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2.1 Chemicals

Pregnant mare serum gonadotropin (PMSG), Norepinephrine, Dopamine, Estradiol, Dehydrepiandrosterone (DHEA), cytochrome c, 17β-estradiol, DHEA 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, , India and Spectrochem Pvt. Ltd., Mumbai, India. Dulbecco's Modified Eagle's Medium (DMEM), TRI Reagent and Caspase 3 Assay Kit, Colorimetric were purchased from Sigma Co., U.S.A. Lyophilized aliquot of antisera to the StAR protein was a kind gift from Dr. Douglas M. Stocco, Dept of Cell Biology and Biochemistry; Texas Tech University, U.S.A. Oligoprimers of HPSF purification grade were purchased from MWG-Biotech AG, Bangalore, India. EIAgen Estradiol and progesterone kit were procured from Adalitis, Italy. Reverse-transcription (RT) and Polymerase chain reaction (PCR) reaction kits were purchased from Fermantas Co., EU.

2.2 Animals

Adult female and 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. The experimental studies were performed after the approval from the Animal Ethical Committee of Department of Biochemistry, The M.S.University of Baroda and were in accordance with the rules of ethical committee.

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 10,000 rpm for 20 minutes at 4^0C.
- Transfer upper aqueous phase carefully to another tube.
- Add 1 ml of 100% isopropanol to precipitate RNA. Incubate at -20^0C for 30 minutes.
- Centrifuge at 10,000 rpm for 20 minutes at 4^0C, 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 -20^0C for 30 minutes, centrifuge at 10000 rpm for 20 minutes at 4^0C 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 4^0C, discard the supernatant.
- Do not over dry the pellet. Dissolve in 100-200µl of DEPC water. Incubate for 10-15 minutes at 55^0C to ensure complete solubilisation of RNA.
- Store the total RNA at -80^0C.

Note: RNAase is very stable enzyme and generally requires no cofactors to function. Therefore a small amount of RNAase 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 un-degraded 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.

Table 1. Sequences of oligonucleotide primers used for PCR amplification

Target gene	GenBank accession no./Reference	Product size (bp)	Target name of gene and primer sequences	Cycle used	Anneal Temp. °C
CYP11a	NM_017286	510	Cytochrome P-450 side chain cleavage(P450scc) 5′-AGA TCC CTT CCC CTG GTG ACA ATG-3′ FP 5′-CCA GGC GCT CCC CAA ATA CAA CA-3′ RP	30	61
StAR	NM_031558	330	Steroidogenic acute regulatory protein(StAR) F-5′-AGG CAG GGG GAT CTT TCT AA-3′ FP R-5′-TGC CTG ACT AGG GTT TCG TT-3′ RP	30	57
CYP19	NM_017085	493	Cytochrome P450 aromatase (P450arom) 5′-GGA ATC CAT CAA GCA GCA TT-3′ FP 5′-TTC CAC CTC CGG ATA CTC TG-3′ RP	30	58
17β-HSD	AF035156	653	17β-hydroxy steroid dehydrogenase type I 5′-CCT CTT TCG CCA CTA TCA GC-3′ FP 5′-GGA GAC AAA TGA GGG CTC-3′ RP	30	55
3β-HSD	M38178	427	3β-hydroxy steroid dehydrogenase type I 5′-ATG CCC AGT ACC TGA GGA GA-3′ FP 5′-TTG AGG GCC GCA AGT ATC A-3′ RP	30	61
FSHR	Holland *et al.*, 2001	311	5′-ACC ATT GTG TCC TCA TCA AGC-3′ FP 5′-GAA ACC TCA TCC GCT ACC CAC-3′ RP	28	65
PR	Svensson *et al.*, 2000	210	5′-CTG CTG GAT GAG CCT GAT GGT G-3′ FP 5′-CAC CAT CCC TGC CAG GAT CTT G-3′ RP	30	57
IGF-1	J02743	277	5′-GAC AGG AAT CGT GGA TGA GTG-3′ FP 5′-AAC AGG TAA CTC GTG ACG AGC-3′ RP	35	59
Bcl-2	Sato *et al.*,1994; Tilly *et al.*,1995	349	5′-CTG GGG ATG ACT TCT CTC G-3′ FP 5′-GGA GAA ATC AAA CAG AGG TC-3′RP	40	55
Bax	Sato *et al.*,1994; Tilly *et al.*,1995	301	5′-AGA CAC CTG AGC TGA CCT TG-3′FP 5′-GTC CCG AAG TAG GAG AGG AG-3′RP	40	55
GnRH	Adelman *et al.*,1986	477	5′-GCA CTA TGG TCA CCA GCG GG-3′FP 5′-CAT GGA TCT CAG CGT CAA TG-3′RP	35	50
FSHβ	D00577	757	5′-TGT ACG AGA CCA TAA GAT TG-3′FP 5′-TTG AGT ATC CTA ACC TTG TG-3′RP	22	60
LHβ	J00749	292	5′-CTG GCT GCA GAG AAT GAG T-3′FP 5′-GAA GGT CAC AGG TCA TTG G-3′RP	20	60
β-actin	V01217	505	5′-CCT GCT TGC TGA TCC ACA-3′FP 5′-CTG ACC GAG CGT GGC TAC-3′RP	30	58

2.5 Western Blotting Analysis

Principle

This method is based on separation of proteins according to size and can also be used to determine the relative molecular mass of proteins. These proteins are then blotted onto the nitrocellulose paper against which specific primary antibody is added so as to separate only protein of our interest. Against this primary antibody, enzyme conjugated secondary antibody is added, which could be detected adding substrates for this enzyme.

Reagents

(1) Running gel (12%)

30% Acrylamide	-	2.0 ml
Running gel buffer (1.875 M)	-	1.25 ml (pH - 8.3)
10% SDS	-	0.05 ml
Water	-	1.675 ml
10% APS	-	60 µl (Freshly prepared)
TEMED	-	6 µl

(2) Stacking gel (5%)

30% Acrylamide	-	0.5 ml
Stacking gel buffer (0.5 M)	-	0.38 ml (pH- 6.8)
10% SDS	-	0.03 ml
Water	-	2.1 ml
10% APS	-	30 µl (Freshly prepared)
TEMED	-	3 µl

(3) 30% Acrylamide solution

(4) 10% APS:

(5) Lysis buffer, (pH - 7.4): -

(6) 10 X Tris (121 M.Wt.)

(7) 10X Transfer buffer (250 mM Tris + 1.92 M Glycine): -

(8) Blocking buffer

(9) TBS (20 mM Tris, 500mM Nacl, pH 7.5)

(10) T-TBS (0.2 % Tween - 20 in TBS)

(11) 6X Sample buffer: 12% SDS, 40% glycerol, 30% β-merceptoethanol, 120 mM EDTA, 1 mg/ml bromophenol blue, and 373 mM Tris HCl (pH 6.8)

Procedure

Ovarian/Testicular homogenate (10%) was prepared with lysis buffer and centrifuged to get mitochondrial fraction following differential centrifugation. The mitochondrial proteins (100 µg) were solubilized in a sample buffer containing 25 mM Tris Cl, pH 6.8, 1% SDS, 5% mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue, and were separated onto 12% SDS-PAGE under reducing conditions, along with M.wt. Markers. The separated proteins were then electrophoretically transferred to nitrocellulose membrane and allowed to run at 38 V, 70 mA for 13-15 hrs. Then add blocking buffer (to prevent non- specific binding). Incubate for 6 to 8 hrs/overnight. 1:1000 diluted primary Ab added (StAR antibody) i.e., 9ml of blocking buffer + 3µlof primary antibody. Incubate for 6-8 hrs. Wash with blocking buffer 3 times for 25 min. 1:10,000 diluted HRP conjugated secondary Ab added (Goat antirabbit IgG-HRP conjugated) i.e., 1 µl: 10ml in blocking buffer and incubated for 10 min. Then wash with TBS for 10 min. Following vigorous washing steps, TMB substrate added in dark conditions. Observe bands on nitrocellulose paper. The bands of interest were quantitated using a Alpha Imager Software. Values obtained were expressed as integrated optical density units (arbitrary units).

2.6 PMSG-hCG induced superovulation in immature rats

Immature Charles Foster female rats were treated *in vivo* with 10 I.U of PMSG (Pregnant Mare's Serum Gonadotropins) subcutaneously, stimulating the FSH receptor, and after 48 h of PMSG injection, 50 I.U of hCG (human Chorionic Gonadotropin) intra-peritoneally, to mimic the endogenous LH-surge with concomitant stimulation of the LH receptor (Svensson *et al.* , 2000).

2.7 Rat Ovarian Granulosa cell isolation (Campbell, 1979)

Reagents

1. Hanks Balanced salt solution (250 ml) contains 2 g of NaCl, 0.1 g of KCl, 0.015 g of Na_2HPO_4, 0.015 g of KH_2PO_4, 0.0625 g of $NaHCO_3$ and 250 mg of glucose in 250 ml D/w. Sterilize and Filter it. Adjust the pH to 7.2.

2. EGTA + BSA Solution: 6.8 mM EGTA + 0.2% BSA.

3. Hypertonic Sucrose Solution: 0.5 M Sucrose with 1.8 mM EGTA in 100 ml.

4. HBSS + EGTA (2mM)

5. Trypan Blue (0.18%)

Protocol

- Ovaries were removed in aseptic condition and immediately transferred into sterile saline and centrifuged at 1000 rpm / 5 minutes, to remove fat.
- Ovaries were re-suspended in 2 ml of EGTA-BSA solution and incubated for 15 minutes at 37° C and centrifuged at 1000 rpm for 5 minutes.
- Ovaries were then re-suspended in 1ml of Hypertonic Sucrose solution and incubated for 5 minutes in ice, followed by centrifugation at 1000 rpm for 5 min.
- Pellet containing ovaries were removed from the solution and the cells were expressed in 2ml of HBSS solution using blunt spatula, kept in ice.
- HBSS containing the cells were centrifuged at 1500 rpm for 5 to 7 min.
- Pellet containing the cells was re-suspended in 2 ml of HBSS-EGTA solution and centrifuged at 1000 rpm for 5 min.
- Above steps were repeated again for 3 to 4 times.
- Final pellet was suspended in 200 µl of HBSS.
- 10 µl of cells were stained with Trypan blue and counted in 16 small squares of 4 corner squares of the Hemocytometer.
- Number of cells was calculated by taking the average of number of cells present per square.
- Number of cells = average of number of cells present in all 4 squares x 10^4 x dilution factor
- Expression Unit = **Number of cells/ mg ovary**

2.8 Estimation of Lactate Dehydrogenase (LDH)

Principle:

Cytosolic LDH is leached into the medium during membrane damage which will catalyze the conversion of sodium pyruvate to lactate with the concomitant oxidation of NADH to NAD shows and absorbance maxima at 340nm. The

activiy of LDH can be measured in terms of decrease in the absorbance of NADH with respect to time.

Reagents:

1. Phosphate Buffer (pH 7.4)
2. NADH Solution (3.5 mM)
3. Sodium pyruvate (21mM)

Protocol:

Reagents	Blank (ml)	Test (ml)
Phosphate buffer	2.5	2.5
NADH	0.1	0.1
Supernatant	-	$5x10^5$
Sodium pyruvate	0.1	0.1

Add the first two reagents and then incubate the cells with them for 10 minutes at 37°C to equilibrate. On adding sodium pyruvate, take O.D at 340 nm.

Unit: % LDH relaese

2.9 Estimation of Caspase activity (Colorimetric assay kit, Sigma)

Principle:

Caspase 3 hydrolyzes the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405nm. The concentration of the pNA released from the substrate is calculated from the absorbance values at 405nm.

Protocol:

1. Pellet the cells from metal exposed and control cells by centrifugation at 600 x g for 5′ at 4°C.
2. Remove the supernatant by gentle aspiration.
3. Wash the cell pellets once with 1 ml of PBS. Centrifuge the cells and remove the supernatant completely by gentle aspiration.
4. Suspend the cell pellets in 1X lysis buffer at a concentration of 100µl per 10^7 cells.

5. Incubate the cells on ice for 15-20 minutes at 4°C.

6. Centrifuge the lysed cells at 12,000 x g for 10-15 minutes at 4°C.

	Cell lysate	Caspase 3 5µg/ml	Assay buffer (µl)	Caspase 3 inhibitor Ac-DEVD-CHO 2mM	Caspase 3 substrate Ac-DEVD-pNA 20mM
Reagent blank	-	-	990 µl	-	µl
Control cells	10 µl	-	980 µl	-	µl
Control cells+ inhibitor	10 µl	-	970 µl	10 µl	µl
Metal exposed cells	10 µl	-	980 µl		µl
Metal exposed cells+ inhibitor	10 µl	-	970 µl	10 µl	µl
Caspase3 positive control	-	10 µl	980 µl	-	µl
Caspase3 positive control + inhibitor	-	10 µl	970 µl	10 µl	µl

7. Transfer the supernatants to new tubes for immediate analysis.

8. Place 10µl of cell lysate or Caspase 3 Positive Control in appropriate tubes.

9. Add 1X Assay Bufer to each of the tubes.

10. Add 10µl of caspase 3 inhibitor to the appropriate tubes.

11. Start the reaction by adding 10µl of caspase 3 substrate to each tube and mix gently. Cover the tubes and incubate at 37°C for 1.5 to 2 hours or more if signal is too low.

12. Read the absorbance at 405 nm.

13. Calculate the caspase 3 activity in µmol of pNA released per min per ml of cell lysate.

Unit: µmol pNA/min/ml

2.10 Comet assay (Single cell electrophoresis) (Collins *et al.*, 1997)

Principle:

Strand breakage of the supercoiled duplex DNA leads to the reduction in size of the large molecule and these strands are stretched out by electrophoresis under alkaline conditions. When viewed under microscope, a cell has the appearance of comet, with head formed by the nucleus and tail containing DNA fragment or strand migrating in direction of the anode. The detection of DNA migration is dependent on various parameters such as concentration of agarose in gel, the pH, temperature, voltage, amperage and duration of eletrophoresis. Two principles in the formation of the comet are:

1. DNA migration is a function of both size and the number of broken ends of the DNA
2. Tail length increases with damage initially and then reaches a maximum that is dependent on the electrophoretic conditions, and not the size of fragments.

Cell Source:

Isolated Granulosa cells suspension ($2\text{-}5\times10^7$ cells/200 µL) obtained from gonadotropin primed immature rat.

Reagents:

1. Low melting point agarose (0.5% LMP Agarose From Sigma)
2. Normal melting point agarose(1% LMP Agarose)
3. Lysis buffer (pH-10)
4. Electrophoresis buffer (pH - 13)
5. Neutralizing solution (pH - 7.5)
6. Staining solution: Ethidium bromide (10 µg/mL)

Method:

(A) Slide preparation

1) 0.5% LMPA and 1% NMPA is prepared in PBS (Ca^{++}, Mg^{++} free)
2) Frosted slide is put in methanol for chilling and wrap slide with tissue paper.

3) First layer is made using 200 µL melted NMPA. Coverslip ((No.1, 24×50) is placed on it to make the layer uniform. Then the slide is kept at 4°C for few minutes. This increases the stability of the gel.
4) Second layer is made after gently removing the coverslip. This layer has 10 µL of granulosa cells suspension (0.6-2×10^6cells) and 90 µL of melted LMPA. From this step onwards all the work is done in dark or in dim light. Again the slide is placed at 4°C for few minutes.
5) Third layer is made using 90 µL of melted LMPA after gently removing the coverslip. Care should be taken to prevent the loss of blood cells while removal of coverslip. It is put at 4°C for few minutes.

(B): Lysis

1) The slide is placed in freshly prepared chilled lysis buffer after gently removing the coverslip.
2) It is left at 4°C for 1 hour in an opaque couplin jar. The slide can be left in this condition for 1 week without damage.

(C): Alkaline unwinding

1) The slide is removed from the lysis buffer and is washed with chilled distilled water to remove residual detergents and salts. This increases assay reproducibility.
2) It is then placed in chilled electrophoresis buffer for 20 minutes for allowing the unwinding of DNA under alkaline conditions.

(D): Electrophoresis

1) After alkali unwinding, the ssDNA in the gel is electrophoresed under alkaline conditions to produce comets. The alkaline buffer used for electrophoresis is same as that used for alkaline unwinding.
2) Voltage ranges from 0.7 to 1.0 volt/cm. We use 20 V and 250 mA and electrophoresed for 20 minutes. Raising or lowering the buffer level sets current (250mA).
3) Electrophoresis is conducted under low temperature conditions to increase reproducibility. Use ice packs surrounding electrophoresis tank to maintain low temperature.

(E) Neutralization

1) After electrophoresis, drop wise cover the slide with neutralization buffer as it neutralizes the alkali in the gel.
2) The slide is given washes by dropping buffer on slide.

(F) Staining

1) 60 µL of ethidium bromide (working solution, 1µg/ml) is used to stain the slide.
2) The slide is placed in dark for about 45 minutes in moist chamber.

(G) Visual Scoring: Each slide was viewed by fluorescence microscopy (Nikon Type 108) and the degree of damage was assessed visually by the appearance of formation of comet tail.

2.11 Measurement of intracellular ROS generation: DCF Fluorescence

Principle: Measurement of intracellular ROS production gives an estimate of the oxidative stress created by any toxic agent inside the cell. The non-fluorescent dye DCF-DA passively diffuses in to the cells where the acetates are cleaved by intracellular esterases. The resulting diol is retained by the cell membrane. With generation of ROS this diol is oxidized to the fluorescent form 2′, 7′-dichlorofluorescein (DCF) which can be qualitatively detected by fluorescent microscopy.

Protocol:

1. Distribute 1.0 ml of cells into each well of 6-well culture plate
2. Incubate the cells for 48 h in CO_2 incubator
3. The cells were washed with HBSS and incubated with 100 µM carboxy-H_2DCF-DA and DAPI in the loading medium in CO_2 incubator at 37 °C for 30 min HEPES buffered salt solution (HBSS), 100 ml : 0.6 g HEPES (25 mM), 0.7 g NaCl (120 mM), 40 mg KCl (5.4 mM), 26.5 mg $CaCl_2$ (1.8 mM), 0.21 g $NaHCO_3$ (25 mM), 0.27 g Glucose (15 mM) → adjust pH 7.4 with NaOH → filter sterilization]
4. [100 mM carboxy-H_2DCF-DA stock solution : 10 mg / 0.188 ml DMSO, stored at -20 °C at dark → For loading the cells with carboxy-H_2DCF-DA,

stock solution is diluted with loading medium (99% basal culture media + 1% FBS) to a final concentration of 100 μM]

5. The cells were washed with HBSS to remove carboxy-H_2DCF-DA and replaced with 1.0 ml of HBSS containing chemical treatments
6. The DCF fluorescence of granulosa cells was measured using fluorescence microscope with excitation and emission wavelengths of 485/530 nm, respectively and DAPI fluorescence with excitation and emission wavelength of 355/460 nm

2.12 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.

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.				

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

Protocol

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.

2.13 Enzyme Linked Immunosorbent Assay (ELISA):

Estradiol and Progesterone (Adaltis kit, Italy)

ESTRADIOL

Principle: -

It is based on the principle of competitive binding between E2 in the test specimen and E2-HRP conjugate for a constant amount of rabbit anti-estradiol in goat anti-rabbit IgG coated wells. The amount of E2-HRP conjugate, immunologically bound to the well progressively decreases as the concentration of E2 in the specimen increases. TMB reagent serves as substrate for E2-HRP conjugate that result in the development of blue color.

Reagents and Materials: -

- Antibody – coated wells
- Reference standard set (0 to 1000 pg/ml)
- Rabbit anti – estradiol reagent
- Estradiol – HRP conjugate reagent
- Estradiol control 1 and 2
- TMB reagent
- Stop solution

Procedure: -

1. Secure the desired number of coated wells in the holder

2. Dispense 25 µl of standards, specimens, and controls into appropriate wells.
3. Dispense 100 µl of estradiol – HRP conjugate reagent into each well.
4. Dispense 50 µl of rabbit anti- estradiol (E2) reagent to each well.
5. Thoroughly mix for 30 second and then incubate at R.T. for 90 min
6. Rinse and flick the microwells 5 times with distilled or deionized water.
7. Dispense 100 µl of TMB reagent into each well. Gently mix for 10 second
8. Incubate at R.T. for 20 min
9. Stop the reaction by adding 100 µl of stop solution to each well.
10. Gently mix for 30 seconds, make sure that all the blue color changes to yellow completely.
11. Read absorbance at 450 nm with a microtiter well reader within 15 min.

Calculations:

Calculate the mean absorbance value (OD 450 nm) for each set of reference standards, controls and samples. Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in pg/ml on a linear-linear graph paper, with absorbance values on the vertical or Y axis, and concentrations on the horizontal or X axis. Use the mean absorbance values for each specimen to determine the corresponding concentration of estradiol in pg/ml from the standard curve.

Units: pg of estradiol/ml

PROGESTERONE

Principle: -

It is based on the principle of competitive binding between progesterone in the test specimen and enzyme labeled progesterone conjugate for a constant amount of anti-progesterone antibody immobilized on the microwell surface. The amount of conjugate that binds to the microwell surface will decrease in proportion to the concentration of progesterone in the patient sample. Progesterone conjugate, immunologically bound to the well progressively decreases as the concentration of progesterone in the specimen increases. TMB

reagent serves as substrate for enzyme labeled progesterone conjugate that result in the development of color.

Reagents and materials: -

- Coated microwells.
- Enzyme conjugate
- Reference standard set
- Quality control set (control)
- Dilution buffer
- Washing buffer
- Substrate reagent A
- Substrate reagent B
- Stopping solution

Test procedure: -

- Secure the desired number of coated wells in the holder
- Dispense 50µl of each reference standard, control and test sample into the appropriate well. Complete pipetting within 5 minutes.
- Dispense one drop of enzyme conjugate into each well.
- Gently rock the wells for twenty seconds then seal (by covering) with parafilm or other film sealant.
- Incubate at 37°C for 60 min.
- Remove and discard cover seal. Decant the incubation mixture thoroughly by flicking into a sink containing disinfectant.
- Rinse or wash the microwells five times with diluted washing buffer.
- Dry the wells by firmly tapping the plate on a clean paper towel to remove excess washing solution.
- Dispense one drop of substrate reagent A and one drop of substrate reagent B into each well. Gently rock the wells fro twenty seconds. Incubate at room temperature (15° to 28°C) for 15 min.
- Stop the reaction by adding one drop of stopping solution to each well and gently rock the wells.

- Read the absorbance at 450 nm of each well within 30 min after stopping the reaction.

Calculations:

❖ Calculate the average absorbance value (A_{450}) for each reference standard, control and test sample.

❖ Divide the average A_{450} value for each standard, control and test sample by the average A450 of Standard 0 ng/ml and multiply by 100 to obtain $\%A/A_0$ for each sample.

❖ Prepare a standard curve by plotting the average absorbances versus the corresponding concentrations of the standards on linear-log graph paper.

❖ Using the absorbance or the $\%\ A/A_0$ value for each control and test sample, determine the corresponding concentration of progesterone in ng/ml from the standard curve.

Units: ng of progesterone/ml

2.14 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°C in a motor driven glass homogenizer. The homogenates were centrifuged at 4000 g at 4°C 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), 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.

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

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

7) Enzyme source:

17β hydroxy steroid oxidoreductase - 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% ovary/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 granulosa cells, 5×10^5 cells were sonicated at 5 cycles for 2 min.

Reagents	Blank (ml)	Test (ml)
Tris-HCl	1.0 ml	1.0 ml
NAD	0.5	0.5
Substrate	0.5	0.5
Sample	----	0.05
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.

2.16 NADPH- and NADH-cytochrome *c* reductase (Williams and Kamin, 1962)

Reagents

1. 0.3 M potassium phosphate + 0.1 mM EDTA buffer, pH 7.7
2. 1 mM KCN
3. 100 µM cytochrome *c*
4. 100 µM NADPH or NADH

Enzyme source: Crude hepatic microsomal fraction (10–50 µg of protein) prepared from 10% liver homogenate.

Reagents	Volume (µl)
Buffer	450
KCN	100
Cytochrome c	200
NADPH or NADH	200
Auto zero and reaction was initiated by adding enzyme	
Sample	50
The linear increase in the absorbance was monitored for about 3 min at 550 nm.	

The results were expressed as nanomoles of reduced cytochrome *c* per minute per milligram of cell protein, using an extinction coefficient of 21 mM^{-1} cm^{-1}.

2.17 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 $MgCl_2$
3. 1.6 mM p-nitrophenol (PNP)
4. 20 mM UDP-glucoronic acid
5. 0.2 N TCA
6. 0.5 N NaOH

Enzyme source: Crude hepatic microsomal fraction

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

2.18 γ-glutamyl transferase (γ-GT) (Persijn *et al.* , 1976)

Reagents

1. Buffered Substrate: - 250 mg L-gama-glutamyl-paranitroanilide + 872 mg glycyl-glycine + 672 mg MgCl2 in 300 ml of 0.05 M phosphate buffer, pH 8.6
2. 0.0075 M NaOH
3. p'-nitroanilide (0.9 µM)

Reagents	Blank (ml)	Test (ml)
Buffered Substrate	0.25	0.25
Enzyme	0.5	0.1
Incubate at 37°C for 45'		
NaOH	1.25	1.25
Enzyme	0.5	-

The rate of formation of 5-amino-2-nitrobenzoate was recorded at 405 nm at 37°C for 2 min.

Unit: The results were expressed as milliunits per milligram of cell protein, using an extinction coefficient of 9.5 mM^{-1} cm^{-1}.

2.19 Glutathione-S-transfarease (GST) (Habig et al, 1974)

Reagents

1. Buffer (pH-6.5); 0.1 M sodium phosphate + 1 mM Na_2EDTA buffer
2. 20 mM GSH
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.

2.20 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 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
Sample	-	-	0.1	0.1
D/W (ml)	0.1	0.1	0.03	-
Keep in ice for 10′, ↺3000 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

2.21 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. 0.1 M Phosphate buffered saline (PBS, pH 7.4)

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

Procedure

Reagents	Blank	Test
Sample (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

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

Principle

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

Reagents

1. KPO_4 (0.2M, pH 8)
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 0.12 O.D./180 sec

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
Take absorbance at O.D. 420 nm		

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

2.23 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 the reaction)

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
Take absorbance at O.D. 240 nm		

Unit: mmoles of H_2O_2 decomposed/mg protein

2.24 Glutathione Peroxidase (GPx) (Paglia and Valentine *et al.*, 1974)

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.

Unit : Δlog (GSH) / min / mg protein

2.25 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.

2.26 Alkaline phosphatase (ALP) (Bowers & McComb, 1975)

These hydrolytic enzymes are present in high concentrations in liver, bone, placenta and intestinal epithelium. Increased serum 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 µmoles

Reagent	Control	Test
Working buffered substrate	0.4 ml	0.4 ml
Placed in water bath at 37C for 5 min		
Serum 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 was done according to the slope calculated from the standard graph.

Unit: µmoles PNP formed/min/g tissue

Acid phosphatase (ACP) (Bowers & McComb, 1975)

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)

Standard range: 0.04-0.16 µmoles

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		

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

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

2.27 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 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		

2.28 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: µg/g tissue

2.29 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: Serum

Protocol

	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/L

2.30 Creatinine (Bonsnes and Taussky, 1945)

Creatinine is the end product of creatine metabolism. It is largely formed in the muscle by irreversible and nonenzymatic removal of water from creatine phosphate. It is a waste product and excreted out from the kidney. Increased serum creatinine level is a clinical evidence of renal disease.

Principle:

Creatinine reacts with picric acid under alkaline conditions to form a characteristic yellow-orange complex. The color intensity is measured at 520 nm.

Reagents

1. Saturated Picric acid (40 mM)
2. 0.75 M NaOH
3. Standard creatinine solution was prepared in the range 10-50 ug.

Stock concentration is 100 mg/dl. Working concentration is 10 mg/ dl

Sample preparation: 1.5 ml of picric acid was added to 0.5 ml of serum and tubes were centrifuged at 3000 rpm for 15 min.

Reagents	Test	Blank
Supernatant	1.0ml	---
Distilled Water	1.0 ml	2 ml
Picric Acid	1.0ml	1ml
0.75 m NaOH	1.0 ml	1 ml
Incubated for 20 min at RT and absorbance was recorded at 520 nm		

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

Unit - mg / dl.

2.31 Cholesterol estimation (Leffler *et al.*, 1963)

Principle

In this method cholesterol is extracted from the tissue using isopropanol. The aceticacid-$FeCl_3$ reagent acts on cholesterol converting it into cholestradiene, which then reacts with concentrated H_2SO_4 to form colored complex. This is estimated colorimetrically at 540 nm. Range of the method is 75-350 mg.

Reagents

1. $FeCl_3$ reagent- 500 mg $FeCl_3.6H_2O$ in 500 ml of glacial acetic acid or phosphoric acid.
2. Standard cholesterol - 200 mg/dl in isopropanol
3. Tissue sample - 10% ovary/testis/liver homogenate

Protocol:

Reagents	Test (ml)	Blank (ml)	Standard (ml)
Standard Cholesterol	-	-	0.5
Sample	0.5	-	-
$FeCl_3$-glacial acetic acid	1.0	1.0	1.0
Conc. H_2SO_4	2.0	2.0	2.0
Mix well, keep at R.T. for 10' & Read the absorbance at 540nm			

Slope: 0.016 O.D. / mg cholesterol
Unit: mg cholesterol/gm tissue

2.32 Vitamin C (Roe & Keuther, 1943)
Principle:

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid, which when treated with 2, 4-dinitrophenylhydrazine form bis-2,4-dinitrophenylhydrazone. The hydrazone in strong sulphuric acid undergoes rearrangement to form a product measurable at 520nm. Thiourea is added to provide a mildly reducing medium, which helps prevent interference from nonascorbic acid chromogens.

Reagents:

1. 2,4-dinitrophenylhydrazine/thiourea/copper (DTC) solution: (0.4g thiourea, 0.05g CuSO4.5H2O, 3g 2,4-dinitrophenylhydrazine) , Make the volume upto 100ml with 9N H2SO4

2. 65% H2SO4 (chilled)
3. TCA (10%)
4. Samle: 10% tissue homogenate

Protocol:

Reagents	Blank	Test
TCA	0.5	-
Sample (homogenate)	-	0.5
DTC solution	0.1	0.1
Incubate for 3 h at 37°C		
65% H2SO4	0.75	0.75
Mix well. Allow to stand at RT for 30'		
Read O.D. at 520nm		

Calculation was done according to the slope calculated from the standard graph (Slope: 0.074).

Unit: µg of vitamin C/100 mg tissue

2.33 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 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.

2.34 Sperm Count and Viability (Eliasson, 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.

2.35 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.

2.36 Estimation of Fructose (Motoshima and Settlage, 1978)

The 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 $ZnSO_4$ 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.

2.37 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 vapors 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.

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

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

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

2.38 Histology

Ovary, liver and testis were removed and fixed in Bouin's 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.

2.39 Statistical analysis

Statistical analyses of data was done by Student's test, one-way analysis of variance (ANOVA) and all groups were compared by means of Bonferroni, with significance set at $p<0.05$. All values represent the mean ± S.E.M. Data were analyzed using PRISM version 3.03 Graph Pad software.