was estimated by using DCF-DA (Dihydrodichlorofluorescein diacetate) as a probe according to the method of Kim et al. (1996).
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Reagents

1. 20 mM Tris–HCl
2. 130 mM KCl
3. 5 mM MgCl₂
4. 20 mM NaH₂PO₄
5. 30 mM glucose
6. 5 µM DCF-DA
7. 1 µmol H₂O₂

Procedure

100 µL of testes homogenates were incubated with the assay media (20 mM Tris–HCl, 130 mM KCl, 5mM MgCl₂, 20 mM NaH₂PO₄, 30 mM glucose and 5 µM DCF-DA) at 37°C for 15 mins. H₂O₂ (1µmol) was added into the mixture at the end of the assay. The formation of DCF was measured at an excitation wavelength of 488 nm and emission wavelength of 510 nm for 10 mins by using fluorescence spectrometer.

35. Assay of total serum Testosterone

Total serum Testosterone was estimated by the method of RIA using the kit for Coat-A-count purchased from Diagnostic Product Corporation, USA (Catalogue No: LKTT).

36. Assay of serum Estradiol

Estradiol was estimated by the method of RIA using the kit for Coat-A-count, Estradiol purchased from Diagnostic Product Corporation, USA. (Catalogue No: LKE2).
37. Assay of serum LH and FSH

Follicle-stimulating hormone (FSH) and Lutenising hormone (LH) were measured using electrochemiluminescence immunoassay using Immuno analyser (Roche Diagnostics) (Catalogue No: LKFS and LKLH). The detection limits were 0·10 mIU/mL for FSH and LH.

38. Histopathological studies

The histopathological studies were carried out according to the method of Gurr E (1962).

A section of the tissue was dissected and was kept in 10% formalin solution for fixing. Fixing prevents autolysis and putrefaction of tissues. The tissues were kept in formalin solution for 1 hr. The tissues were then dipped in different concentrations of alcohol in ascending order and finally in absolute alcohol (10 mins each) for removing water. They were then kept in methyl benzoate until it sank and then dipped in benzene for removing alcohol. After this, the tissues were infiltered with molten paraffin and the tissues were placed in it. The paraffin was cooled till it hardened, enclosing the tissues.

Using a rotary microtome, thin sections (4-5 µm) of paraffin infiltered tissues were made. The tissues were deparaffinised with xylene and heated with 100%, 90% and 70% alcohol (10 mins each) for removing undesirable pigments and other materials. The sections were then stained with heamatoxylin (nucleus stainer) and counter stained with eosin (cytoplasm stainer) and dehydrated with 70%, 90% and 100% alcohol (10 mins each). The sections were mounted using DPX (Dibutyl phthalate in xylene) and examined under a microscope.
39. Assay of Hexokinase [EC.2.7.1.1]

Activity of hexokinase was assayed by the method of Crane and Sols (1953).

Reagents

1. ATP-Magnesium chloride mixture- 0.2 mL solution of ATP and magnesium chloride of appropriate strength were required to pH 7 and diluted to final concentration of 0.075 M ATP and 0.04 M magnesium chloride.

2. Buffer mixture- 0.2 mL solution of tris, histidine, EDTA and magnesium chloride were mixed, neutralized to pH 7, and diluted to the concentration of 0.1 M and 0.01 M respectively.

3. Glucose (0.01 M)- 0.2 mL

4. Water- 0.3 mL

Procedure

The chilled tissue was homogenized at 0°C with three volume of buffer of the following composition- Tris-0.1M (pH 7), histidine-0.1 M, EDTA-0.1 M and magnesium chloride-0.01 M (pH 7). The homogenate was centrifuged at 3000 g at 0°C for 5 mins and supernatant was used as the enzyme source.

The tubes were pre-incubated at 37°C and the reaction was started by the addition of the enzyme preparation (0.1 mL). Immediately after the addition of the enzyme, an aliquot (0.2 mL) was withdrawn to ice-cold solution of 5% zinc sulphate (1 mL). It was then neutralized with 0.3 N barium hydroxide using phenolphthalein as indicator, centrifuged and glucose in the supernatant estimated by the method of Asatoor and King (1954). After 30 mins, another sample (0.2 mL) was withdrawn
and glucose estimated as before. One unit of enzyme activity is defined as mg glucose phosphorylated/min/mg protein.

40. Assay of Pyruvate kinase [EC.2.7.1.40]

Pyruvate kinase was assayed by the method described by Bucher and Pfleiderer (1955).

Reagents

The composition of assay mixture is shown below

1. NADH (1.5 M)
2. ADP (2.3 M)
3. PEP (phosphoenol pyruvate) (7.8 M)
4. Magnesium sulphate (8 M)
5. KCl (7.5 M)
6. Lactate dehydrogenase (0.035 M)
7. Triethanolamine-HCl buffer (0.05M, pH 7.5)

Procedure

The chilled tissue was homogenized at 0 C in 0.05 M triethanolamine-HCl buffer. The reaction was started by pipetting enzyme solution into the assay mixture (5 mL). The enzyme solution was diluted in distilled water (0.1 mL) and the optical density was measured at 340 nm for 2 mins at 15 sec interval. One unit of enzyme activity is defined as micromoles of NADH oxidized/min.

41. Isolation of mitochondria from rat testes

Isolation of mitochondria from rat testes was described by Sahoo et al. (2008).
Homogenate of testis (20%) was prepared in ice-cold phosphate buffer (50 mM, pH 7.4) containing sucrose with the help of a Potter-Elvejhem motor driven glass Teflon homogenizer. The crude homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged at 600 g for 10 mins at 4°C to precipitate nuclei and cell debris. The supernatant was centrifuged at 10,000 g for 20 mins at 4°C to separate mitochondrial pellet. The supernatant was the post mitochondrial supernatant. The mitochondrial pellet was washed three times with phosphate buffer (50 mM, pH 7.4) and each time the mitochondrial pellet was obtained at 10,000 g for 5 mins at 4°C. To assess the purity of mitochondrial fraction obtained by differential centrifugation, we checked the specific activity of the marker enzyme, succinate dehydrogenase enzyme in crude homogenate, nuclear, mitochondrial and post mitochondrial fractions.

41.1 Mitochondrial ROS Levels

Reactive oxygen species (ROS) generation in testis mitochondria was quantified using dihydrodichlorofluorescein diacetate (DCF-DA), a non-polar compound which after hydrolysis by intracellular esterases, reacts with ROS to form a highly fluorescent dichlorofluorescein (DCF) (Driver et al. 2000). Briefly, testis mitochondria (0.2 mg protein) were incubated with Locke’s solution [(NaCl 154 mM, KCl 5.6 mM, NaHCO₃ 3.6 mM, HEPES 5.0 mM, CaCl₂ 2.0 mM, glucose 10 mM/mL, pH 7.4 containing DCF-DA (5 mM/mL)] for 30 mins at 37°C. The fluorescence was measured with excitation and emission wavelengths at 480 and 530 nm. ROS levels were quantified from a dichlorofluorescein standard curve and expressed as pmol DCF formed/min/mg protein.
41.2 Mitochondrial enzyme activities

41.2.1 Assay of Citrate synthase [EC. 2.3.3.1]

The activity of citrate synthase was determined by monitoring the oxidation of DTNB (Srere 1969).

Reagents

1. Tris–HCl buffer (0.1 M, pH 8.1)
2. 0.1% Triton X-100
3. DTNB (5, 5’-dithiobis-(2-nitrobenzoic acid) (0.2 mM)
4. Acetyl CoA (0.1 mM)
5. Oxaloacetate (10 mM)

Procedure

Mitochondrial protein (0.05 mg) was added to Tris–HCl buffer (0.1 M, pH 8.1), 0.1 % Triton X-100 containing DTNB (0.2 mM) and acetyl CoA (0.1 mM). The reaction was started by the addition of oxaloacetate (10 mM), absorbance monitored at 412 nm for 3 mins and the activity was expressed as gmol thiol oxidized/ min/mg protein.

41.2.2 Assay of Isocitrate dehydrogenase [EC.2.7.1.41]

Isocitrate dehydrogenase activity was estimated by the method of Kornberg (1955).

Reagents

1. 0.05 M Potassium phosphate buffer (pH 7)
2. 0.1 M magnesium chloride
3. 0.025 M ADP
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4. 0.05 M NAD

5. 0.005 M DL-isocitrate

Procedure

The reaction mixture contains phosphate buffer (2 mL), magnesium chloride (1 mL), ADP (0.2 mL), NAD (0.2 mL), the enzyme solution (0.1 mL), dl-isocitrate (1 mL) and water (1 mL). The assay was run at room temperature. A control containing all reaction components except isocitrate was incubated. The increase in optical density at 340 nm resulting from the reduction of NAD was observed at 30 secs intervals for 3 mins. One unit of enzyme activity is defined as microgram of NAD reduced/30 seconds/mg protein.

41.2.3 Assay of Succinate dehydrogenase [EC.1.3.99.1]

Succinate dehydrogenase activity was measured by the method of Arrigoni and Singer (1962).

Reagents

1. Potassium phosphate buffer (0.5 M)
2. Succinate (0.5 M)
3. DCIP (2,6-dichlorophenol-indophenol) (1.5 mg/20 mL water)
4. KCN (190 mg/20 mL water)
5. 2% PMS
6. Calcium chloride
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Procedure

The reaction mixture contained 100 µM of buffer pH 7.0, 3 mM of KCN, 80 µM of calcium chloride, 0.04 µM of DCIP and 0.1-0.2 mg of enzyme in total volume of 2.8 mL; 50 µM of succinate and 0.05 mL of 2% PMS were added. The decrease in absorbance at 600 nm was determined using a spectrophotometer for two mins at 15 seconds time interval. The amount of DCIP reduced was calculated using the relationship

\[ \frac{OD \times 10000}{16.2 \times \text{protein (mg)} \times \text{time (mins)}} = \text{Nanomoles of dye reduced/min/mg protein} \]

Where, 16.2 is the milli extinction coefficient. The enzyme activity is defined as unit/mg protein where one unit of enzyme activity represents one nanomoles of dye reduced/min.

41.2.4 Assay of Malate dehydrogenase [EC.1.1.1.37]

Malate dehydrogenase was assayed by the method of Mehler et al. (1948).

Reagents

1. 0.25 M glycylglycine buffer, pH 7.0
2. 0.0015 M NADH
3. 0.00076 M oxaloacetate

Procedure

The reaction mixture contains the following, glycylglycine buffer, pH 7 (0.6 mL), NADH (0.2 mL), distilled water (4.9 mL) and enzyme (0.12 mL). The reaction was carried out at room temperature and was started by the addition of either oxaloacetate or enzyme. Optical density was taken at 340 nm against a blank
Materials and Methods

containing all components except NADH, at intervals of 15 sec for 1-2 mins. One unit of enzyme activity is defined as mM of NADH oxidized/mg protein/15 sec.

41.2.5. Estimation of ATP

Determination of ATP was done with Hexokinase and Glucose-6-phosphate dehydrogenase and was estimated by the method of Williamson and Corkey (1967).

Reagents

1. Buffer: 50 mM triethanolamine-HCl (TRA), 10 mM MgCl₂, 5 mM EDTA, pH 7.4. Adjusted the pH of the buffer with KOH and stored at 2-4°C.
2. Glucose: 1.0 M
3. NADP⁺ - 10 mg/mL
4. Adenosine 5’-triphosphate standard: 0.1 mM. A stock solution of 10 mM ATP (sodium salt) may be prepared and stored frozen for several weeks. This stock solution is diluted 1:100 with distilled water.
5. Enzymes:
   a. Hexokinase, 2 mg/mL (140 U/mg). Diluted commercial hexokinase (10 mg/mL) 1:5 with distilled water.
   b. Glucose-6-phosphate dehydrogenase, 0.2 mg/mL (140 U/mg). Diluted the commercial enzyme (1 mg/mL) 1:5 with distilled water.

Procedure

The concentrations of ATP in the standard solution was determined spectrophotometrically by following the optical density change at 340 nm using the following reaction mixture: buffer, 1.97 mL; glucose, 0.01 mL; NADP⁺, 0.10 mL;
ATP standard or distilled water, 0.50 mL; glucose-6-phosphate dehydrogenase, 0.01 mL.

After mixing the sample, read the optical density at 340 nm ($R_1$). Added 0.01 mL of hexokinase and took the readings until the reaction has reached completion ($R_2$). The optical density change upon the addition of 0.01 mL of hexokinase to a blank cuvette containing distilled water instead of ATP standard solution is subtracted from the difference $R_2 - R_1$. In the case of test samples, 0.50 mL of tissue homogenate is added and took the absorbance.

41.2.6 Assay of NADH-cytochrome c reductase [EC 1.10.2.2] and succinate-cytochrome c reductase [EC 1.3.99.1]

Assay of NADH-cytochrome c reductase and succinate-cytochrome c reductase as described by Navarro et al. (2002, 2004).

Reagents

1. Phosphate buffer (0.1 M, pH 7.4)
2. NADH (0.2 mM)
3. Potassium cyanide (1 mM)
4. Cytochrome c (0.1 mM)
5. Succinate (20 mM)

Procedure

The mitochondria (0.1 mg protein) were added to phosphate buffer (0.1 M, pH 7.4) containing NADH (0.2 mM), KCN (1 mM) and the reaction was initiated by the addition of cytochrome c (0.1 mM). The decrease in absorbance was monitored for 3 mins at 550 nm and the activity was expressed as 1 mol cytochrome c reduced/
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min/mg protein. To determine the activity of succinate-cytochrome c reductase, the substrate succinate (20 mM) was added instead of NADH to the reaction mixture.

41.2.7. Determination of ATPase activity

Total ATPase activity in testis and mitochondrial Mg\textsuperscript{2+} ATPase activity was measured by the method of Desaiah and Ho (1979).

Reagents

1. 50 mM Tris–HCl (pH 7.4)
2. 100 mM NaCl
3. 5 mM ATP
4. 20 mM KCl
5. 5 mM MgCl\textsubscript{2}
6. 0.1 mM Ouabain
7. 20% TCA

Procedure

Total ATPase activity in testis was assayed in medium containing Tris-HCl buffer (50 mM), NaCl (100 mM), KCl (20 mM), MgCl\textsubscript{2} (5 mM) final concentration. Mg\textsuperscript{2+} ATPase activity in mitochondria was initiated by adding mitochondrial protein (0.05 mg) to a medium containing Tris–HCl (50 mM, pH 7.4) containing ATP (5 mM), KCl (20 mM), MgCl\textsubscript{2} (5 mM) and Ouabain (0.1 mM). Following addition, the reaction mixture was incubated for 15 mins at 37°C. Terminally, the reaction was stopped by the addition of 20% TCA. After brief centrifugation, the inorganic phosphate formed in the protein free supernatant was determined according to Fiske
and Subbarow (1925) and the enzyme activity was expressed as inorganic phosphate liberated/mg protein for 15 min.

41. 2.8. Measurement of Mitochondrial respiration

Mitochondrial respiration was measured polygraphically using a Clark oxygen electrode connected to computer operated oxygraph unit (Hansatech, Norfolk, UK) (Yang et al. 2004).

Procedure

1. 15 mM KH$_2$PO$_4$
2. 15 mM KCl
3. 50 mM Tris–HCl
4. 0.225 M sucrose
5. 5 mM MgCl$_2$
6. 0.1 mM EDTA
7. 0.25 mM ADP
8. 5 mM malate
9. 2.5 mM glutamate
10. Freshly prepared mitochondrial suspension

Procedure

A freshly prepared mitochondrial suspension was added to 1 mL of assay buffer (containing 15 mM KH$_2$PO$_4$, 15 mM KCl, 50 mM Tris–HCl, 0.225 M sucrose, 5 mM MgCl$_2$, 0.1 mM EDTA, pH 7.4) at 30°C in a sealed chamber equipped with a magnetic stirrer. Oxygen consumption was measured in the absence (state 4 activity) and presence of 0.25 mM ADP (state 3 activity) and succinate
(final concentration 5 mM) or malate/glutamate (2.5 mM) as substrate, respectively. The Respiratory control ratio (RCR) was expressed as the ratio of state 3–4 activity.

41.2.9. Mitochondrial permeability transition (MPT) pore opening

MPT was measured by the method of Masaki et al. (1989).

Reagents

1. 215 mM mannitol
2. 71 mM sucrose
3. 3 mM HEPES
4. 100 mM CaCl₂

Procedure

Mitochondria (0.25 mg protein) were suspended in a swelling medium (mannitol 215 mM, sucrose 71 mM, HEPES 3 mM, pH 7.4 containing 5 mM succinate). The absorbance was monitored at 540 nm immediately following the addition of CaCl₂ (100 mM).

41.2.10. Mitochondrial membrane potential

Mitochondrial membrane potential was determined by measuring the uptake of Rhodamine 123 (Shimizu et al. 1999).

Reagents

1. Phosphate buffered saline (0.1 M, pH 7.4)
2. Rhodamine 123 (1.5 mM/ mL)

Procedure

Mitochondria (0.05 mg protein) were added to phosphate buffered saline (0.1 M, pH 7.4) containing Rhodamine 123 (1.5 mM/ mL) and incubated at 37°C for 30
mins. Following centrifugation at 10,000 g for 10 mins, the fluorescence was measured at excitation and emission wavelengths of 490 and 520 nm, respectively.

41.2.11. Intracellular Ca\(^{2+}\) levels

Intracellular calcium in testicular cytosolic fraction was estimated by O-cresolphthalein complex one method kits procured from Accurex Biomedical Pvt. Ltd. (Batch No: 51028: code No: A-4).

42. Total RNA Isolation

Total RNA was isolated from testis/isolated Leydig cell/microsomes by using TRI Reagent (Sigma Aldrich) by the modified method of chomoznsky and Sacchi (2006).

Procedure

1. Homogenized tissue samples in TRI Reagent (1 mL per 100 mg tissue).

2. After homogenization, centrifuged the homogenate at 12,000 g for 10 mins at 2-8°C to remove the insoluble material. Transferred the clear supernatant to fresh tube.

3. Allowed the sample to stand for 5 mins at room temperature. Added 0.2 mL of chloroform per mL of TRI Reagent used. Covered the sample tightly, shook vigorously for 15 seconds, and allowed to stand for 2-15 mins at room temperature. Centrifuged the resulting mixture at 12,000 g for 15 mins at 2-8°C.

4. Transferred the colorless upper aqueous phase to fresh tube and added 0.5 mL of isopropanol per mL of TRI Reagent used in sample preparation. Allowed the sample to stand for 5-10 mins at room
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temperature. Centrifuged at 12,000 g for 10 mins at 2-8°C. The RNA precipitate formed a pellet on the side and bottom of the tube.

5. Removed the supernatant and washed the RNA pellet by adding a minimum of 1mL of 75% ethanol per 1 mL of TRI Reagent used in the sample preparation, vortexed the sample and then centrifuged at 7,500 g for 5 mins at 2-8°C.

6. Briefly dried the RNA pellet for 5-10 mins by air drying. Added an appropriate volume of water to the RNA pellet.

7. The concentration and purity of total RNA was determined by absorbance at 260/280 nm in a UV spectrophotometer. If the ratio of $A_{260/280}$ is 1.8-2.0, then the purity is maximum.
43. Two step RT-PCR Set up of the first step RT reaction

RT-PCR reaction was carried out using two step RT-PCR kit from Masterscript. Prepared two mastermix and kept them on ice. Mastermix 1 contained the RT primer (Oligo (dT), random primers or gene specific antisense primer), dNTPs and template RNA. Mastermix 2 contained the enzymes, and the RT-PCR Buffer.

<table>
<thead>
<tr>
<th>Components</th>
<th>RT with Oligo(dt) primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mastermix 1</strong></td>
<td></td>
</tr>
<tr>
<td>RNase-free water</td>
<td>4 µL</td>
</tr>
<tr>
<td>dNTP mix, 10 mM each</td>
<td>2 µL</td>
</tr>
<tr>
<td>Oligo (dT) primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>Template RNA</td>
<td>3 µL</td>
</tr>
<tr>
<td><strong>Mastermix 2</strong></td>
<td></td>
</tr>
<tr>
<td>RNase-free water</td>
<td>6 µL</td>
</tr>
<tr>
<td>RT-PCR Buffer with 25mM Mg</td>
<td>2 µL</td>
</tr>
<tr>
<td>Masterscript RT Enzyme</td>
<td>2 µL</td>
</tr>
</tbody>
</table>
ii. Set up for the second step PCR reaction

Set up mastermix 3 and 4 for the PCR step in separate nuclear-free microcentrifuge tubes mixed well, centrifuged and placed on ice.

<table>
<thead>
<tr>
<th>Components</th>
<th>RT with Oligo(dt) primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mastermix 3</strong></td>
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</tr>
<tr>
<td>RNase-free water</td>
<td>4 µL</td>
</tr>
<tr>
<td>dNTP mix, 10 mM each</td>
<td>2 µL</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>First step RT reaction mix</td>
<td>3 µL</td>
</tr>
<tr>
<td><strong>Mastermix 4</strong></td>
<td>40 µL</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>36 µL</td>
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<tr>
<td>RT-PCR Buffer with 25mM Mg</td>
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</tr>
<tr>
<td>Masterscript PCR Enzyme mix</td>
<td>2 µL</td>
</tr>
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</table>

iii. Incubation programme for first strand cDNA synthesis (RT) with random hexamers

<table>
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<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65°C</td>
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</tr>
<tr>
<td>2</td>
<td>4°C</td>
<td>1 min</td>
</tr>
<tr>
<td>3</td>
<td>58°C</td>
<td>90 mins</td>
</tr>
<tr>
<td></td>
<td>Hold at 4°C</td>
<td></td>
</tr>
</tbody>
</table>
iv. Incubation programme for second step PCR

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>2 mins</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>3</td>
<td>58°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>4</td>
<td>72°C</td>
<td>2.5 mins</td>
</tr>
<tr>
<td>5</td>
<td>72°C</td>
<td>7 mins</td>
</tr>
</tbody>
</table>

Go to Step 3, Repeat 39 cycles.

44. Quantitative Real Time PCR

Total RNA with A260/A280 ratios between 1.8 and 1.9 was used for real-time PCR analyses. Complementary DNA was synthesized from four µg of total cellular RNA using first strand cDNA synthesis kit (Eppendorf, Germany). cDNAs from samples for different genes were quantified using 8 µL of cDNA, 12.5 µL of SYBR Green Master mix (Eppendorf, Germany), 1µL each of forward primer and reverse primer in a Real-time PCR system. Real-time PCR was conducted with a program that consisted of 3 min at 95°C followed by 40 cycles of 95 °C for 15 s, 56°C for 20 s and 72°C for 30 s. The primer concentration for qPCR was 10pm/20µL. After 40 amplification cycles, a melting analysis was carried out to verify the correct product by its specific melting temperature (T_m). The fold change in expression was calculated using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittigen, 2001).
45. DNA Gel Electrophoresis

By the method of Beldler et al. (1982).

Reagents.

50 X TAE Buffer

i. Tris base - 242 g
ii. Glacial acetic acid - 57.1 mL
iii. 0.5 M EDTA

Made up to 1000 mL with distilled water (Filtered and autoclaved). 50 X TAE were diluted to 1X prior to use.

DNA loading dye

i. Sucrose - 60 %
ii. EDTA, pH 8.0 - 100 mM
iii. Bromophenol blue - 0.25 %
iv. Ethidium bromide:

Prepared as 10 mg/mL solution in distilled water and stored in a screw cap vial, wrapped with a thin foil at 4°C. This was added to the gel at 0.5 µg/mL final concentration prior to pouring.

Procedure

Added 0.24 g of agarose and 3 mL of TAE buffer with 30 mL distilled water. Heated the mixture for 5 mins for the agarose to dissolve. Cooled to 45°C and add 2 µL of ethidium bromide. Set the gel former and poured the agarose mixture. After solidification, 10 µL of DNA sample and 5 µL of DNA loading dye were added.
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Loaded the sample to each well with a marker and filled the tank with 1X TAE buffer. Applied 200V current until the bromophenol blue run three fourth of the gel.

46. Western blot

Reagents

10X PBS

i. 80 g NaCl

ii. 2 g KCl

iii. 14.4 g Na₂HPO₄

iv. 2.4 g KH₂PO₄

Dissolved in 800 mL double distilled water. pH adjusted to 7.4. Autoclaved and 1X working solution was prepared

Lysis Buffer (4°C)

i. 50 mM Tris–HCl (pH 7.4)

ii. 150 mM NaCl

iii. 1 mM EDTA

iv. 1% Triton X-100

v. 1% sodium deoxycholate

vi. 0.1% SDS

1 mM phenyl methyl sulfonyl fluoride (PMSF): Added right before use. (10 mL: 1 mM PMSF (in iso-propanol at ~20°C). Diluted 1:10 with double distilled water. pH will be 8.3.)
10X Towbin’s Electrotransfer Buffer

i. 30.3 g Tris Base

ii. 144 g Glycine

Made upto 1000 mL double distilled water

1X Towbin’s

i. 100 mL 10X stock

ii. 200 mL (20%) Methanol

Made upto 1000 mL double distilled water

Blocking Buffer

i. 0.1% Tween 20

ii. 5% powdered milk in 1X PBS

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970).

Reagents

1. Separating gel (20%):

i. Acrylamide-Bis (29.2:0.8) - 6.75mL

ii. 1.5 M Tris-HCl (pH-8.8) - 2.5 mL

iii. 10% SDS - 0.1 mL

iv. Deionized water - 0.75 mL

v. 10% APS (Ammonium persulphate) - 0.1 mL

vi. TEMED (Tetramethylethylenediamine) - 8 μL

2. Stacking Gel (5mL):

i. Acrylamide-Bis (29.2:0.8) - 830 μL

ii. 1.5 M Tris-HCl (pH-8.8) - 630 μL
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iii. 10% SDS - 50 µL
iv. Deionized water - 3.4 mL
v. 10% Ammonium persulphate - 50 µL
vi. TEMED - 5 µL

3. Acrylamide-Bis (30 %):
   - Acrylamide - 29.2 g
   - Bis acrylamide - 0.8 g

Made up to 100 mL with deionized water. The solution was filtered with Whatmann 1 mm filter paper and stored in a brown bottle at 4°C.

4. 10X TGS Buffer:
   i. Tris - 3 g
   ii. Glycine - 14.4 g
   iii. SDS - 1 g

Made up to 100 mL with deionized water. Stock was diluted to 1X before use.

5. 4X Sample Buffer:
   i. 1M Tris-HCl (pH- 6.8) - 2.1 mL
   ii. 20 % SDS - 1 mL
   iii. 100 % Glycerol - 1 mL
   iv. β- Mercaptoethanol - 0.5 mL
   v. Bromophenol blue - 2.5 mg

Made up to 5 mL with deionized water. Bromophenol blue was first dissolved in methanol before adding other components.
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Procedure

Testicular samples/Leydig cell (cells can be directly lysed in lysis buffer after isolation and purification by percoll centrifugation) were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 1 mM PMSF. Samples were then centrifuged at 15,000 g for 15 mins. Supernatants from each sample were added to a gel loading buffer (100 mM Tris, pH 6.8, 20% glycerol, 200 mM DTT, 4% SDS, 0.03% bromophenol blue) and boiled for 5 mins. The protein content of the supernatant from centrifugation was determined by spectrophotometry using bovine serum albumin as standard. Approximately, 40 mg total protein per lane was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The gel was transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore Corp. Bedford, Massachusetts, USA) and blocked in 5% nonfat milk in PBS containing 0.1% Tween-20 (pBST) overnight at 4°C. The membranes were then incubated for 2 h with rabbit polyclonal antibody against LHR, StAR, P450scc, 17β-HSD, 3β-HSD and GAPDH (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, 1:1,000 dilution). The membranes were washed three times and then incubated with horseradish peroxidise (HRP)-conjugated secondary antibodies (1:2500) at room temperature for 1 h. The membranes were washed and then incubated in chromogenic substrate, diamino benzidiene (DAB) until the bands are visible.
47. Isolation and Purification of Leydig cells

Leydig cells were isolated from testes of adult male guinea pigs by enzymatic digestion and purified on a discontinuous Percoll gradient (Rigaudiere et al. 1988).

Reagents

1. Dulbecco's Modified Eagle's Medium, Nutrient Mixture, F-12 Ham (DMEM/F12 1:1 mixture), pH 7.4: 12 g DMEM/F-12 and 1.2 g sodium bicarbonate were dissolved in 800 mL of autoclaved Milli Q water. 10 mL of penicillin-streptomycin solution and 1 mL of amphotericin-B solution were added and pH of the medium was adjusted to 7.4 with 1 N NaOH and the volume was made up to 1 L.

2. Collagenase (Type IV) (0.25 mg/mL): 25 mg collagenase was dissolved in 100 mL DMEM/F-12 (pH 7.4).

3. 100% Percoll: 100% Percoll solution was prepared by dissolving 3.9 mg DMEM/F-12, 75 mg bovine serum albumins (BSA) and 0.75 mg sodium pyruvate in 22.5 mL of Percoll (113%). The pH was adjusted to 7.4 with 0.01 N HCl and finally made up to 25 mL with autoclaved Milli Q water.

Preparation of Percoll gradient: Different Percoll gradients were prepared by mixing 100% Percoll and DMEM/F-12 (pH 7.4) in different ratios: i.e., 75% (7.5 mL + 2.5 mL), 60% (6 mL + 4 mL), 45% (4.5 mL + 5.5 mL), 30% (3 mL + 7 mL), 15% (1.5 mL + 8.5 mL) and 5% (0.5 mL + 9.5 mL). 2 mL of 75% Percoll gradient was added to a graduated centrifuge tube and above this layer, 60%, 45%, 30%, 15% and 5% gradients of Percoll (2 mL each) were laid gently one over the other, taking care to avoid mixing. Testes were decapsulated aseptically with fine forceps without
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breaking the seminiferous tubules and incubated in polypropylene tubes with culture medium containing HEPES and collagenase (0.25 mg/mL). Incubation was performed by shaking the tubes in their long axis in thermostated shaking water bath at 34°C for 15 mins. After the incubation, tubes were gently shaken and 10 mL of DMEM/F-12 (pH 7.4) without collagenase was added and allowed to stand for 10 mins. The supernatant was then aspirated using a Pasteur pipette and transferred to sterile centrifuge tubes. This procedure was repeated once again to increase the yield of Leydig cells. The supernatants were combined and centrifuged at 2,500 g for 10 mins at 4°C. After discarding the supernatant, the pellet obtained was resuspended in 1 mL of DMEM/F-12 representing a crude testicular interstitial cell suspension. Discontinuous Percoll gradients were used to obtain purified Leydig cells from this crude preparation. 1 mL of crude Leydig cell suspension was applied on top of this discontinuous gradient and centrifuged at 3,000 g for 30 mins at 4°C. After centrifugation, most of the Leydig cells were observed in between 30% and 45% gradients. These Leydig cells were aspirated carefully using a Pasteur pipette and transferred to centrifuge tubes containing 5 mL medium. After mixing thoroughly, the tubes were centrifuged at 2,500 g for 10 mins at 4°C and the supernatant obtained was discarded. To remove excess percoll, the cell pellets were washed thrice with excess medium and then finally suspended in 1 mL of medium and counted using haemocytometer.

47.1 Identification of Leydig cells

The purified Leydig cells were identified by histochemical localization of 3β-hydroxysteroid dehydrogenase (3β-HSD) performed according to the method of Aldred and Cooke (1983).
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Reagents

1. Phosphate buffer (pH 7.2)

   Solution A: 1.56 g monobasic sodium phosphate (0.1 M) was dissolved in 100 mL distilled water.

   Solution B: 1.78 g dibasic sodium phosphate (0.1 M) was dissolved in 100 mL distilled water.

   28 mL of solution A and 72 mL of solution B were mixed well, pH was adjusted to 7.2 and the final volume was made up to 200 mL with distilled water.

2. Phosphate buffer containing NAD- 3 mg/mL and nitroblue tetrazolium (NBT: 1 mg/mL).

3. Dehydroepiandrosterone (DHEA): (2 mg/mL).

Procedure

To 20 μL of Leydig cell suspension, 490 μL NAD-NBT solution and 10 μL DHEA were added and incubated at 37°C for 60 mins. After incubation, cells were washed with phosphate buffer (pH 7.2). The percentage of positively stained cells was counted using a Neubauer haemocytometer under the microscope. The Neubauer haemocytometer has a grid containing five major squares, A, B, C, D and E. In the E square, the four small squares in the corner are called E1, E2, E3, E4 and the central small square is E5. 10 μL of 3β-HSD stained Leydig cell suspension was added to both sides of the haemocytometer. While performing the count, all the Leydig cells within the designated squares and those cells, which fall over the lines, were included. Major Square is 1 mm long, 1 mm wide and the thickness of the fluid between the coverslip and the haemocytometer is 0.1 mm. Leydig cells in the
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squares El, E2, E3, E4, and E5 were counted. The basic formula applied to obtain the Leydig cell concentration using the haemocytometer is given below:

\[
\text{Leydig cell concentration} = \text{Number of Leydig cells} \times \text{Multiplication factor} \\
(50,000) = "x" \text{ cells/mL}
\]

Approximately more than 90% of the cells stained positive for 3\(\beta\)-HSD by haemocytometer counts, reflecting the purity of Leydig cell preparation.

47.2 Viability of Leydig cells

Viability of purified Leydig cells was assessed by trypan blue exclusion method described by Aldred and Cooke (1983).

Reagent

0.4% trypan blue containing 0.1% BSA.

Procedure

100 \(\mu\)L trypan blue solution was mixed with 100 \(\mu\)L Leydig cell suspension (- 50,000 cells) and incubated for 5 mins at 37°C. The cells were then washed twice with saline and 10 \(\mu\)L suspensions was placed in the haemocytometer and viewed under the microscope. The number of unstained and stained cells represents viable and damaged cells, respectively. The cells were counted and the percentage of viable cells was calculated using the formula:

\[
\% \text{ of viable cells} = \frac{\text{Number of cells unstained} \times 100}{\text{Total number of cells}}
\]

The viability of purified Leydig cells was 90-95%.
Leydig cells ($1 \times 10^5$) were sonicated in ice-cold Tris-HCl buffer (pH 7.4) and centrifuged and the supernatant was collected and used for the assay of various biochemical parameters.

47.3. Estimation of ascorbic acid content

Vitamin C in isolated Leydig cells was determined by the method of Roe and Kuether (1943).

Reagents

1. 6% Metaphosphoric acid
2. 0.2% 2,6 Dichlorophenol indophenol dye
3. 2% Thiourea
4. 5% Metaphosphoric acid
5. 2% Dinitrophenyl hydrazine (DNPH) in 9 N H$_2$SO$_4$
6. 8% H$_2$SO$_4$

Procedure

Leydig cells ($1 \times 10^6$) extract from testis of control and treated animals were homogenised with 6% metaphosphoric acid containing 2 N acetic acid and centrifuged for 20 mins at 5000 g. The supernatant was used for ascorbic acid estimation. 1 mL supernatant was pipetted into stoppered tube. 5 mins after the addition of 0.05 mL of 0.2% 2, 6-dichlorophenol indophenol dye, 0.05 mL water was added. After 1 h, all the tubes were incubated for 3 h with 1 mL of 2% thiourea in 5% metaphosphoric acid and 0.5 mL of DNPH in 9 N H$_2$SO$_4$ at 60°C. The reaction was stopped by chilling. The osazone formation was then dissolved by the addition of 2.5 mL of 85% H$_2$SO$_4$. The absorbance at 540 nm was read against a
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blank. The blank was prepared exactly the same way except for the omission of DNPH until the addition of 85% H₂SO₄.

48. Isolation of microsomes

Microsomal fractions were prepared and processed as described by Schenkman and Jansson (1999) and isolated by differential ultracentrifugation with some modifications.

Reagents

1. Isotonic saline

2. Sucrose-TKM buffer (sucrose 0.25 M, Tris 80 mM, KCl 25 mM, MgCl₂ 5 mM, pH 7.4)

Procedure

The connective tissues surrounding the testes were removed following which they were washed in chilled isotonic saline to remove excess blood. Each testis was carefully decapsulated and the residual organ was cut into small pieces using a sterile scalpel blade. Subsequently, the samples of cut testis were minced separately with a fine pair of scissors and thoroughly mixed to obtain a homogenous mixture of minced tissue samples per animal. One-gram of each minced sample was chilled in isotonic saline for 5 mins prior to being homogenized in two volumes of sucrose-TKM buffer. Each homogenate was centrifuged at 10,000 g for 10 mins; supernatant harvested and subjected to centrifugation at 15,000 g for 15 mins to pellet down nuclei and mitochondria. The resultant supernatant was centrifuged at 100,000 g for 60 mins at 4°C following which, the cytosolic supernatant and microsomal pellet were separated. The pellet was rinsed twice with 5 mL of sucrose-TKM buffer and
resuspended in 5 mL of the same buffer. Testicular microsomes were aliquoted into
cryovials (Wheaton Science Products, Millville, NJ), and stored at -80°C.

48.1 CYP2E1 activity

CYP2E1 activity was performed according to the method of Reinke and
Moyer (1985).

Reagents

1. 0.1 mg of protein
2. 100 mM potassium phosphate buffer, pH 6.8
3. 200 mM p-nitrophenol
4. 1.0 mM NADPH
5. 70% perchloric acid

Procedure

Microsomal preparations from guinea pig testis (975 mg of protein) were
incubated in 100 mM potassium phosphate buffer, pH 6.8, with 200 mM p-
nitrophenol, a specific substrate for CYP2E1. After equilibration at 37°C for 3 mins,
reactions were initiated with NADPH (1.0 mM), incubated at 37°C for 30 mins, and
then terminated with 20 mL of 70% perchloric acid. Denatured proteins were
removed by centrifugation (10 mins at 3000 rpm), and the supernatant fraction was
analyzed for p-nitrocatechol formation at 510 nm. The p-nitrophenol hydroxylase
(p-NPH) activity was expressed as nanomoles of p-nitrocatechol formed per min per
milligram of microsomal protein.
48.2 Assay of Microsomal NADPH-cytochrome P450 reductase [EC 1.6.2.4]

This activity was performed according to the method of Dignam and Strobel (1975).

Reagents

1. 0.1 mg of protein
2. 0.3 M potassium phosphate buffer, pH 7.8
3. 75 µM Cytochrome c
4. 0.1 mM NADPH

Procedure

The method is based on the reduction of cytochrome c in a medium containing NADPH at 550 nm. Cytochrome c (75 µM, 0.3M potassium phosphate buffer, pH 7.8), 0.1 mM NADPH and 0.1 mg of protein were added to the reaction mixture. The decrease in absorbance was measured for 3 mins and the activity was expressed as µmol cytochrome c reduced/min/mg protein.

49. Statistical analysis

All the analyses were carried out using the SPSS/PC (version 17.0; SPSS Inc. Chicago, IL, USA) software package program. Data are presented as means with their standard errors. One-way ANOVA and post hoc Tukey-honestly significant difference test were used to determine the differences among the groups. The degree of significance was set at p≤0.05.