

Chapter -II
MATERIALS AND METHODS

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❖ **Chemicals**

Dihydroepiandrosterone (DHEA), 17 β Estradiol, Bovine Serum Albumin (BSA), Ethylene Glycol Tetra Acetic Acid (EGTA), Chloramine-T, Dithiothreol (DTT), peroxidase were purchased from Sigma Inc, USA. Tris-HCl, Potassium Phthalate, Iodonitroterazolium (INT), Tween -20, NAD, NADH, reduced Glutathione (GSH), Phenazane metho sulfate (PMS), Folin-Ciocalteu's were purchased from Sisco Research Laboratories Ltd, Mumbai, India.

Trypan blue was obtained from Hi-Media Labs, India. Sephadex G-25 column was procured from Amersham Inc, U.K. Na- 125 I (2 mCi) was purchased from BRIT, India. Rat Luteinizing Hormone (AFP 115368) and rat Follicle Stimulating Hormone (AFP 19896) were obtained from National Health Pituitary Program (NHPP), NIDDK, Bethesda, U.S.A, as a kind gift from Dr. Parlow.

➤ **Animals**

Adult virgin female rats of Charles-Foster strain weighing 180-220 g kept under controlled conditions of light (lights on from 07.00 to 20.00 h) and temperature ($24\pm 2^\circ\text{C}$) with ad libitum access to food and water were used. Ovarian cycle was checked daily by vaginal cytology. Animals displaying atleast three 4-day cycles were selected for the experiment.

❖ METHODS

➤ 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 containing 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 the proestrous stage and kept in ice.
- Centrifuged at 1000 rpm / 5 minutes, to remove fat.
- Ovaries were resuspended 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 resuspended 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 resuspended 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.

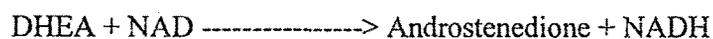
- Final pellet was suspended in 200 μ l of HBSS.
- 10 μ l of cells were stained with Trypan blue and counted in 16 small squares of 4 corner squares of the Hemocytometer.
- Number of cells was calculated by taking the average of number of cells present per square.
- Number of cells = average of number of cells present in all 4 squares $\times 10^4 \times$ dilution factor

Expression Unit = Number of cells/ mg ovary

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

Principle: Ovarian or placental 3 β HSD converts DHEA to Androstenedione and ovarian or placental or granulosa cellular 17 β HSD converts 17 β Estradiol to estrone. In both reactions, NAD is reduced to NADH, which is coupled to the reduction of the tetrazolium via diaphorase present in the cells or tissue.

3 β HSDH



17 β HSDH



Reagents

1. Phthalate buffer: (pH =3.0, 50 mM) 2.55g of potassium hydrogen phthalate in a mixture of 51 ml N/10 HCl and add 2.5 ml of Tween 20. pH is adjusted to 3.0 and volume is adjusted to 250ml with distill water.

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2. Tris -HCl (0.1 M) (pH =7.8)
3. NAD (0.5mM)
4. Color reagent: 40 mg of Iodonitroterazolium (INT), 10 mg Phenazane metho enzyme assay, PMS was omitted from the reagent.
5. Substrate: DHEA is dissolved in 0.3 ml to 0.5 ml of DimethylFormamide. 17β Estradiol is dissolved in minimum amount of ethanol and then diluted in distill water. Stock of both substrates were prepared in 50/100 ml Tris-HCl (pH 7.8). DHEA is used as substrate for 3β HSDH and estradiol for 17β HSDH.

Sample Preparation: 10% ovarian or placental homogenate was prepared in 0.1 M Tris HCl buffer (pH =7.8) These were then centrifuged at 12000 g at 4° C in Sorvall, high-speed centrifuges. Supernatant was used as a source of the enzyme. For granulosa cells, 4×10^5 cells were sonicated at 5 cycles for 2 min.

Protocol

| Reagents | Blank | Test |
|--------------------------|--------|--------|
| Tris-Hcl | 1.1 ml | 1.0 ml |
| NAD | 1.0 ml | 1.0 ml |
| Substrate | 1.0 ml | 1.0 ml |
| Enzyme | ---- | 0.1 ml |
| INT | 0.5 ml | 0.5 ml |
| Incubate at 37° for 1 hr | | |
| Pthalate buffer | 2.0 ml | 2.0 ml |

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

Units: nmol of NAD reduced/ min/ mg protein

➤ **Radio Immuno Assay of 17 β Estradiol/ Progesterone**

Principle: The Coat-A Count Estradiol / Progesterone estimation is based on antibody coated tubes. I ¹²⁵ labelled estradiol/ progesterone competes with estradiol/ progesterone present in the serum / tissue for antibody sites. After incubation, separation of bound from free is achieved by decanting, where the antibody is immobilized to the wall of a polypropylene tube. The tube is then counted in a gamma counter, the counts being inversely related to the amount of estradiol present in the sample. The quantity of estradiol in the sample is determined by comparing the counts to a calibration curve.

Reagents

1. I ¹²⁵ Estradiol/ I ¹²⁵ progesterone
2. Progesterone/ Estradiol calibrators
3. Progesterone standards- 0-40 ng / mL, Estradiol standards –0-3600 pg/mL

Sample- Serum , ovarian or placental homogenate (10 % homogenate in PBS)

Protocol

| Reagents | Total counts | Standard | Test |
|--|--------------|----------|-------|
| Standards | ----- | 100µl | ----- |
| Serum/ tissue Sample | ----- | ----- | 100µl |
| Tracer | 100µl | 100µl | 100µl |
| Incubate for 3 hrs at room temperature | | | |
| Decant thoroughly | | | |
| Count for 1 min in gamma counter | | | |

For Progesterone: Standard of various concentrations (0-65 ng/ml) has been used for calibration curve. .

For Estradiol: Standard of various concentrations (0-5000 pg/ml) has been used for calibration curve.

Calculation:

T- Total counts of 100µl of I¹²⁵-estradiol/ I¹²⁵-progesterone

B₀- CPM of bound with I¹²⁵-estradiol/ I¹²⁵-progesterone in absence of estradiol/progesterone (zero binding)

B- CPM of bound with I¹²⁵-estradiol/ I¹²⁵-progesterone in presence of estradiol/progesterone standard.

%B/T and % B/B₀ was calculated for estradiol/ progesterone standards and serum/ tissue samples. A logit-log graph was plotted against % B/B₀ and concentration of

progesterone/estradiol standards. Serum/tissue estradiol/ progesterone concentrations were calculated from the graph.

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

pg of progesterone present in ml serum or mg/g tissue.

➤ **RADIO RECEPTOR ASSAY**

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. LH-R has a molecular weight of 30 KDa while FSH-R has molecular weight of 32KDa. These are present on the membrane of granulosa cells and the interaction of gonadotropins with their receptors mediates further events of granulosa cell proliferation.

○ **IODINATION OF LUTEINISING HORMONE (r-LH) / RAT FOLLICLE STIMULATING HORMONE (r-FSH) (Greenwood et al., 1963)**

Reagents

1. Phosphate Buffered Saline (PBS) pH =7.4

0.05 M Dipotassium Hydrogen Phosphate and 0.05 M Potassium Dihydrogen Phosphate in saline

2. Sephadex G-25 Column.

3. 1% BSA.

4. Chloramine-T (1.5 mg/ml) in 0.05 M Phosphate Buffer.

5. Phosphate Buffer: 700 mg NaOH, 1.26 g of Di Sodium Hydrogen Phosphate, 1.8 g NaCl, 2.92 g EDTA, 0.334 g Sodium Di hydrogen Phosphate in 200 ml D/w. Add to this, 0.2% BSA. Adjust pH to 7.4
6. Sodium Meta-bisulfite: 24 mg/ 5ml in 0.05 M phosphate buffer.
7. 1% Potassium Iodite (KI), 8% Sucrose.

Protocol for Iodination

50 μ l / 5 mg of r-LH or r-FSH, 5 μ l Na-I¹²⁵ and 7.5 μ l of Chloramine-T was incubated together for 45 seconds at 25 ° C and reaction was terminated by a mixture of 50 μ l Sodium- Meta bisulfite, 100 μ l 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 μ l of the reaction mixture was loaded into the column.
- Ten drops per eppendroff was collected.
- Aliquot of 10 μ l of the each fraction was counted.
- Fraction that had maximum counts is used as iodinated gonadotropins.

o SATURATION KINETICS

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)
4. Unlabelled FSH or Unlabelled LH
5. Labelled LH or labelled FSH

Protocol

- 1ml of Glycine-HCl buffer was added to the tube containing 1×10^5 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 labelled 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 were 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 granulosa cells (Guerrero et al., 1993)**

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)

- 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 25,000cpm (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

➤ **METALLOTHIONEIN FRACTION PREPARATION** (Bayne et. al., 1985)

Metallothionein (MT) is a family of proteins with molecular weight of approximately 6.5KDa, rich in cysteine and with 7 metals distributed in two domains., the α - and β clusters. Major Metallothionein that exist in almost in all tissue is MT-1.

Reagents

1. 0.9% NaCl
2. Ammonium bicarbonate buffer (pH 7.8)

2. Ammonium bicarbonate buffer (pH 7.8)

Protocol

Placenta was homogenized in 0.9% NaCl (10%). Centrifuged at 11000 g for 30 min and the supernatant obtained was heated at 70⁰ C for 10 min. Centrifuged at 2500 rpm for 10 min and supernatant obtained was loaded in a Sephadex G-75 column (0.6X60 cm). The fractions were eluted using ammonium bicarbonate buffer (pH 7.8). They were then monitored at 254 and 280 nm in a UV-Visible spectrophotometer. The absorbance obtained at 254 nm was converted into equivalent amounts of protein by interpolating from a standard graph of bovine serum albumin in ammonium bicarbonate buffer read at the same wavelength. The fractions having high absorbance at 254 nm and a comparatively low absorbance at 280 nm, which correspond to the metallothionein, were used for metal analysis.

> GRANULOSA CELL MEMBRANE PREPARATION (Riordan and Ling, 1979)

Granulosa cells were isolated from ovaries immediately and placed in HBSS solution. The cells were then sonicated at 5 cycles for 2 minutes. Sonicated cells were spun at 1000 g for 10 minutes. Supernatant was centrifuged at 12000g for 20 min. The supernatant was removed and recentrifuged at 20,000 g for 1 h twice. The pellet obtained contains the partially purified membrane fraction, which is resuspended in 10 mM Tris. Aliquot was used for protein estimation.

➤ **PLACENTAL MEMBRANE PREPARATION** (Parkkila et al., 1997)

Placenta were removed from the animal and placed in 0.25 mM Sucrose/20 mM Tris-HCl solution (pH = 7.5) and washed thoroughly to remove the blood. 10% homogenate was prepared in 0.25 mM Sucrose/20 mM Tris-HCl solution (pH = 7.5). Centrifuged at 1000g for 10 min to remove debris and nuclear fraction. The supernatant was spun at 1,05,000g for 1 h. The Pellet obtained was resuspended in homogenizing buffer with 1% Triton-X 100 and centrifuged again at 1,05,000g for 30 min. The pellet thus obtained contains the membrane. This is resuspended in 20 mM Tris-HCl and aliquot of membranes was used for the assay.

➤ **MEMBRANE FLUIDITY** (Shinitzky and Barenholz, 1978)

Fluidity specifically refers to the property of the hydrophobic region of the membrane and has been used to express the increased disorder of the fatty acyl chains. Fatty acid chains develop kinks with every double bond. With increase in the number of kinks the packing density of the membrane decreases and subsequently the fluidity of the membrane. Lipid fluidity was assessed by steady state fluorescence polarization of the lipid soluble probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). Depending on the availability of space in the lipid bilayer, DPH gets differentially lodged and when light passes through it, it gets depolarized. The amount of depolarization gives the fluidity of the membrane.

Reagents

1. 0.6 μ M 1,6-diphenyl-1,3,5-hexatriene (DPH)
2. 10mM Tris HCl (pH 7.4)

3. 0.25 M sucrose buffer in 20mM Tris HCl (pH 7.4)

Protocol

A suspension of granulosa cell or placental membrane was incubated for 60 min with 0.6 μ M 1,6-diphenyl-1, 3,5-hexatriene (DPH) in 0.32 M sucrose buffer containing 10mM Tris HCl, pH 7.4. The excitation and emission wavelengths were 360 and 430 nm, with bandwidth 5 nm and 10 nm, respectively. Polarisation values for parallel (vertical) and perpendicular (horizontal) were taken with a Shimadzu RF-540 spectrofluorimeter. The polarisation value (P) was calculated from the equation

$$P = \frac{I_{VV} - G I_{VH}}{I_{VV} + G I_{VH}}$$

where I_{VV} and I_{VH} are vertical and horizontal components of emitted light, respectively, when emitted with vertically polarised light and G is the correction factor for the emission monochromator.

➤ $\text{Na}^+\text{K}^+\text{ATPase}$ (Floreani et al., 1981)

Principle

$\text{Na}^+\text{K}^+\text{ATPase}$ is an integral membrane bound enzyme and used as a marker for studying membrane changes. It contains two subunits- α subunit that contains the enzyme's catalytic activity and ion binding sites and β subunit whose function is unknown. The enzyme pumps out Na^+ and K^+ into the cell against the concentration gradient with a concomitant hydrolysis of intracellular ATP.



ATPase was measured by the release of inorganic phosphorus from ATP. The Pi was assayed according to Fiske and Subbarow (1925). To determine the basal Mg^{2+}

ATPase activity 0.2 mM ouabain was added to the incubation medium; the Na⁺K⁺ATPase activity was calculated as the difference between total ATPase and Mg²⁺ATPase activity.

Reagents

1. 50 mM Tris HCl (pH7.4)
2. 100 mM NaCl
3. 20 mM KCl
4. 3 mM MgCl₂
5. 3 mM ATP
6. 0.2 mM ouabain
7. protein (50-100 µg of granulosa cell membrane or placental membrane)
8. 10%trichloroacetic acid (TCA)

| Reagents | Test | Test | Blank | Blank |
|--|------------|------------|------------|------------|
| | (-ouabain) | (+ouabain) | (-ouabain) | (+ouabain) |
| Tris HCl | 0.1 ml | 0.1 | 0.1 | 0.1 |
| NaCl | 0.1 | 0.1 | 0.1 | 0.1 |
| KCl | 0.04 | 0.04 | 0.04 | 0.04 |
| MgCl ₂ | 0.12 | 0.12 | 0.12 | 0.12 |
| Protein | 0.1 | 0.1 | ----- | ---- |
| D.W | 0.42 | 0.32 | 0.52 | 0.42 |
| ATP | 0.12 | 0.12 | 0.12 | 0.12 |
| Ouabain | ----- | 0.1 | ---- | 0.1 |
| Incubated for 20 min, at 37 ⁰ C in water bath | | | | |
| TCA | 0.1 | 0.1 | 0.1 | 0.1 |

The tubes were centrifuged at 3000 rpm for 10 min and the aliquot from the supernatant was taken out for phosphorus estimation.

➤ **INORGANIC PHOSPHORUS** (Fiske and Subbarow, 1925)

Principle

Orthophosphate reacts with molybdate form phosphomolybdic acid. This product gets reduced to blue coloured molybdenum by a strong reducing agent like ANSA, which is estimated colorimetrically at 660 nm.

Reagents

1. Ammonium molybdate: 4.0 g Ammonium molybdate dissolved in minimum amount of concentrated H_2SO_4 and then made upto 100 ml with distilled water.
2. 1-amino 2-naphthol 4-sulphonic acid (ANSA): 20 mg triturate (mixture of 0.2 g ANSA, 1.2g sodium sulfite, 1.2 g sodium sulfite) in 1ml distilled water.
3. Standard : Dissolve 3.51 mg KH_2PO_4 in minimum amount of DW. Add 100 μ l concentrated H_2SO_4 . Make the volume to 10 ml with DW. Dilute this to obtain appropriate working standard concentration (0.8 mg/ μ l)

Protocol

| | Test | Blank |
|---------------------|--------|--------|
| Supernatant | 0.5 ml | ---- |
| Distill Water | 3.0 ml | 3.5 ml |
| Ammonium Molybdate | 0.4 ml | 0.4 ml |
| Incubate for 10 min | | |
| ANSA | 0.1 ml | 0.1 ml |

Absorbance was recorded at 660 nm

Standard graph for inorganic phosphate was prepared with KH_2PO_4 (range 2-40 μg).

Slope- 0.02 O.D./ μg Pi

Unit- μ mol Pi liberated/min/mg protein

➤ **SCHIFFS BASE** (Tappel, 1975)

The formation of Schiff's base ($\text{R}_1\text{-N}=\text{CH}-\text{CH}=\text{CH}-\text{NH}-\text{R}_2$) was measured to assess the lipid peroxidation in placental membrane samples.

Reagents

1. chloroform
2. chloroform-methanol (2:1 v/v)

Protocol

Lipids were extracted from placental membrane samples with chloroform-methanol (2:1 v/v), dried and redissolved in chloroform. Fluorescence emission was determined at 420 nm (excitation at 360 nm) on spectrofluorometer.

➤ **INORGANIC PEROXIDES** (Bernt and Bergmeyer, 1965)

This procedure measures the oxidation of o-dianisidine after treatment of samples with peroxidase to liberate molecular oxygen. The optical density (436 nm) of the samples was compared with that of H_2O_2 as standard.

Reagents

1. Buffer-enzyme mixture (0.12 M PO_4 buffer (pH 7.0); 40 μg peroxidase

(POD)/ml) : Dissolve 2.07 g Na_2HPO_4 ; 1.09 g NaH_2PO_4 and 6 mg POD in DW and make upto 150 ml.

2. Chromogen (5 mg o-dianisidine HCl /ml DW)

3. Peroxidase reagent – With vigorous stirring add 0.5 ml solution II to 50 ml solution I. Store the mixture in dark bottle. Prevent the growth of bacteria by addition of a few drops of chloroform.

4. H_2O_2 standard solution (20 μg H_2O_2 /ml)

a.. Dilute 1.0 ml 35% v/v H_2O_2 solution to 250 ml with DW. Check the H_2O_2 content : dilute 20 ml of the solution with 30 ml DW and 5 ml 1 N H_2SO_4 and titrate with 0.1 N KMnO_4 to a permanent pink solution. 1.0 ml of 0.1 N KMnO_4 solution is equivalent to 1.70 mg H_2O_2 .

b. According to the results of the titration, dilute appropriate volume (between 10 ml and 20 ml) of the solution to 1000 ml with DW.

5. Perchloric acid 0.6 M - Dilute 5.2 ml 70% Perchloric acid to 100 ml DW.

Stability- Solution 3 should be freshly prepared each day. If the solution 2 becomes turbid, it can be filtered. Always prepare the H_2O_2 standard solution just before use.

Sample- Placental membrane

Protocol

| Reagent Standard | Test | Blank | |
|---|------|-------|-----|
| Peroxide reagent | 5 | 5 | 5 |
| DW | -- | 0.2 | -- |
| Standard (H ₂ O ₂) | -- | --- | 0.2 |
| Sample | 0.2 | --- | --- |
| Mixed well, kept at RT for 5 min; Read the absorbance at 436 nm | | | |

Unit - $\mu\text{g}/\text{H}_2\text{O}_2/\text{ml}$

➤ 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.

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

I_t

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

Both tissue and blood 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 µg/ml | 2.5 – 20 µg/ml | 0.2 – 1.6 |
| 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 |

Pb or Cd µg/ml :

Total vol. in test tube × conc. in ppm (as obtained in AAS) × total residual
volume

Amount of sample taken before dilution × volume of blood taken (or weight of tissue)

Unit: $\mu\text{g/ml}$ or $\mu\text{g/g}$ or ng wet weight

➤ **REDUCED GLUTATHIONE (GSH)** (Beutler & Gelbart, 1985)

Glutathione (γ -glutamylcysteinylglycine, GSH) is highly concentrated intracellular antioxidant, accounts for 90% intracellular non-protein thiol content. Highest concentration of GSH is present in liver. Glutathione exists in two forms: reduced glutathione(GSH) and the oxidized form glutathione disulfide (GSSG). The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (Parris MK 1997). Glutathione status is homeostatically controlled both inside the cell and outside, being continually self-adjusting with respect to the balance between GSH synthesis (by GSH synthetase), its recycling from GSSG (by GSH reductase), and its utilization (by peroxidases, transferases, transhydrogenases, and transpeptidases).

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: glacial metaphosphoric acid (1.67g), EDTA (0.20g), NaCl (30g) and total volume was made up to 100 ml with distilled water (D/W)
2. 0.3M Na_2HPO_4 .
3. DTNB : 40 mg DTNB dissolved in 100 ml 1% sodium citrate.
4. PBS (0.1M, pH 7.4).
5. Standard GSH solution : 2mM GSH.

(Standard range 10-100µg)

Sample preparation – 3×10^5 granulosa cells were taken in 100 µl and sonicated for 2 minutes with 5 cycles per minute. 10% Placental homogenate in Phosphate buffered saline (PBS) (0.1M, pH 7.4).

Protocol

| Reagents | Blank (Cell) | Test (Cell) | Blank (Tissue) | Test (Tissue) |
|----------------------------------|--------------|-------------|----------------|---------------|
| Sample | -- | 100 µl | --- | 1 ml |
| Precipitating reagent | 250µl | 250µl | 1.5 ml | 1.5 ml |
| Distill water | 100 µl | --- | 1 ml | --- |
| | | | | |
| Supernatant | 100 µl | 100 µl | 0.5 ml | 0.5 ml |
| Na ₂ HPO ₄ | 0.5 ml | 0.5 ml | 2 ml | 2 ml |
| DTNB | 100 µl | 100 µl | 0.25 ml | 0.25 ml |

Absorbance was read at 412 nm within a minute after the addition of DTNB.

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

Units –GSH mg/g of tissue or GSH µg/ 3×10^5 cells

➤ LIPID PEROXIDATION LEVELS (LPO) (Ohkawa et al., 1979)

Polyunsaturated fatty acids (PUFA) are vulnerable to oxidative damage. ROI generated during various biochemical reactions initiates a chain reaction by abstracting H atom from PUFA and forms primary stable peroxy radical and lipid hydroperoxide. Lipid peroxides generate secondary stable products lipid aldehydes,

malondialdehydes, 4-OH alkenals, alkanals, 2-alkanals and 2-4 alkanals etc. Compared to free radicals these aldehydes are stable and can diffuse within or even escape from the cell and attack targets far from the site of their generation. LPO is a good indicator of oxidative damage to the tissues, especially the membrane lipids.

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 that can be measured colorimetrically at 532 nm.

Reagents

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 .

2. 0.1M Phosphate buffered saline (PBS) - pH 7.4 :

3. 10mM Tetra methoxy propane (TMP) for standard solution.

Standard range (1-10 nmoles)

Sample Preparation – 4×10^5 granulosa cells were taken in 100 μ l of HBSS and washed with PBS twice, to remove HBSS. The cells are resuspended in 100 μ l of PBS and sonicated for 2 minutes with 5 cycles/min. 10 % placental homogenate was prepared in PBS (pH-7.4).

Protocol

| Reagents | Blank | Tissue | Granulosa cells |
|-------------|-------|--------|-----------------|
| Sample | - | 1.0ml | 100 μ l |
| D/w | 1.0ml | - | 100 μ l |
| TBA reagent | 1.0ml | 1.0ml | 1.0 ml |

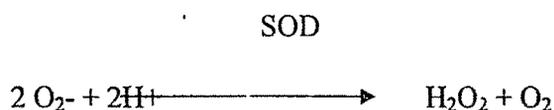
Kept in a boiling water bath for 20 min. After cooling, centrifuged at 3000 rpm/20 min. Absorbance were taken against tissue blank at 540 nm.

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

Unit – nmoles of MDA formed/ g tissue or nmoles of MDA formed/ 4×10^5 cells

➤ **SUPEROXIDE DISMUTASE (SOD)** (Kakkar et al, 1984)

SOD is present in all the aerobic organisms. It provides an essential defense against the potential toxicity of molecular oxygen (Beyer et al 1991, Bowler et al 1992). SOD helps to prevent tissue damage by superoxide radicals($O_2^{\bullet-}$). It is a metalloenzyme which catalyzes dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen (O_2).



Two isoenzymes i.e. Cu-Zn SOD(cytosol) and Mn-SOD(mitochondria) are present in eukaryotic animals which are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments but how it is communicated at molecular level is unknown.

Reagents

1. 0.89%KCl
2. PBS(0.1M, pH 7.4)
3. Sodium pyrophosphate (pH 8.3) - 0.052 mM
4. PMS - 186 μ M
5. NBT - 300 μ M

6. NADH - 780 μ M

Sample Preparation – 4×10^5 granulosa cells suspended 100 μ l of PBS was sonicated for 2 minutes with 5 cycles /min. Sonicated cells were used as a source of enzyme. 4% tissue homogenate in 0.89% KCl was prepared and centrifuged at 3000 rpm for 15 min. Supernatant was used for SOD estimation.

Principle

Mixtures of NADH and phenazine methosulfate (PMS) generates superoxide under non – acidic conditions via the univalent oxidation of reduced PMS. NBT serve as a detector molecule for superoxide through reduction in to a stable, blue coloured formazone product, which can be measured at 560nm.

Protocol

| Reagents | Test | Control |
|-----------------------------|--------------------|---------|
| Sodium pyrophosphate buffer | - 1.2 ml | 1.2 ml |
| PMS | - 0.1 ml | 0.1 ml |
| NBT | - 0.3 ml | 0.3 ml |
| Diluted enzyme | - 0.01ml /0.02ml | --- |
| D / W | - 1.2 ml / 1.18 ml | 1.2 ml |
| NADH | - 0.2ml | 0.2 ml |

All tubes were incubated for 90 seconds at 37°C then reaction was terminated by adding 1.0 ml glacial acetic acid and shaken vigorously. Reduced NBT was extracted in 4 ml of n-Butanoi. Tubes were centrifuged and absorbance was read at 560 nm against butanol blank.

Calculation –

$$\text{SOD (IU/g)} = \frac{\text{OD}^{\text{control}} - \text{OD}^{\text{test}}}{\text{OD}^{\text{control}}} \times \frac{100}{0.01} \times \frac{60}{90} \times \frac{1}{4 \times 10^5 \text{ cells or tissue wt (g/dl)}}$$

Unit – One unit of SOD is defined as the amount of enzyme required to inhibit NBT reduction by 50% as compared to control.

➤ CATALASE (CAT) (Hugo, 1987)

Catalase (CAT) is a heme protein contains four ferriprotoporphyrin groups per molecules. This enzyme is also found in all aerobic organisms and is important in removal of H₂O₂ generated in peroxisomes (microbodies). Highest CAT activity is found in liver and kidney and lowest in connective tissue. In tissue it is mainly present bound to the membranes of mitochondria and peroxisomes, whereas it exist in soluble state in erythrocyte (Hugo Aebi).

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.



Reagents

1. Phosphate Buffer : (50 m mol/ L , pH 7.0)

a. 36 g KH₂PO₄ in 250 ml Distill Water.

b. 13 g Na₂HPO₄ in 300 ml Distill Water.

Mix both in the ratio 1:1.5 and adjust pH to 7.0

2. Hydrogen peroxide (30 m mol/ L)

3. Absolute alcohol (ethanol)

4. Triton X-100 (10%)

4. PBS (0.1M, pH 7.4)

Sample Preparation – Sample preparation was done according to the method of Cohen et.al., 1970. 10% placental homogenate was prepared in PBS (pH 7.4), centrifuged at 1000 rpm to remove cell debris. Supernatant was used for enzyme analysis. 3×10^5 granulosa cells were taken in 200 μ l of PBS (pH-7.4) and sonicated for 2 min at 5 cycles/min. Sonicated cells is used as enzyme source. 10 μ l ethanol was added to 1.0 ml supernatant and these tubes incubated in ice water bath for 30 minutes. Just before the assay 10 μ l of Triton X-100 and 9 ml of phosphate buffer were added.

Protocol

| Reagents | Blank | Tissue /Granulosa cells |
|-------------------------------|-------|-------------------------|
| Sample | 0.2ml | 0.2ml |
| Phosphate buffer | 2.8ml | 1.8ml |
| H ₂ O ₂ | - | 1.0ml |

Decrease in absorbance was monitored at 240 nm for 5 sec

Calculation – $\frac{2.303 \times \log E_1 \times \text{dilution factor}}{\Delta t}$

$$\frac{2.303 \times \log E_1 \times \text{dilution factor}}{\Delta t}$$

Units- μ moles of H₂O₂ decomposed /sec/g tissue or 4×10^5 cells

> CHOLESTEROL ESTIMATION (Leffler and McDougald, 1963)

Principle

In this method cholesterol is extracted from the tissue using isopropanol. The acetic acid-FeCl₃ reagent acts on cholesterol converting it into cholestradiene, which then reacts with concentrated H₂SO₄ to form colored complex. This is estimated colorimetrically at 540 nm. Range of the method is 75-350 mg.

Reagents

1. FeCl₃ reagent – 500 mg FeCl₃.6H₂O in 500 ml of glacial acetic acid or phosphoric Acid.
2. Standard cholesterol – 200 mg/dl in isopropanol
3. Tissue sample – 10% placental membrane or granulosa cell membrane

Protocol

| | Test | Blank | Standard |
|--|------|-------|----------|
| Standard Cholesterol | --- | ---- | 0.5 ml |
| Sample | 0.5 | ---- | --- |
| FeCl ₃ -Glacial Acetic acid | 1.0 | 1.0 | 1.0 |
| Conc. H ₂ SO ₄ | 2.0 | 2.0 | 2.0 |

Mixed well, kept at room temp for 10 min. and read the absorbance at 540 nm.

Range of the method – 75-350 µg

Slope- 0.016 O.D. / µg cholesterol

> TOTAL PHOSPHOLIPIDS (Folch et al., 1957)

Extraction was done by the method given by Folch et al. using organic solvents to dissolve the phospholipids. MgCl₂ was added to precipitate the proteins. The extracted

phospholipids were digested by heating in the presence of strong acid so that only Pi remains which is estimated by Bartlett's method which is an improvement over the Fiske Subbarao method with an increased sensitivity and reliability. The absorption at 830nm is increased by heating the mixture in strong acid. The range of the method is 0.4-4 μ g.

Reagents

1. Chloroform: methanol mix (2:1 v/v) prepared fresh.
2. MgCl₂ 0.017gm% in Distill Water
3. Digestion mixture: 25 ml perchloric acid, 25 ml H₂SO₄ in 100ml Distill Water
4. Ammonium molybdate: 4 gm ammonium molybdate was dissolved in minimum volume of conc. H₂SO₄ and then made upto 100 ml with Distill Water.
5. 1-amino 2-naphthol 4-sulphonic acid (ANSA) 20 mg triturate (mixture of 0.2 g ANSA, 1.2g sodium sulfite, 1.2 g sodium sulfite) in 1ml distilled water.
6. 10 N H₂SO₄.

Protocol

The method was standardized with respect to cell number and finally 4×10^5 cells were used. To this 2ml of chloroform-methanol mix was added, the tubes were vortexed and centrifuged. The last layer was drawn out and 1ml chloroform-methanol mix was added to the remaining denatured protein. This step is repeated twice. All the extracts are pooled and 0.4 ml of 0.017% MgCl₂ is added to this. The reaction tube is again vortexed and extracted. The extract is evaporated to zero volume. To this phospholipids coated tube, 1 ml of extraction mix and 0.5 ml digestion mix is added and the tubes are digested overnight in a sand bath till they do

not smell of chlorine. The digest thus obtained is used for Pi estimation by Bartlett's method.

Protocol

| Reagents | Test | Blank |
|--|-------------|---------|
| Digest | 100 μ l | -- |
| 10 N H ₂ SO ₄ | 0.25 ml | 0.25 ml |
| Ammonium Molybdate | 0.2 ml | 0.2 ml |
| Distill water | 3.9 ml | 3.9 ml |
| ANSA | 0.1 ml | 0.1 ml |
| Vortexed, keep it in boiling water bath for 8-10 min | | |
| Cool it and read at 830 nm. | | |

The standard graph was obtained with slope=0.25 O.D./ μ g.

Formula used for calculation = OD (test)/OD (std) x conc. of std. x 0.5/0.05 x 25

Unit: μ g of total lipid/mg protein

➤ GLYCOGEN CONTENT (Seifter et al., 1950)

Principle

Glycogen reacts with strong alkali and decomposed into glucose, which on further reaction with Anthrone reagent forms coloured complex.

Reagents

1. 30%KOH
2. 0.2% Anthrone reagent
3. Sample- To one gm of placental tissue, 3 ml 30%KOH is added. Heat it in a boiling water bath for 30 min. Take aliquot for glycogen estimation.

Protocol

| Reagent | Test | Blank |
|------------------|------|-------|
| Sample | 1.0 | --- |
| KOH | ---- | 1.0 |
| Anthrone reagent | 2.0 | 2.0 |

The tubes were covered with glass marble, kept for 10 min. in a boiling water bath.

After cooling, absorbance was read at 620 nm.

Standard range - 15-150 μg .

➤ ESTIMATION OF DNA (Burten, 1956)

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 Diphenylamine to give blue colored complex which is estimated colorimetrically.

Reagents

1. Phosphate buffered saline (PBS)(pH=7.4)
2. Diphenylamine (DPA) Reagent : Mixture of 0.75 g of DPA, 50 ml glacial acetic acid, 0.75 ml conc. Sulfuric acid, 0.25 ml distill water and 5 μl of acetaldehyde.
3. Ethyl alcohol 95%
4. Trichloro acetic acid (TCA) 10%

Sample preparation- 10% placental 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. The supernatant containing acid solution fraction was discarded and pellet was suspended again in 2.5 ml 10% TCA. Centrifuged at 4000 rpm for 15 min and the pellets were dissolved in 5 ml 95% ethyl alcohol. Centrifuged at 4000 rpm for 15 min. and repeated the same procedure twice. The pellet was then dissolved in 5 ml 5% TCA, heated at 90⁰ C for 30 min. Cooled and centrifuged at 4000 rpm for 20 min. The supernatant was used for nucleic acid estimation.

| Protocol | 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 | | |

Standard - range 10-200 µg/ml.

Unit: µg/g tissue

➤ 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 (PBS)
2. Standard RNA
3. Orcinol reagent

4. Ethyl alcohol 95%

5. Trichloro acetic acid (TCA) 10%

Sample preparation-as described for DNA estimation

| | 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 | | |

Standard – Standard graph for RNA was prepared in the range 10-80 $\mu\text{g/ml}$.

Unit – $\mu\text{g/g}$ tissue

➤ **TOTAL LIPIDS** (Folch et al., 1957)

Principle

Method is based upon the extraction of lipids from the tissue by chloroform-methanol extraction, which gives coloured compound with dichromate.

Reagents

1. Chloroform-methanol (3:1 volume/volume)
2. Potassium dichromate :15 mg/ml

Sample Preparation: 5% homogenate in PBS(pH=7.4)

Protocol

To 5 ml homogenate, 10 ml of chloroform: methanol mixture is added and kept in water bath at 60 C until zero volume is made. 1.8 ml of potassium dichromate solution is added and kept in boiling water bath for 40 min. Solution is cooled and volume is made to 3.5 ml with distill water. Absorbance is read at 620 nm.

Standard – Standard graph was prepared in the range 50-500 µgm.

Unit: µg/gm tissue.

➤ **CATHEPSIN-D** (Anson, 1937)

This enzyme is a lysosomal endopeptidase and known to play a role in physiological and pathological breakdown of intracellular and extracellular proteins.

Principle

Cathepsin-D activity is measured by degradation of hemoglobin, which leads to liberation of acid soluble tyrosine and tryptophan containing peptides. These peptides are then quantitatively measured by sensitive reaction of Folin-Ciocalteu reagent and giving the absorbance at 660 nm.

Reagents

1. Acetate buffer (pH=3.5)
2. Substrate solution (100g/L): Dissolve 2 g of hemoglobin in 20 ml Acetate buffer. And adjust the pH to 3.5
3. Trichloro acetic acid (0.3 M)
4. NaOH (0.5M)
5. FC reagent: Dilute 1 volume to 2 volumes with distill water.

Standard solution: Dissolve 181.2 mg in min amt of HCl and make upto 1 litre.

Sample preparation: 10% uterine homogenate

Protocol

| Reagents | Test | Blank |
|--|--------|--------|
| Sample | 0.2 ml | --- |
| Distill water | --- | 0.2 ml |
| Substrate | 1 ml | 1 ml |
| Shake vigorously at 37°C for 10 min | | |
| TCA | 2 ml | 2 ml |
| Shake and stand the tubes at RT for 30 min; Filter through Watmann No. 3 | | |
| Filtrate | 1 ml | 1 ml |
| NaoH | 2 ml | 2 ml |
| FC reagent | 0.6 ml | 0.6 ml |
| Mix and wait for 5 min and note the absorbance at 750 nm against blank | | |

Standard – Standard graph for Cathepsin-D was prepared in the range 4-40 ~~µmoles~~ ^{ug. (Correction)}

Units: nmols of tyrosine formed/min/ mg protein

➤ ALKALINE PHOSPHATASE (ALP) (Bowers & Mc Comb, 1975)

The hydrolytic enzymes are present in high concentrations in liver, bone, and placenta. Elevations of ALP are of most diagnostic significance in the evaluation of hepatic and bone disorders.

Principle

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

Reagents

1. P-Nitro phenyl phosphate (PNPP) (0.4%)
2. 0.05 M NaOH.
3. Glycine buffered substrate: Mix 5.7 g of glycine, 0.095 g of Magnesium Chloride (anhydrous), in 750 ml of Distill water and 85 ml of 1N NaOH and made to 1 litre.
4. Working substrate: Mix equal amount volume of glycine buffer substrate and stock PNPP . Adjust pH =10.3 to 10.4
5. Standard PNP solution - Stock 1 mM in 0.05 N NaOH (Prepared fresh)
Standard range: 0.04 to 0.16 μ mols

Sample preparation – serum/ plasma or uterine lysosomal fraction

| Reagents | Test | Blank |
|--|---------|-------|
| Buffered substrate | 1 ml | ---- |
| Placed in water bath at 37 C for 5 min | | |
| Serum /Tissue | 0.05 ml | ---- |
| Incubate for 30 minutes/37° C | | |
| 0.05 N NaOH | 10 ml | 10 ml |
| Absorbance was recorded at 405 nm | | |

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

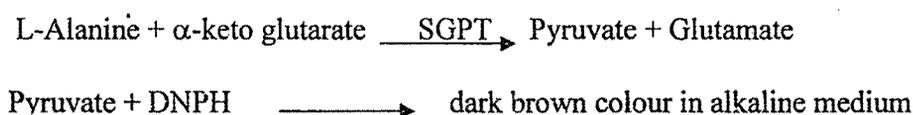
Units: μ moles of PNP formed/ min/l of serum or g tissue

➤ **SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT)** (Reitman and Frankel ,1957)

The enzyme belongs to the class of transferases. GPT is distributed in many tissues and high concentrations present in the liver. High GPT activity is seen in liver disorders and hepatocellular disorders.

Principle

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



Reagents

1. Buffered substrate (pH 7.4): Dissolve 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 distill water and made up the volume to 1 litre. Adjusted the pH to 7.4 with NaOH.
2. 2,4 Di Nitro Phenyl Hydrazine (DNPH). Dissolve 200 mg in 250 ml of 1 N HCl and made up the volume to 1 litre with 1 N HCl.
3. 16 g/l (400nM) NaOH.
4. Sodium pyruvate (44 mg%)
5. Sample – serum sample

(Standard range – 22-110 μ g)

| Reagents | Test | Blank |
|-------------------------------|---------|---------|
| Serum | 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.

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

Units: μ moles of Pyruvate formed/ min/ litre

➤ HEMOGLOBIN (Drabkin and Austin, 1932)

A decrease in hemoglobin below normal range is an indication of anemia. An increase in hemoglobin concentration occurs in hemoconcentration due to loss of body fluid in severe diarrhea and vomiting. High values are also observed in congenital heart disease in emphysema and also in polycythemia. Hemoglobin concentration drops during pregnancy due to hemodilution.

Principle

When blood is mixed with Drabkin's reagent containing potassium cyanide and potassium ferricyanide, hemoglobin reacts with ferricyanide to form methaemoglobin, which is converted to stable cyanmethaemoglobin by the cyanide. The intensity of the

colour is proportional to hemoglobin concentration and it is compared with a known cyanmethaemoglobin standard at 540 nm.

Reagents

Drabkin's reagent - A mixture of 200 mg potassium ferricyanide, 50 mg of Potassium cyanide, 140 mg of potassium dihydrogen phosphate and one ml of Triton-X 100 dissolved in 1L DW.

Cyanmeth haemoglobin (Hb standard) standard: 15 g/dl (O.D. of this standard at 540 nm corresponds to 15 g/dl hemoglobin)

| | Test | Blank |
|-------------------|------|-------|
| Drabkin's reagent | 5.0 | 5.0 |
| Blood | 0.02 | 0.02 |

Mixed well, kept at room temperature for 10 min and read the absorbance at 540 nm.

Absorbance of the standard (15g/dl) was read by pipetting it directly in a cuvette.

Unit: g/dl

➤ 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 | --- |
| Distill 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.

➤ HISTOLOGY

Ovary, Uteri and placental tissues 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.

➤ SDS Polyacrylamide Gel Electrophoresis (SDS PAGE) (Laemeli method)

Electrophoresis is a method where charged molecules like proteins migrate in response to electric field. The rate of migration depends upon strength of the electric field; net charge, size and shape of the molecules and also on the ionic strength, viscosity and the temperature of the medium in which molecules are moving.

Principle

In SDS PAGE, migration is not dependent on intrinsic charge of the polypeptide but on the molecular weight. Sodium Dodecyl Sulfate (SDS) is an anionic detergent, which denatures the protein by wrapping around the polypeptide backbone (i.e., 1.4 g of SDS binds per g of protein) and gives the polypeptide a negative charge in proportion to its length. When treated with SDS and a reducing agent (DTT or 2-mercaptoethanol), the polypeptides become rods of negative charges with equal charge densities. This system is efficient in separation of proteins according to molecular weight as they move towards the anode. Protein mobility, a quantitative measure of migration rate of the charged species is intermediate between the mobility of the buffer ion in the stacking gel (leading ion) and mobility of the buffer ion in the upper tank (trailing ion). When electrophoresis is started, the ions and the protein start to migrate into the stacking gel. The protein concentrate in a very thin zone, called the stack, between the leading ion and the trailing ion. The protein continues to migrate in the stack until they reach the separating gel.

Reagents

- 30 % Acrylamide/ 0.8% Bisacrylamide (Soln A): Mix 30 g Acrylamide and 0.8 g of NN' Methylene Bis Acrylamide in a total volume of 100 ml Distill Water. Filter through watman paper and at 4 °C in dark bottle. Discard after 30 days, since Acrylamide gets degraded to acrylic acid and ammonia.

NOTE: *Acrylamide is neurotoxic. Gloves should be worn during handling of solution. Avoid pipetting thro' mouth.*

- 4X Tris-Cl/ SDS pH=6.8 (0.5 M Tris-Cl containing 0.4% SDS) (Soln. C) [Stacking gel buffer: 6.05 g of Tris base was dissolved in 40 ml of Distill water. pH was adjusted to 6.8 with HCl. And total volume was made up to 100 ml. 0.4 g of SDS was added and stored at 4°C.

- 4X Tris-Cl/ SDS pH=8.8 (1.5 M Tris-Cl containing 0.4%SDS) (Soln. B) [Separating Gel buffer]

91 g of Tris base was dissolved in 300 ml of Distil water. pH was adjusted to 8.8 with 1N HCl and made up to 500 ml with distill water. 0.4g of SDS was added and store at 4 °C.

- 10% Ammonium Per Sulfate (APS): Dissolve 100mg of APS in 1ml water. (Prepared Fresh)

- Preparation of separating gel (10%)

| | |
|--|-----------|
| 30% Acrylamide solution/0.8% bisacrylamide solution (Soln A) | : 5 ml |
| 4X Tris-Cl/ SDS pH=8.8 (Soln. B) | : 3.75 ml |
| Distill water | : 6.25 ml |

10% Ammonium per sulfate : 0.05 ml

TEMED : 0.01 ml

Mix all the constituents and pour immediately into gel apparatus.

- Preparation of stacking gel: In 25 ml beaker, 0.65 ml of 30%acrylamide/ 0.8% bisacrylamide, 1.25 ml of 4x Tris-Cl /SDS (soln. C), pH=6.8 and 3.05 ml distill water, 0.025 ml of 10% ammonium per sulfate and 0.005 ml of TEMED was added and mixed gently. Mixture was poured into the gel apparatus immediately.
- 2X Treatment Buffer: 2.5 ml of Tris-Cl (pH= 6.8), 4 ml SDS (4%), 2 ml of Glycerol(20%), 100 µl of bromophenol blue (marker) and 1 ml of 2- mercapta ethanol was mixed together and volume was made to 10 ml with distill water. Divide the aliquots, freeze at -70°C.
- Tank Buffer(0.025 M Tris, .192 M glycine, 0.1% SDS, pH =8.3)
Tris Base 2 g, Glycine 57.6 g, SDS 40 ml, make volume with water upto 4 litres.
- Stain (0.025 % Commassie blue R-250, 40% Methanol, 7% Acetic acid)
Commassie Blue R-250 0.5 g, Methanol 800 ml, Stir until dissolved Add Acetic acid 140 ml make up volume to 1 litre with Distill water.
- Destaining Solution (50% Methanol, 10% acetic acid)
Methanol 500 ml, Acetic acid 100 ml, Make up the volume to 1 litre.

Protocol

- Pouring the separating gel: Assemble glass plate sandwich using clean glass plates and two 0.75mm spacers. Pour the separating gel as mentioned earlier.

Using pipette, slowly cover the top the gel with water saturated with isobutyl alcohol.

Allow the gel to polymerize for 30 to 60 minutes at RT.

- Pouring the stacking gel: Pour off the layer of water saturated with isobutyl alcohol and rinse with Tris-Cl/ SDS (1X). Rinse well 2 to 3 times.
- Pour the stacking gel as mentioned earlier.
- Pour the solution in to glass plates immediately else it will get polymerized.
- Do not introduce air bubbles into the stacking gel.
- Insert the Teflon comb into the layer of stacking gel solution. If necessary, add additional stacking gel to completely fill the spaces in the comb. Do not introduce air bubbles while insertion of comb.
- Allow the stacking gel to polymerize 45 minutes to 1 hr.

Preparation of sample and loading the gel:

Dilute the aliquot of the protein sample to be analyzed (1:1) v/v with 2x SDS/ sample buffer and heat for 3 to 5 minutes at 100 ° C in a sealed screw cap eppendroff. If the sample is precipitated protein pellet, dissolve the protein to be separated in 50 to 100µl of 1X SDS/ sample buffer and boil for 5 minutes at 100 ° C. Dissolve protein molecular weight standards mixture 1x sample buffer as per supplier instruction.

For dilute samples, take 5 parts of protein solution to 1 part of 6X SDS sample buffer to increase the amount of protein loaded. Proteins can be concentrated by precipitation in acetone, ethanol, TCA but losses occur. For 0.8 cm well, 25 to 50 µg total protein is recommended when stained with Commassie blue staining and 1 to 10 µg total protein is needed for samples containing one or few proteins.

- Carefully remove the Teflon comb, rinse well with 1X SDS electrophoresis buffer to remove unpolymerised gel.

- Fill the lower tank with 1X SDS electrophoresis buffer and partially fill the upper tank with 1X SDS electrophoresis buffer.
- Using micropipette load the protein samples into the wells. Protein samples are mixed with bromophenol blue before loading. Unloaded well can be loaded with 1X SDS electrophoresis buffer, so that protein sample do not diffuse.
- Now fill remainder upper tank with 1X SDS electrophoresis buffer, so that electrode is in contact with buffer.

Run the gel

- Connect the power supply to cell and run at 10mA of constant current until bromophenol blue -Tracking dye enters the separating gel. Then increase the current to 15 mA.
- After bromophenol blue tracking dye reaches at the end of the separating gel, disconnect the power supply.

Disassemble and analyze the gel

- Discard the electrode buffer and remove the upper buffer and attached sandwich.
- Carefully remove the spacers from edge of sandwich along its length.
- Cut edge of the gel in form of triangle and put in the staining solution for 2 hours.
- Remove the gel and then place in destaining solution until background of the gel becomes clear.

➤ Statistical analysis

Results were subjected to Student's-test, one way analysis of variance (ANOVA); ANOVA followed by Neumans-Keuls multiple comparison test using prism 3.03 software to test the difference among different treated and untreated groups.