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2.1 Chemicals

5-Hydroxy Tryptamine (serotonin), Norepinephrine, Dopamine, Estradiol, Dehydrepiandrosterone (DHEA), Peroxidase (POD) were procured from Sigma Chemical Co., U.S.A. and all other chemicals were of AR grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India, Qualigens Fine Chemicals, Mumbai, India, Suvidhanath Laboratories, Vadodara, India and Spectrochem Pvt. Ltd., Mumbai, India. Luteinizing hormone (LH, AFP115368)) and Follicle stimulating hormone (FSH, AFP19896) were kind gifts from Dr. Parlow, NIDDKs National Hormone and Pituitary Program (NHPP), USA.

2.2 Animals

Adult virgin male rats of the Charles-Foster strain weighing 180-220 g kept under controlled conditions of light (lights on from 07.00 to 20.00 h) and temperature (24 ± 2°C) and having access to food and water were used.

2.3 **Total RNA extraction** (Chomczynski and Sacchi, 1987) :

Total RNA extraction kit is based on widely used guanidine thiocynate phenol : chloroform procedure of Chomoczynsky and Sacchi. Samples are lysed in highly denaturing condition inactivating RNAses, followed by phenol : chloroform extraction wherein DNA and proteins are removed and then precipitated with isopropanol. An additional step of suspending and reprecipitating ensures further purity of the RNA.

Reagents

1. Denaturing solution.
2. Water saturated phenol.
3. 1X TE buffer.
4. DEPC water.
5. Chloroform-isoamyl alcohol mix (49:1).
6. Isopropanol.
7. 75% ethanol (prepared with DEPC treated water).

Procedure

- Homogenize the sample (100 mg) in 1 ml of denaturing solution.
- Add 1 ml of water saturated phenol followed by 200µl of chloroform-isoamyl alcohol mix (freshly prepared in the ratio of 49:1).
- Mix thoroughly and incubate in ice for 20 minutes.
- Centrifuge at 10000 rpm for 20 minutes at 4°C.
- Transfer upper aqueous phase carefully to another tube.
- Add 1 ml of 100% isopropanol to precipitate RNA. Incubate at -20°C for 30 minutes.
- Centrifuge at 10000 rpm for 20 minutes at 4°C, discard the supernatant.
- Resuspend the pellet in 0.3 ml of denaturation solution, precipitate RNA by adding 0.3 ml of 100% isopropanol, incubate at -20°C for 30 minutes, centrifuge at 10000 rpm for 20 minutes at 4°C and discard the supernatant.
- Resuspend the RNA pellet in 75% ethanol. Incubate at room temperature for 10-15 minutes to dissolve residual amounts of guanidine.
- Centifuge at 10000 rpm for 20 minutes at 4°C, discard the supernatant.
- Do not over dry the pellet. Dissolve in 100-200µl of DEPC water. Incubate for 10-15 minutes at 55°C to ensure complete solubilisation of RNA.
- Store the total RNA at -70°C.

Note: Rnases are very stable enzymes and generally require no cofactors to function. Therefore a small amount of Rnase in an RNA preparation will degrade entire RNA. Hence sample should be handled very carefully.

2.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR essentially has two parts: (1) Using AMV Reverse Transcriptase enzyme one can generate high yields of full-length, first-strand cDNA from RNA and (2) the cDNA thus generated acts as the template for the subsequent PCR amplification. The isolation of undegraded intact mRNA is very essential for successful first strand synthesis and subsequent PCR amplification. Care has been taken to avoid any RNase contamination in the buffers and containers used in RNA preparation.

Reagents

1. Random hexamer (1μg/μl)
2. Oligo $(dT)_{18}$ primer (0.2μg/μl)
3. Rnase inhibitor from human placenta (10U/μl)
4. 5X RT buffer
5. 30mM dNTP mix (7.5 mM each)
6. AMV Reverse Transcriptase (10U/μl)
7. 0.1 M DTT
8. 10X PCR buffer
9. Taq DNA Polymerase (3U/μl)
10. Dilution buffer for Taq. Pol.
11. Total RNA from Hela cells for control reaction
12. G3PDH Forward primer (0.1μg/μl)
13. G3PDH Reverse primer (0.1μg/μl)
14. Sterile water
15. 0.5 M EDTA

Procedure

Step 1 : First strand cDNA synthesis

- 1-5 μl of mRNA sample (10-100ng) of mRNA can be used. If total RNA is to be used, use 5-10 times more total RNA than mRNA) was added to a sterile Rnase-free tube (DEPC treated).
- Sterile water was added to bring volume to 9μl.
- 1 μl of random primer or oligo $(dT)_{18}$ primer was added.
- 5 μg of RNA and 1 μl of oligo $(dT)_{18}$ was used for control reaction.
- The vial was placed at 65^0C for 10 minutes and then at room temperature for 2 minutes to remove any secondary structure.
- Spin the vial briefly.
- Then reagents was added in the following order: (1) 1 μl Rnase inhibitor (2) 1 μl 0.1 M DTT (3) 4 μl RT Buffer (5X) (4) 2.0 μl 30 mM dNTP mix (4) 0.5μl Reverse Transcriptase (5) 1 μl sterile water.

Step 2 : PCR amplification

Each PCR reaction mixture (50µl) contained 0.2 mM of each dNTP mixture, 1 µM of each primer, 2mM $MgCl_2$, Taq polymerase 0.025 units and 1 µl of the cDNA reverse-transcribed as described above. PCR was performed by denaturing at 94°C for 60 sec, annealing for 60 sec, and extension at 72°C for 60 sec. Cycles performed for the amplification were 35.

2.5 17β-hydroxy steroid oxidoreductase, 3α-hydroxy steroid dehydrogenase (3α-HSD) and 3β-HSD/17β-HSD (Shivanandappa and Venkatesh, 1997)

Principle

The assay is based on the formation of NADH due to enzymatic oxidation of the steroid alcohol, which is coupled to the reduction of the tetrazolium via diaphorase present in the tissue.

Reagents

1. 50 mM Phthalate buffer (pH 3.0): 2.55 g of potassium hydrogen phthalate dissolved in a mixture of 51 ml N/10 HCl and 2.5 ml Tween 20; pH adjusted to 3.0 and the volume made upto 250 ml with distilled water.
2. 0.1 M Tris-HCl buffer (pH 7.8)
3. 0.25 M of sucrose buffer (pH 7.4)
4. 5 mM NAD
5. Color reagent : 40 mg INT, 10 mg PMS, and 0.5 ml Tween 20 were dissolved in 50 ml distilled water for the standard curve. For the enzyme assay, PMS was omitted from the reagent.
6. The substrate

   17β hydroxysteroid oxidoreductase: 17β estradiol was first dissolved in minimum amount of alcohol and the stock solution (1 mM) prepared in 50 ml Tris-HCl buffer.

3α hydroxy steroid dehydrogenase: DHEA was first dissolved in minimum amount of diformamide and the stock solution (1 mM) prepared in 50 ml Tris-HCl buffer.

3β-HSD/17β-HSD: DHEA is dissolved in 0.3 ml to 0.5 ml of Dimethyl Formamide (DMFO). 17β estradiol is dissolved in minimum amount of ethanol and then diluted in distil water. Stocks of both substrates were prepared in 50/100 ml Tris-HCl (pH 7.8). DHEA is used as substrate for 3βHSDH and estradiol for 17 βHSDH.

7. Enzyme

17β hydroxy steroid oxidoreductase (liver was used as the source of enzyme): Liver microsomal fraction preparation- 10% homogenate was prepared in cold 0.25 M of sucrose buffer (pH 7.4). The homogenate was centrifuged at 1000 g for 15 min at 4°C. The sediment was discarded and supernatant was centrifuged for 8000 g for 30 min at 4°C. For further fractionation the above supernatant was centrifuged at 15000 g for 45 min and the pellet thus obtained was dissolved in minimum amount of 0.25 M of sucrose buffer (crude microsomal preparation).

3α hydroxy steroid dehydrogenase : 10% homogenate of hypothalamus/ pituitary in 0.25 M of sucrose buffer was centrifuged at 3000 rpm for 20 min and the supernatant was used as enzyme source.

3β-HSD /17β-HSD : 10% testis homogenate was prepared in 0.1 M Tris HCl buffer (pH =7.8). These were then centrifuged at 12000 g at 4° C. Supernatant was used as a source of the enzyme. For leydig cells, 4 x 10^5 cells were sonicated at 5 cycles for 2 min.

Reagents	Blank (ml)	Test (ml)
Tris-HCl	1.06 ml	1.0 ml
NAD	0.5	0.5
Substrate	0.250	0.250
Sample	----	0.06
INT	0,250	0.250
Incubate at 37°C for 1 hr		
Phthalate buffer	1.0	1.0
Centrifuged at 3000 rpm for 20 min; supernatant was read at **490 nm** against blank.		

Standard curve: 1 mM solution of NADH was prepared in distilled water. Aliquots of graded concentrations of NADH (0 to 150 nmol) were reacted with the color reagent (0.5 ml) and after color formed, 1.0 ml of phthalate buffer was added to each tube and absorbance read at 490 nm. A standard curve was prepared by plotting NADH concentration vs absorbance.

Unit: nmols of NADH formed/min/mg protein

2.6 UDP-Glucoronyl transferase (UDPGT) (Gorski and Kasper, 1977)

Principle

UDP-Glucoronyl transferase catalyses transfer of D-glucoronic acid from UDP-α-glucoronuic acid to an acceptor compound with inversion at the C-1 carbon of the sugar to yield the β-glucuronide. The degree of conjugation was based on the disappearance of p-nitrophenol absorption in the visible region. The rate of glucoronide formation was calculated directly from the absorbance change.

Reagents

1. 0.4 M phosphate buffer (pH 7.4)
2. 40 mM MgCl2
3. 1.6 mM p-nitrophenol (PNP)
4. 20 mM UDP-glucoronic acid
5. 0.2 N TCA
6. 0.5 N NaOH

Enzyme: Crude hepatic microsomal fraction, prepared as discussed above.

Protocol

Reagents	Blank(ml)	Test(ml)
Buffer	0.26 ml	0.18 ml
UDPG	----	0.04ml
$MgCl_2$	0.04ml	0.04ml
PNP	-----	0.04ml
Sample	0.1ml	0.1ml
Incubated at 37^0 C for 10 min with shaking		
TCA	0.4ml	0.4ml
Centrifuged at 2500 rpm for 20 min and 0.5 ml supernatant was taken		
NaOH	1.5ml	1.5ml
Read the absorbance at 450 nm		

A molecular extinction coefficient for p-nitrophenol of 18,200 was used to calculate the amount of phenol conjugated.

Unit: Units of PNP conjugated/min/mg protein

2.7 Leydig cell isolation and purification (Bermúdez et al., 1988)

Reagents

1. DMEM/F12, HEPES (250 ml, pH 7.4): 3.6g DMEM, 15 mg MgCl3, 12.5 mg MgSO4, 4.25 mg L-Methionine, 300 mg sodium bicarbonate, 38.6 CaCl2, 91.25 mg L-glutamine, 15 mg leucine, 25 mg L-lysine, 40 µg/ml (Add 250 µl of 40 mg/ml) were dissolve in 200 ml sterile double distilled water with continue stirring. Adjust pH, below 0.2-0.3 than required pH for assay and make up to 250 ml with sterile distilled water. Filter the mixture using 0.22-µ filter and store at 2-8^{0}C.
2. Leydig cell separating medium (Medium-L, pH 7.4): 1.75 mg collagenase (Sigma type-IV) + 5 mg BSA were dissolved in 5 ml of DMEM. Filter the mixture using 0.22-micron filter and store at 2-8^{0}C
3. Pro-100% percoll (pH 7.6): 20 mg DMEM/F12 + 390 mg BSA + 3.9 mg sodium pyruvate dissolved in 13 ml distilled water. Adjust pH to 7.6 and filter it. Store at 2-8^{0}C.
4. 100% percoll (pH 7.6): 1 ml pro-100% percoll + 9 ml 113% percoll (Stock percoll).
5. Percoll gradients were prepared ranges from 5% to 75 %.
6. Phosphate buffer (pH 7.2, 0.1 M).
7. 1 mM Nitrosoblue tetrazolium (NBT) in phosphate buffer.
8. 1.5 mM β-NAD.
9. Dehydroepiendrosterone (DHEA) 0.05 mM in 35% methanol
10. 0.4% Trypan blue solution, containing 0.1% BSA
11. Trypsin inhibitor (35 mg) in 100 ml DMEM.

Isolation procedure

Testis was decapsulated with fine forceps without breaking the semineferon tubules under an aseptic condition. These were incubated with 5 ml medium in 25 ml flasks for 15 minutes at room temperature. The flasks were gently shaken without damaging semineferon tubules and then 10 ml of cold DMEM was added to stop collegenase activity. Allow it to stand for 5 min at RT. Supernatant were transferred to sterilized centrifuge tubes (Repeat this procedure once again to remove the remaining leydig cells in the testis). Supernatant were combined and centrifuge (1000 X g at 4^{0}C for 15 minutes). Pellet was resuspended in 5 ml DMEM (Supernatant was discarded) centrifuge it 1000 X g at 4^{0}C for 15 minutes (Repeated timer for washing the cells). The pellet was resuspended in 5 ml DMEM. This cell suspension is called crude leydig cell suspension.

Leydig cell purification

Leydig cells were purified by using discontinuous percoll density gradient method. For that 2ml of 75% percoll gradient was added slowly to graduated centrifuge tube. Above this layer, 60%, 45%, 30%, 15% and 5% gradients of percoll (2 ml each) were loaded gently one over other, taking care to avoid mixing in an aseptic condition. 1 ml crude leydig cell suspension was added above the percoll gradients. The tubes were centrifuge 1000 X g at 4^{0}C for 20 minutes. Purified leydig cells were observed in between 30% and 45% percoll gradients. This layer of leydig cells was removed carefully and mix with 5 ml of DMEM. These were centrifuge at 800 X g, 4^{0}C for 15 minutes and pellet was resuspended in 2 ml DMEM (Supernatant was discarded). Again centrifuge it 800 X g at 4^{0}C for 15 minutes (Repeated 3 times for to remove percoll present). The pellet was resuspended in 2 ml DMEM for in vitro solution.

Identification of leydig cells (Aldred and Cooke, 1983):

The purified leydig cells were identified by histo-chemical localization of 3β-hydroxysteroid dehydrogenase. For this 100µl leydig cells suspension was incubated with 100µl of β-NAD, 100µl NBT and 100µl DHEA at 37^{0}C for 60

minutes. After incubation the cells were washed trice with phosphate buffer and suspended in the same buffer. The percentage of the positively stained cells was counted using a heamocytometer under the microscope.

2.8 Radio Immuno Assay (RIA) of Testosterone (Tohda et al., 2001)

Principle : The procedure follows the basic principle of radioimmunoassay where there is competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of I^{125} labeled testosterone bound to the antibody is inversely proportional to the concentration of unlabeled testosterone present. The separation of the free and bound antigen is achieved by decanting or aspirating the antibody-coated tubes.

Reagents

1. Testosterone standards (Lyophilized): Six vials, labeled A-F, containing concentrations of approximately 0, 0.1, 0.5, 2.5, 10.0 and 25.0 ng/ml testosterone in serum with sodium azide as a preservative. Vials A and B-F was reconstituted with 1.0 ml and 0.5 ml of deionized water respectively.
2. I^{125} Testosterone
3. Testosterone controls (Lyophilized)
4. Anti-Testosterone-coated tubes.

Sample- Serum, testis homogenate (10 % homogenate in PBS).

Protocol

Reagents	Total counts	Standard	Controls	Test
Standards	---	50µl	----	-----
Controls	----	----	50µl	----
Serum/ tissue Sample	---`	----	----	50µl
Tracer(I^{125} Testosterone)	500µl	500µl	500µl	500µl
Incubate for 1 hrs at 37^0 temperature in a water bath				
Decant thoroughly and blot the tubes to remove any droplets adhering to the rim				
Count for 1 min in gamma counter.				

Calculation

T = Total counts of 50µl of I^{125}-testosterone. B_0 = CPM of bound with I^{125}-testosterone in absence of testosterone (zero binding). B = CPM of bound with I^{125}-testosterone in presence of testosterone standard. %B/T and % B/B_0 was

calculated for testosterone standards and serum/ tissue samples. A logit-log graph was plotted against % B/B_0 and concentration of testosterone standards. Serum/tissue testosterone concentrations were calculated from the graph.

Units: ng of testosterone present in ml serum or mg/g tissue.

2.9 Radio Receptor Assay (RRA)

Luteinising Hormone Receptor [LH-R] and the follicle-stimulating hormone receptor [FSH-R] belongs to the class of transmembrane receptors, having seven membrane spanning regions. The ligand-binding domains of gonadotropin receptors-LH-R and FSH-R are thought to be composed of the β strands of the nine leucine-rich repeats of their N-terminal extracellular domains. These are present on the membrane of leydig cells and the interaction of gonadotropins with their receptors mediates activation of testosterone synthesis and spermatogenesis.

Iodination of Luteinising Hormone (r-LH) / Rat Follicle Stimulating Hormone (r-FSH) (Greenwood et al., 1963)

Reagents

1. Phosphate Buffered Saline (0.05 M PBS, pH =7.4)
2. Sephadex G-25 Column.
3. 1% BSA
4. Chloramine-T (1.5 mg/ml) in 0.05 M Phosphate Buffer.
5. 25 mM Sodium Meta-bisulfite in 0.05 M phosphate buffer.
6. 1% Potassium Iodide (KI), 8% Sucrose.

Protocol for Iodination

50 µl/5 mg of r-LH or r-FSH, 5 µl Na-I^{125} and 7.5 µl of Chloramine-T was incubated together for 45 seconds at 25 ° C and reaction was terminated by a mixture of 50 µl Sodium- Meta bisulfite, 100 µl 1% KI and 8% Sucrose.

Purification of Iodinated Gonadotropins by Gel filtration

- Sephadex G- 25 column was washed with 20 ml of PBS (pH=7.4) and then equilibrated using the PBS.
- 200 µl of the reaction mixture was loaded into the column.
- Ten drops per eppendroff was collected.
- Aliquot of10 µl of the each fraction was counted.
- Fraction that had maximum counts is used as iodinated gonadotropins.

Saturation Kinetics

Reagents

1. 0.5 M NaOH
2. 0.3 M PBS (pH =7.4) containing 0.2% BSA
3. 50 mM Glycine-HCl Buffer (pH =3.0)
4. Unlabelled FSH or Unlabelled LH
5. Labelled LH or labelled FSH

Protocol

- 1ml of Glycine-HCl buffer was added to the tube containing 1x 10^5 leydig cells and was incubated for 2 min at room temperature.
- Tubes were then centrifuged at 1500 rpm for 10 min.
- Unlabelled r-LH/ r-FSH was added in excess to the non- specific binding tubes followed by incubation for 1-1.5 h, which was followed by addition of labeled hormone.
- Various concentrations of iodinated LH (10,000, 20,000, 30,000, 40,000 counts) or iodinated FSH (10,000, 20,000, 30,000 counts) was added to the tubes.
- All tubes were incubated at 4 ° C for 18 hours.
- 1ml cold PBS containing BSA was added to all tubes and was shaken well.
- All the tubes were then centrifuged at 1500 rpm / 4° C for 10 min.
- Pellet was washed with cold PBS for 3 to 4 times and counts were taken in the pellet.
- Plot the graph counts of labelled hormone versus % B/F (% Bound hormone/ Free hormone)

Binding of LH and FSH in leydig cells (Thanki and Steinberger, 1976)

Reagents

1. 0.5 M NaOH
2. 0.3M Phosphate Buffer Saline (pH =7.4) containing 0.2% BSA
3. 50 mM Glycine-HCl Buffer (pH =3.0)

Protocol

- 1ml of Glycine-HCl buffer was added to the tubes containing 1x 10^5 cells and incubated for 2 min at room temperature and centrifuged at 1500 rpm for 10 min.
- Non-specific binding tube containing the excess of unlabelled LH/ FSH incubated for 1-1.5 hrs.
- I^{125}-r-LH/ I^{125}-r-FSH (30,000 counts/20,000 counts) was added to the tubes and incubated at 4 ° C for 18 h.
- 1ml cold PBS was added to the tubes, shaken well and followed by centrifugation at 1500 rpm/4 ° C for 10 min.
- Cold PBS (pH=7.4) was added and the pellet was washed thrice and then counted in the gamma counter.

Calculation

For LH-receptor: 0.033nMoles corresponds to 23,000 cpm (calculated from total counts of iodinated fraction in relation to bio-reactivity of iodinated LH)

For FSH receptor: 0.033nMoles corresponds to 25,000 cpm (calculated from total counts of iodinated fraction in relation to bio-reactivity of iodinated FSH)

Units: Femtomoles of LH-R/ Femtomoles of FSH-R per 100,000 cells

2.10 Amine measurements

Amines were estimated by the flourimetric method of Shellenberger and Gordon, 1971.

Reagents

1. 0.4 M/0.2 M perchloric acid
2. 0.1% (wt/vol) Na_2S_2O5 in perchloric acid
3. 0.025% Na_2EDTA in perchloric acid
4. Alumina
5. 10 M NaOH
6. 0.5 M / 0.1 M borate buffer (pH 10):
7. Saturated NaCl solution
8. Butanol
9. 0.05 M phosphate buffer
10. N-heptane
11. 0.1 M phosphate buffer-EDTA solution
12. 2.5 % alkaline sodium sulfite solution
13. Iodine reagent (2.0 g KI and 0.5 g Iodine in 40 ml DW)
14. 0.1 M Ninhydrin solution

Tissue sample preparation

The hypothalamus was dissected out immediately, weighed and homogenized in chilled 0.4 M perchloric acid solution containing 0.1% (wt/vol) Na_2S_2O5 and 0.025% Na_2EDTA at 4^0C in a motor driven glass homogenizer. The homogenates were centrifuged at 4000 g at 4^0C for 15 min. The supernatant was taken and mixed with 25 mg Alumina. The solution was shaken well, centrifuged at 500 g for 5 min and the supernatant was used for 5-HT estimation. Alumina was further washed four times with 5 ml of H_2O, and extracted with 150 µl 0.2 M $HClO_4$ solution. The supernatant was used for the estimation of norepinephrine and dopamine.

Estimation of Norepinephrine (NE) and Dopamine (DA)

1 ml aliquot of the perchloric acid eluate was made to pH 6.5±0.2 with 1.5 ml of 0.1 M phosphate buffer-EDTA solution. 0.2 ml of the iodine reagent was added (shaking immediately to mix) kept for exactly 2 min, after which 0.5 ml of the alkaline sodium sulfite solution was added. After 2 min. 0.4 ml of glacial

acetic acid was added to bring the pH to 4.4 - 4.8 and kept in hot air oven at 100°C for 3-4 min. Tubes were placed in ice bath for cooling and the NE fluorescence was read at 380/495 nm. Returned the samples to oven and heated at 100°C for an additional 40 min to develop DA fluorescence. After cooling the tubes DA fluorescence was read at 325/380 nm. The accuracy and precision of the assay were checked by running, in parallel, different concentrations of external and internal standards (125-1000 ng) and blanks with tissue samples. The fluorescent intensity was proportional to standard concentration over the range 0.008-0.5 ug/ml.

2.11 Metal analysis

Principle

The absorption of energy by atoms in the ground state forms the basis of atomic absorption spectroscopy. When a solution containing metallic species is introduced in the flame the vapours of the metallic species is formed. Most of the metal atoms remain in the ground state and absorb light of their own wavelength having specific resonance. The amount of light absorbed will be directly proportional to the concentration of the free atoms in the flame given by the Beer- Lambert Law.

$$\textbf{Absorbance} = \log_{10} \frac{I_0}{I_t} = \textbf{K.C.L}$$

I_0 = intensity of incident radiation emitted by light, I_t = intensity of transmitted radiation (amount not absorbed), C = concentration of sample, K = constant (can be obtained experimentally), L = path light.

Sample preparation

Tissue samples were digested in reagent grade nitric acid-perchloric acid (2:1) mixture. The digestion was continued till samples become colorless. Then the acid mixture was evaporated and the precipitate thus obtained was dissolved in a few drops of concentrated HCl. The samples were diluted to one ml. with distilled water and then read in GBC 902 double beam atomic

absorption spectrophotometer. Sensitivities of the assay for lead and cadmium were calculated.

	Lead (Pb)	**Cadmium (Cd)**
Range of standard concentration	2.5 - 20 ug/ml	0.2 - 1.6 ug/ml
slit width	1.0 mm	0.5 mm
wavelength	217 nm	228.8 nm
flame	air acetylene	air acetylene
sensitivity	0.06	0.009
cathode lamp	lead	cadmium

$$\text{Ug/ml} = \frac{\text{Total volume in test tube} \times \text{conc. in ppm (as obtained in AAS)} \times \text{total residual volume}}{\text{Amount of sample taken before dilution} \times \text{volume of blood taken (or weight of tissue)}}$$

2.12 Oxidative parameters

Tissues (testis, liver, prostate and pituitary) will weighed, 10% homogenized in chilled (4°C) isolation medium: 0.25 M sucrose, 10 mM Tris-HCl buffer pH 7.4, 1 mM EDTA and 250 µg BSA/ml. The isolation of mitochondria and post-mitochondrial fractions will according to the procedures describe previously (Swegert et al., 1999; Kaushal et al., 1999). The mitochondrial and post-mitochondrial fractions obtained will subjected to biochemical estimations/analysis of reactive oxygen species related parameter. ROS parameters include **(a)** Reduced Glutathione **(b)** Lipid Peroxidation Levels **(c)** Superoxide Dismutase **(d)** Catalase **(e)** Glutathione Peroxidase **(f)** Glutathione Reductase **(g)** Glucose 6-phosphate dehydrogenase **(h)** Glutathione-S-transfarease. The activity of Alkaline phosphatase (ALP) and Acid phosphatase (ACP) will be estimated in post-mitochondrial fraction. Protein estimation will be carried out to express enzyme activity in terms of specific activity (Lowry et al., 1951).

(a) Reduced Glutathione (GSH) (Beutler and Gelbart, 1985)

Principle : 5-5′ Dithiobis (2 nitrobenzoic) acid (DTNB) is a disulfide compound which is readily reduced by sulfhydryl compounds forming a highly colored yellow anion, which can be read at 412 nm.

Reagents

1. Precipitating (ppting) reagent: 1.67g metaphosphoric acid (MPA), 0.20g EDTA, 30g NaCl, make volume to 100ml with D/W)
2. 0.3M Na_2HPO_4.
3. DTNB: 40 mg DTNB dissolved in 100 ml 1% sodium citrate (freshly prepared).
4. PBS (0.1M, pH 7.4).
5. Standard GSH solution: 2mM GSH. (Standard range 10-100µg)

Protocol

Reagents	Reagent Blank	Substrate Blank	Enzyme Blank	Test
MPA (ml)	1.0	1.0	1.0	1.0
Aliquot (cyto/mito)	-	-	0.1	0.1
D/W (ml)	0.1	0.1	0.03	-
Keep in ice for 10′, Ʊ3000 rpm/15′, take supernatant				
Supernatant (ml)	0.4	0.4	0.4	0.4
Na_2HPO_4 (ml)	0.6	0.6	0.6	0.6
DTNB (ml)	-	0.03	-	0.03
Immediately take O.D. $_{412}$				

Unit : pmole / mg protein

(b) Lipid Peroxidation Levels (LPO) (Ohkawa and Ohishi, 1979)

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA) which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substance (TBARS). TBARS gives a characteristic pink color which can be measured colorimetrically at 532 nm.

Reagent

1. TBA reagent: TBA(100mg),EDTA(46mg),20%TCA(10ml), 2.5 N HCl (5 ml) total volume was made up to 20ml with D/W (freshly prepared) .
2. 0.1M Phosphate buffered saline (PBS, pH 7.4)

3. 10mM Tetra methoxy propen (TMP) for standard solution. Standard range (1-10 nmoles)

Procedure

Reagents	Blank	Test
Aliquot (cyto/mito) (ml)	-	0.1
D/W (ml)	1.0	0.9
TBA reagent (ml)	1.0	0.9
Keep in boiling water bath for 20', afterwards cool tubes, ↺3000 rpm/15', take supernatant & measure O.D. $_{532}$		

Unit : nmoles of MDA formed / mg protein

(c) Superoxide Dismutase (SOD) (Marklund and Marklund, 1974)

Principle

The superoxide dismutase activity inhibitis pyrogallol auto-oxidation under standard assay condition, the reaction being measured at 420 nm.

Reagents

1. KPO_4 (0.2M, pH 8)($k2HPo_4$ +k_2HPO_4)
2. Pyrogallol: a pinch of pyrogallol is dissolved in 0.5N HCl, so as to achieve auto-oxidation within 0.1 to 0.120 O.D./180 sec (freshly prepared)

Protocol

Pyrogallol auto-oxidation: 0.5ml buffer + 0.450 ml D/W (autozero)
+ 0.050 ml pyrogallol (start)

Auto-oxidation should be between 0.1 to 0.120 O.D. /180 secs

Now, proceed as per protocol, reaction starts by addition of pyrogallol.

Reagents	Blank	Test
Aliquot (cytosol/mitochondria) (ml)	-	0.03
KPO_4 (ml)	0.5	0.5
D/W (ml)	0.45	0.42
Pyrogallol (ml)	0.05	0.05
O.D. $_{420}$		

Unit : Enzyme required for 50% inhibition of pyrogallol auto-oxidation

(d) Catalase (CAT) (Hugo, 1987)

Principle: Catalase is a heme containing enzyme which catalyzes dismutation of hydrogen peroxide into water and oxygen. Decomposition of hydrogen peroxide by catalase is measured spectrophotometrically at 240 nm, since hydrogen peroxide absorbs UV light maximally at this wavelength.

$$H_2O_2 \xrightarrow{CAT} H_2O + O_2$$

Reagents

1. KPO_4 (50mM, pH 7)
2. H_2O_2 (10.3mM) (freshly prepared)

Protocol

Blank : KPO_4 buffer + D/W (autozero) + H_2O_2 (start)

Test : KPO_4 buffer + D/W + Aliquot (autozero) + H_2O_2 (start)

Reagents	Blank	Test
Aliquot (cyto) (ml)	-	0.03
KPO_4 (ml)	0.5	0.5
D/W (ml)	0.45	0.42
H_2O_2 (ml)	0.05	0.05
$O.D._{240}$		

Unit : mmoles of H_2O_2 decomposed/mg protein

(e) Glutathione Peroxidase (GPx) (Hafeman et al., 1994)

Principle : Glutathione peroxidase leads to the H_2O_2 mediated oxidation of reduced glutathione, which reacts with 5-5′ Dithiobis (2-nitrobenzoic) acid (DTNB) to give yellow colour measurable at 412 nm.

Reagents

1. KPO_4 (0.4M, pH 7)
2. 30 mM Metaphosphoric acid (MPA)
3. Glutathione (2mM) (freshly prepared)
4. H_2O_2 (10mM) (freshly prepared)
5. NaN_3 (10mM) (freshly prepared)
6. Na_2HPO_4 (0.4M)
7. DTNB (40mg DTNB dissolved in 100ml 1% sodium citrate) (freshly prepared)

Protocol

Reagents	Reagent blank	Substrat blank	Enzyme blank	Test
KPO_4 (ml)	0.1	0.1	0.1	0.1
Glutathione (ml)	-	0.1	-	0.1
NaN_3 (ml)	0.1	0.1	0.1	0.1
Aliquot (cyto/mito)	-	-	0.02	0.02
D/W (ml)	0.2	0.1	0.18	0.08
H_2O_2 (ml)	0.1	0.1	0.1	0.1
Incubate for 5' at 37°C (water bath)				
MPA (ml)	0.4	0.4	0.4	0.4
Keep in ice for 10', ↺2000 rpm/10', take supernatant				
Supernatant (ml)	0.6	0.6	0.6	0.6
Na_2HPO_4 (ml)	0.6	0.6	0.6	0.6
DTNB (ml)	0.03	0.03	0.03	0.03
Immediately take O.D. $_{412}$				

Calculation was done according to the slope calculated from the standard graph.

$$\text{GPx Activity} = \frac{[\{\log (SB) - \log (EB)\} - \log (Test)] \times \text{Dilution}}{\text{Slope} \times \text{Aliquot volume (ml)} \times \text{Protein (mg/ml)}}$$

Slope = 0.001

Unit : Δlog (GSH) / min / mg protein

(f) Glutathione Reductase (GR) (Smith et al, 1988)

Reagents

1. potassium phosphate buffer(0.2 M, pH-7.5) + 1 mM Na_2EDTA buffer
2. 3 mM DTNB in 0.01 M phosphate buffer
3. 2mM NADPH in 10 mM Tris-HCL buffer
4. 20 mM GSSG

Protocol

Reagents	Volume (µl)
Buffer	500
DTNB	250
DW	175
NADPH	50
Sample	25
Auto zero and reaction was initiated by adding substrate (GSSG)	
GSSG	50
The increase in absorbance was monitored for about 3 min at **412 nm.**	

The enzyme activity was calculated using the extinction coefficient (E_{412} = 13.6 L mmol $^{-1}Cm^{-1}$).

Unit : Amount of enzyme that catalyzes the reduction of 1 µmole of NADPH per minute per mg protein.

(g) Glucose 6-phosphate dehydrogenase (G6PDH) (Cohen and Rosemeyer, 1975)

Reagents

1. 0.2 M Tris buffer (pH-7)
2. 0.01 M $MgCl_2$
3. 6 mM Glucose 6-phosphate
4. 3mM NADP

Protocol

Reagents	Volume (µl)
Buffer	500
G-6-P	100
$MgCl_2$	100
Sample	50
DW	150
Auto zero and reaction was initiated by adding substrate (NADP)	
NADP	100
The increase in absorbance was monitored for about 3 min at **340 nm.**	

The enzyme activity was calculated using the extinction coefficient (E_{412} = 6.22 L mmol $^{-1}Cm^{-1}$).

Unit : Amount of enzyme that catalyzes the formation of 1 µmole of NADPH per minute per mg protein.

(h) Glutathione-S-transfarease (GST) (Pabst et al, 1974)

Reagents

1. Buffer (pH-6.5); 0.1 M sodium phosphate + 1 mM Na_2EDTA buffer
2. 20 mM reduced glutathione in deionized water
3. 20 mM CDNB (1-Chloro-2,4-Di Nitro Benzene) in 95 % ethanol.

Protocol

Reagents	Volume (µl)
Buffer	850
Reduced glutathione	50
CDNB	50
Auto zero and reaction was initiated by adding enzyme	
Sample	50
The increase in absorbance was monitored for about 3 min at **340 nm.**	

The enzyme activity was calculated using the extinction coefficient (E_{412} = 9.6 L $mmol^{-1}Cm^{-1}$).

Unit: Amount of enzyme that catalyzes the conjugation of 1 µmole of CDNB per minute per mg protein.

2.13 Alkaline phosphatase (ALP) and Acid phosphatase (ACP) (Bowers & McComb, 1975)

These hydrolytic enzymes are present in high concentrations in liver, bone, placenta and intestinal epithelium. Increased ALP levels are frequently encountered as a clinical evidence of liver damage.

Principle : The enzyme reacts with p-Nitrophenyl phosphate (PNPP) and converts it into p-nitrophenol (PNP), which is yellow in color. PNP in alkaline medium gives bright yellow coloured phenolic ions, which can be measured at 405 nm.

Reagents

1. PNPP -0.4% (freshly prepared)
2. 0.05 N NaOH
3. 0.05 M Glycine buffer/0.05 M Citrate buffer
4. Working buffred substrate- equal volume of glycine/ citrate buffer and stock PNPP mixed and pH was adjusted to 10.4.
5. Standard PNP solution - Stock- 1 mm in 0.05 N NaOH (freshly prepared);
   Working standard - 0.04 mM made in 0.05 NaOH
   Standard range - 0.04-0.16 umols

Reagent	Control	Test
Working buffered substrate	0.4 ml	0.4 ml
Placed in water bath at 37C for 5 min		
Sample	—	0.05 ml
0.05 N NaOH	4.0 ml	4.0 ml
Serum/ tissue	0.05 ml	—
Absorbance was recorded at 405 nm		

Calculation : Calculation was done according to the slope calculated from the standard graph.

Unit : μ mols PNP formed/min/g tissue

Acid phosphatase (ACP)

Principle

The enzyme reacts with p-Nitrophenyl Phosphate (PNPP) and converts it into p-Nitrophenol (PNP), which is yellow in colour. PNP in acidic medium gives bright yellow coloured phenolic ions, which can be measured at 405 nm.

Reagents

1. Acid buffer/substrate solution (50mM citrate buffer, pH 4.8, 5.5mM PNPP)
2. Tartarate (0.4M)
3. NaOH (0.1N)

   Standard PNP solution: 1 mM stock in 0.1 N NaOH (freshly prepared)

   Working standard: 0.04 mM made in 0.1 NaOH

   Standard range: 0.04-0.16 μmols

Protocol

Reagent	Sample A	Sample B
Buffer/Substrate solution (ml)	1.0	1.0
Tartarate solution (ml)	—	0.05
Sample (ml)	0.2	0.2
Mix & incubate for exactly 30' in water bath		
Sample	2.0	2.0
Absorbance was recorded at 405 nm		

Read against blank (without tartarate) to which the homogenate is added post NaOH. The increase in extinction, ΔE over the blank is used for calculations. The

activity of Prostatic ACP is obtained by the difference between the activities of Sample A and Sample B (the total ACP activity is measured in Sample A). Yellow colour stable for hours. Calculation was done according to the slope calculated from the standard graph.

Unit : IU (Enzyme required to hydrolyse 1µmole of PNP/min at pH 4.8 at 37°C).

2.14 Estimation of DNA (Burton, 1968)

Principle

The method of estimation of nucleic acid content in tissue is based on the extraction of nucleic acid by hot acid and further hydrolysis in alkali. In hot acid structural chain formed of deoxy pentose is converted into highly reactive β hydroxy levulaldehyde, which reacts with Diphenyl amine to give blue colored complex which is estimated colorimetrically.

Reagents

1. Phosphate buffered saline (0.1M PBS; pH 7.4)
2. Diphenylamine
3. Ethyl alcohol 95%
4. Trichloro acetic acid (TCA) 10%

Sample preparation : 10% liver homogenate was prepared in PBS. From this homogenate 1 ml sample was pipetted out and to this 2.5 ml cold 10% TCA was added. Centrifuged at 4000 rpm for 15 min. Pellet was suspended again in 2.5 ml 10% TCA. Centrifuged at 4000 rpm for 15 min and the pellet was dissolved in 5 ml of 95% ethyl alcohol. Centrifuged at 4000 rpm for 15 min. and repeated the same procedure twice. The pellet was then dissolved in 5 ml of 5% TCA, heated at 90°C for 30 min. Cooled and centrifuged at 4000 rpm for 20 min. The supernatant was used for nucleic acid estimation.

Standard : Standard graph for DNA was prepared in the range 10-200 ug/ml.

Protocol

Reagent	Test	Blank
Sample	1 ml	—
PBS	—	1.0 ml
DPA solution	2.0 ml	2.0 ml
Kept in boiling water bath for 20 min. and read the absorbance at **660 nm**		

2.15 Estimation of RNA (Schneider, 1957)

Principle

Under acidic conditions the ribose sugar present in the RNA is converted into furfural, which then reacts with Orcinol reagent to form, blue/green colored complex.

Reagents

1. Phosphate buffered saline (0.1M PBS; pH 7.4)
2. Standard RNA
3. Orcinol reagent
4. Ethyl alcohol 95%
5. 10%Trichloro acetic acid (TCA)

Sample preparation-as described for DNA estimation

Standard : Standard graph for RNA was prepared in the range 10-80 ug/ml.

Protocol

Reagent	Test	Blank
Sample	1 ml	—
PBS	—	1.0 ml
Orcinol Reagent	1.5 ml	1.5 ml
Kept in boiling water bath for 25 min. and read the absorbance at 660 nm		

Unit : ug/g tissue

2.16 Glutamate Pyruvate Transaminase (GPT) (Reitman and Frankel, 1957)

Principle

GPT transfers amino group from Alanine to α-keto glutarate (α-KG) and convert it into pyruvate. The pyruvate thus formed is reacted with 2,4 Di Nitro Phenyl Hydrazine. The resulting hydrazone of pyruvate is highly colored and its absorbance at 540 nm is proportional to GPT activity.

$$\text{L-Alanine} + \alpha\text{-keto glutarate} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{Glutamate}$$

$$\text{Pyruvate} + \text{DNPH} \longrightarrow \text{dark brown colour in alkaline medium}$$

Reagents

1. Buffered substrate (pH 7.4): Dissolved 15 g Di potassium Hydrogen Phosphate, 2 g Di Hydrogen Potassium phosphate, 300 mg of α keto glutarate, 17.8 g Alanine in 800 ml DW and made up the volume to 1 litre. Adjusted the pH to 7.4 with NaOH.
2. 2, 4 Di Nitro Phenyl Hydrazine (DNPH). Dissolved 200 mg in 250 ml of 1 N HCl and made up the volume to 1 litre with 1 N HCl.
3. 400nM NaOH.
4. Sodium pyruvate (44 mg %)
5. Sample -10% liver homogenate in PBS

   (Standard range - 22-110 ug)

Protocol

Reagents	Test	Blank
Sample	0.05 ml	0.05 ml
Substrate	0.25 ml	-----
Distill water	-----	0.25 ml
Incubate at 37 °C for 30 min.		
DNPH	0.25 ml	0.25 ml
Keep at RT for 20 min.		
0.4 M NaOH	2.5 ml	2.5 ml
Kept at RT for 5 minutes and absorbance was read at 540 nm.		

Unit : μmoles of Pyruvate formed/ min/ litre

2.17 PSA level (Monobind USA Kits)

The commercial PSA assays measure this glycoprotein in the serum using immunoassays. The discrepancy of results between different commercial assays is well recognized and causes a lot of problems in diagnosing and properly staging CaP. The standardization of these assays will eliminate or at least minimize most of these problems. The Monobind USA Kits works on the principle of ELISA. The kit consists of antibody-coated tubes. The PSA antigen obtained from blood serum is added into the antibody-coated tube, which binds to the antibody. A substrate is added which leads to colour formation. PSA is reported in terms of nanograms of protein per milliliter of serum (ng/ml). The normal PSA range is 0 to 4ng/ml serum PSA values above 4ng/ml indicate a

prostate disorder. The scale is open-ended. Men with advanced and widespread prostate cancer can have PSA readings over 2,000 ng/ml.

2.18 Histology

Testis and liver were removed and fixed in Bouins fixative. Histological examination was carried out by standard histological techniques. Sections of 5μm thickness were cut and stained with hematoxylin : eosin. Histological observations were made under the light microscope.

2.19 Rat sperm isolation

After animal sacrificed by cervical dislocation, testes were removed with adherent epididymis and vas deferens. Epididymis and vans deferens were separated from testis and were put into 2 ml prewarmed PBS, pH 7.4. Sperm were allowed to diffuse after the epididymal tubule was pierced with a no. 11 scalpel blade and sperm was forced out of the vas deferens with fine forceps by putting pressure on the lower region of the cauda epididymis and "walking" the forceps down the vas deferens, not forcing out excess material, i.e., immature cells. The dish was shaken gently and, after 5 min of dispersion, an aliquot of sperm was used for sperm count, viability and motility.

2.20 Sperm Count and Viability (Eliasson R., 1977)

An aliquot of sperm was diluted 1:100 with fixative (10% formalin in PBS, pH 7.4) and counted using a haemocytometer. Sperm viability was performed by the eosin nigrosin staining. One drop of semen was mixed with two drops of 1% eosin Y. After 30 s, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of mixture on a clean glass slide and allowed to air dry. The prepared slide was examined using a phase contrast microscope. Pink-stained dead sperm were differentiated from unstained live sperm, and there numbers were recorded.

2.21 Sperm Motility

Epididymal sperm motility was evaluated in the PBS, pH 7.4. A 50-µl aliquot was diluted 20 times in PBS 37°C, and transferred to a glass slide. Under a light microscope (10X magnification), a random field was chosen, and sperm classified as motile or immotile. Sperm motility was expressed as the percentage of motile sperm per field.

2.22 Determination of fructose concentration from prostate and seminal plasma (Motoshima and Settlage, 1978)

The prostate and seminal vesicle was removed, weighed and stored at -20°C to determine the content of fructose. Fructose in seminal plasma reacts with resorcinol in concentrated HCl solution to form red compound under heating. 0.1 ml of fresh sample was mixed with 2.9 ml of distilled water. Then, 0.5 ml of Ba(OH)2 solution (0.15 mol/L) and 0.5 ml of ZnSO4 solution (0.175 mol/L) were added, mixed, and then stood for 5 min to remove seminal proteins. After centrifugation at 3000 g for 15 min, 1 ml of the supernatant was collected for determination of fructose level. The supernatant was replaced with standard fructose solution (0.28 mmol/L) to serve as standard and replaced with distilled water as blanks. Subsequently, 1 ml of resorcinol solution (8.47 mmol/L) and 3 ml of HCl (10 mol/L) were added into tubes, and maintained at 90°C for 10 min. Lastly, absorbance (A) values were read at 490 nm against blanks. Fructose concentration in seminal plasma was expressed as mmol/L: A value of test / A value of standard × 11.12.

2.23 Statistical analysis

Statistical analyses of data was done by one-way analysis of variance and all groups were compared by means of Dunnett's test, with significance set at $p<0.05$. All values represent the mean ± S.E.M. Data were analyzed using PRISM version 3.0 Graph Pad software.

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