Chapter 3

Materials and Methods
MATERIALS AND METHODS

70 healthy young females were selected for the present study. They were the students of School of Medical Education, Kottayam, Kerala and Academy of Medical Sciences Pariyaram, Kannur, Kerala. The following criteria were observed during the selection of the subjects.

- Only those young females who had regular menstrual cycle were selected.
- Those who take any kinds of medication were excluded.
- Those having any previous history of respiratory, cardiovascular or reproductive diseases were excluded.
- All the subjects were non-smokers.

Physical Characteristics

Age

All the female subjects taken for this study were young healthy girl students in the age group of 18-24 year.

Height

Measuring tape was used for measuring the height of the subjects. It was measured when the subject stood straight on a uniform horizontal surface without shoes, with heels together, shoulders relaxed and arms by the side. Measurement was made to the nearest millimetre with the examiner’s eye at the level with the head of the subject to avoid any possible error.
Weight

Weight was measured using lever type Balance (Krupp's India) up to 100 g accuracy. It was measured as far as possible, the same time and uniform conditions of the day to avoid the fluctuation due to the effect of food and other factors. The weighing machine was adjusted to zero before each measurement.

Body Surface Area (BSA)

The body surface area of the subjects was calculated from height and weight using Dubois and Dubois formula (1916).

\[
\text{Body Surface Area (BSA)} = \frac{Wt \times \text{Ht}^{0.425} \times \text{Ht}^{0.725} \times 71.84}{10000}
\]

Respiratory Function Tests

Respiratory function tests during the 4 different phases of menstrual cycles namely the menstrual phase (1 - 4 days), follicular phase (5 - 10 days), midcycle (11 - 16 days) and luteal phase (17 - 28 days) were done using a computerized advanced form of electronic Spirometer called Vitallograph compact II (Vitallograph Ltd., Buckingham, U.K.). Vital Capacity (VC), Forced Vital Capacity (FVC) and the Inspiratory Vital Capacity (IVC) manoeuvres were done by the subject. Both inspiratory and expiratory flow rates were obtained from FVC test. All the subjects were adequately instructed about the test and trial tests were done before the actual test. Each subject was asked to perform three tests and the best performance was recorded. All the tests were performed in the standing position with the nose clipped. The values were automatically converted in the spirometer into BTPS Basal Temperature (37°C), ambient pressure, saturated with water vapour. All the spirometric indices were automatically recorded for the 'best' test as defined by American Thoracic Society, i.e., greatest sum of spirometric indices.
The following respiratory function tests selected for the present investigation.

<table>
<thead>
<tr>
<th>No.</th>
<th>Test</th>
<th>Abbreviations</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Lung Volumes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>VC</td>
<td>Vital capacity</td>
<td>L</td>
</tr>
<tr>
<td>3.</td>
<td>IV C</td>
<td>Inspiratory vital capacity</td>
<td>L</td>
</tr>
<tr>
<td>4.</td>
<td>FVC</td>
<td>Forced vital capacity</td>
<td>L</td>
</tr>
<tr>
<td>5.</td>
<td>FEV(_{0.5})</td>
<td>Forced expiratory volume in 0.5 sec.</td>
<td>L</td>
</tr>
<tr>
<td>6.</td>
<td>FEV(_{1})</td>
<td>Forced expiratory volume in 1 sec.</td>
<td>L</td>
</tr>
<tr>
<td>7.</td>
<td>FEV(_{0.5}/\text{FVC}) %</td>
<td>Percentage of FEV(_{0.5}/\text{FVC})</td>
<td>%</td>
</tr>
<tr>
<td>8.</td>
<td>FEV(_{1}/\text{VC}) %</td>
<td>Percentage of FEV(_{1}/\text{VC})</td>
<td>%</td>
</tr>
<tr>
<td>9.</td>
<td>FEV(_{1}/\text{FVC}) %</td>
<td>Percentage of FEV(_{1}/\text{FVC})</td>
<td>%</td>
</tr>
<tr>
<td>10.</td>
<td>MVV(_{0.5})</td>
<td>Maximum voluntary ventilation</td>
<td>L/min</td>
</tr>
</tbody>
</table>

**Flow rates**

<table>
<thead>
<tr>
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<th>Test</th>
<th>Abbreviations</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>PEF</td>
<td>Peak expiratory flow rate</td>
<td>L/min</td>
</tr>
<tr>
<td>2.</td>
<td>FEF(_{0.2-1.2})</td>
<td>Forced expiratory flow rate bet. 0.2-1.2L of FVC</td>
<td>L/sec</td>
</tr>
<tr>
<td>3.</td>
<td>FEF(_{25-75})%</td>
<td>Forced expiratory flow rate bet. 25-75%</td>
<td>L/sec</td>
</tr>
<tr>
<td>4.</td>
<td>FMFT</td>
<td>Forced mid flow time</td>
<td>L/sec</td>
</tr>
<tr>
<td>5.</td>
<td>FEF(_{75-85})%</td>
<td>Forced expiratory flow rate bet. 75-85%</td>
<td>L/sec</td>
</tr>
<tr>
<td>6.</td>
<td>FEF(_{25})%</td>
<td>Forced expiratory flow rate at 25%</td>
<td>L/sec</td>
</tr>
<tr>
<td>7.</td>
<td>FEF(_{50})%</td>
<td>Forced expiratory flow rate at 50%</td>
<td>L/sec</td>
</tr>
<tr>
<td>8.</td>
<td>FEF(_{75})%</td>
<td>Forced expiratory flow rate at 75%</td>
<td>L/sec</td>
</tr>
<tr>
<td>9.</td>
<td>PIF</td>
<td>Peak inspiratory flow rate</td>
<td>L/sec</td>
</tr>
<tr>
<td>10.</td>
<td>FIF(_{75})%</td>
<td>Forced inspiratory flow rate at 75%</td>
<td>L/sec</td>
</tr>
<tr>
<td>11.</td>
<td>FIF(_{50})%</td>
<td>Forced inspiratory flow rate at 50%</td>
<td>L/sec</td>
</tr>
<tr>
<td>12.</td>
<td>FIF(_{25})%</td>
<td>Forced inspiratory flow rate at 25%</td>
<td>L/sec</td>
</tr>
</tbody>
</table>

(L - Litres, Sec = seconds, ind = indirect, min. = minutes, % = percentage)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Measurement</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC</td>
<td>Vital capacity</td>
<td>The maximum volume of air that can be exhaled after a maximum inspiration</td>
</tr>
<tr>
<td>IVC</td>
<td>Inspiratory vital capacity</td>
<td>IVC is performed by conducting normal tidal respiration until stability is attained, then a full expiration followed by a full inspiration.</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
<td>The volume of air which can be 'squeezed' from the lung when the subject makes a maximum expiratory effort from a position of full inspiration.</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;0.5&lt;/sub&gt;</td>
<td>Forced expiratory volume indicating the time interval in seconds</td>
<td>The volume of air expelled from the lung over a timed period during expiration.</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in percentage</td>
<td>The ratio of timed forced expiratory volume to vital capacity and forced vital capacity expressed in percentage.</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;0.5&lt;/sub&gt;/FVC%</td>
<td>Forced expiratory volume as a percentage of FVC</td>
<td>Maximum volume of air which the subject can move as expiration, during the repetitive maximum respiratory effort.</td>
</tr>
<tr>
<td>MVV</td>
<td>Maximum voluntary ventilation</td>
<td>The maximum flow rate observed during the entire forced expiratory manoeuvre.</td>
</tr>
<tr>
<td>PEF</td>
<td>Peak expiratory flow rate</td>
<td>The maximum flow rate observed during the entire forced expiratory manoeuvre.</td>
</tr>
<tr>
<td>FEF&lt;sub&gt;0.2-1.2&lt;/sub&gt;</td>
<td>Forced expiratory flow between 0.2-1.2 litres</td>
<td>The volume of air exhaled between 0.2 - 1.2 litres of FVC, expressed in litres/sec. (BTPS).</td>
</tr>
<tr>
<td>FEF&lt;sub&gt;25-75%&lt;/sub&gt;</td>
<td>Forced mid expiratory flow rate</td>
<td>The mean expiratory airflow rate measured over the middle half of forced vital capacity, i.e. between 25% and 75% of the forced vital capacity.</td>
</tr>
<tr>
<td>FEF&lt;sub&gt;75-85%&lt;/sub&gt;</td>
<td>Forced late expiratory flow rate</td>
<td>The mean expiratory airflow rate measured between 75% and 85% of the forced vital capacity.</td>
</tr>
<tr>
<td>FMFT</td>
<td>Forced mid expiratory flow time</td>
<td>The time taken to expire the air during middle half of the forced vital capacity, i.e. the time difference between 25% and 75% of the FVC.</td>
</tr>
<tr>
<td>FEF&lt;sub&gt;75%&lt;/sub&gt;</td>
<td>Maximum expiratory flow qualified by volume at different percentage</td>
<td>Instantaneous maximum expiratory flow at specified lung volume, i.e. (a) at 75% of FVC; (b) at 50% of FVC; (c) at 25% of FVC.</td>
</tr>
<tr>
<td>FIF&lt;sub&gt;25%&lt;/sub&gt;</td>
<td>Peak inspiratory flow rate</td>
<td>The maximum inspiratory flow achieved during inspiratory phase of FVC.</td>
</tr>
<tr>
<td>PIF</td>
<td>Peak inspiratory flow rate</td>
<td>The maximum inspiratory flow achieved during inspiratory phase of FVC.</td>
</tr>
<tr>
<td>FIF&lt;sub&gt;25%&lt;/sub&gt;</td>
<td>Maximum inspiratory flow qualified by volume at different percentage</td>
<td>Instantaneous maximum inspiratory flow at specified lung volume, i.e. (a) at 25% of FVC; (b) at 50% of FVC; (c) at 75% of FVC.</td>
</tr>
</tbody>
</table>

Ref.: Bouhays (1977); Kaemarck, Mack and Dimas (1985); Garbe and Chapman (1988)
Estimation of biochemical parameters

Total serum cholesterol (Allain et al., 1974)

Principle

Cholesterol esters are hydrolysed by cholesterol esterase to free cholesterol and fatty acids. Free and bound cholesterol are oxidised by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide is liberated. The hydrogen peroxide produced couples with 4-aminoantipyrine and phenol in the presence of peroxidase to form a coloured compound. The intensity of the colour so developed is proportional to cholesterol concentration and is measured photometrically at 500 nm wave length (490 to 550 nm) or with green filter.

Cholesterol esterase

\[
\text{Cholesterol esters} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol + Fatty acids}
\]

Cholesterol oxidase

\[
\text{Cholesterol} \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one + H}_2\text{O}_2
\]

Peroxidase

\[
\text{H}_2\text{O}_2 + \text{Phenol + 4-Amino antipyrine} \xrightarrow{\text{Peroxidase}} \text{Red Quinone + H}_2\text{O}
\]

Reagents

1. **Buffer**
   - Phenol
2. **Enzymes**
   - Cholesterol esterase
   - Cholesterol oxidase
   - Peroxidase
   - 4-Amino antipyrine
   - Sodium cholate
3. **Standard**
   - Cholesterol: 200 mg/dl

**Procedure**

Transfer and dissolve the enzymes to the buffer mix well and store at 2 to 8°C. This is the chromogen reagent.

Put approximately 1 ml chromogen reagent in all the three test-tubes labelled as Blank (B), Standard (S) and Test (T). Mix and incubate at 37°C for 10 min and read absorbance of test ($A_T$) and standard ($A_S$) against the reagent (B) at 500 nm wavelength (40 to 550 nm) or with Green filter. The colour developed is stable for 1 hour at room temperature.

**Calculations**

Cholesterol concentration (mg/dl) = $\frac{A_T}{A_S} \times 200$

**HDL-Cholesterol (Allain et al., 1974; Izzo et al., 1981)**

**Principle**

Chylomicrons, very low-density lipoprotein (VLDL) and low density lipoprotein (LDL) of serum are precipitated using buffered polyethylene glycol (PEG 600). After centrifugation, high-density lipoproteins remain in the supernatant. The cholesterol and HDL fraction is then estimated by an enzymatic
method using cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine and phenol.

**Reagent 1**

Glycine buffer : pH 10.2

PEG 6000 : 10 g/dL

**Reagent 2**

Cholesterol standard : 50 mg/dL

**Procedure**

**Step I: HDL-Cholesterol separation**

<table>
<thead>
<tr>
<th>Pipette into centrifuge tubes</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>0.3 mL</td>
</tr>
<tr>
<td>Precipitating reagent</td>
<td>0.3 mL</td>
</tr>
</tbody>
</table>

Mix well, keep at room temperature for 10 min and then centrifuge at 4000 rpm for 10 min or at 2000 rpm for 20 min, to obtain a clear supernatant.

**Step II: HDL-Cholesterol Estimation**

Estimate the cholesterol content in the supernatant from step I using cholesterol reagent.
Mix and incubate at 37°C for 10 min or at room temperature (25°C ± 2°C) for 20 min. Mix and read absorbance of the Test (A_T), Standard (A_S) and reagent Blank (A_B) at 500 nm (490-550 nm) or with Green filter.

**Calculations**

\[
\text{HDL cholesterol (mg/dL)} = \frac{A_T - A_S}{A_B} \times 50 \times 2 \quad \text{(Dilution factor of sample)}
\]

To convert mg/dL to mmol/L:

\[
\text{Mmol/L} = \text{mg/dL} \times 0.0258
\]

- A_T - Absorbance of the test
- A_S - Absorbance of standard
- A_B - Absorbance of blank

**LDL-Cholesterol (Allain et al., 1974; Izzo et al., 1981)**

LDL-C can be calculated by using Friedwald and Fredrickson’s formula.

\[
\text{LDL-C} = \text{Total cholesterol} - \frac{\text{Triglycerides} + \text{HDL-C}}{5}
\]
VLDL-Cholesterol (Allain et al., 1974; Izzo et al., 1981)

VLDL-C can be calculated from the total cholesterol, HDL-C and LDL-C using the following equation by the above said authors.

\[
\text{VLDL-C} = \text{Total cholesterol} - (\text{HDL-C} + \text{LDL-C})
\]

Serum Triglycerides (Fossati et al., 1982; McGowan et al., 1983)

Principle

Serum triglycerides are hydrolyzed to glycerol and free fatty acids by lipase. Glycerol is then phosphorylated by ATP to glycerol -3-phosphate (G3P) and adenosine -5-di phosphate in a reaction catalysed by glycerol kinase (GK). G3P is then converted to dihydroxyacetone phosphate (DAP), a hydrogen peroxide by glycerol phosphate oxidase (GPO). The hydrogen then reacts with 4-amino anti pyrene (4 -APP) and 3-hydroxy-2,4,6-Tribromobenzoic acid (TBHB) in a reaction catalysed by peroxidase to yield a red colored quinoneimine dye. The intensity of the colour produced is directly proportional to the concentration of triglycerides in the sample when measured at 540 nm.

\[
\begin{align*}
\text{Triglycerides} & \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty Acids} \\
\text{Glycerol} + \text{ATP} & \xrightarrow{\text{GK}} \text{G}_{3}\text{P} + \text{ADP} \\
\text{G}_{3}\text{P} + \text{O}_{2} & \xrightarrow{\text{GPO}} \text{DAP} + \text{H}_{2}\text{O}_{2} \\
\text{H}_{2}\text{O}_{2} + \text{TBHB} & \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + \text{H}_{2}\text{O}
\end{align*}
\]
**Reagents**

- Buffer 50 mM
- ATP 0.5 mM
- 4-Aminoazophenone 0.3 mM
- HRP > 2 U/mL
- Chlorophenol 3.5 mM
- Magnesium 12 mM
- Glycerol Kinase 0.3 U/mL
- Glycerol Phosphate Oxidase 4.5 U/mL
- Lipase 200 U/mL
- Surfactant, Stabilisers and Preservatives

**Procedure**

Label test tubes Blank, Standard, Controls, etc. Pipette 1.0 ml of reagent into each cuvette. Place all tubes in incubator and bring reagent up to 37°C. Pipette 0.01 ml of sample into respective tubes. Incubate all tubes at 37°C for five minutes. Zero spectrophotometer at 540 nm with reagent blank. Read and record absorbances of all tubes.

**Calculation**

If Absorbance sample = 0.300, Abs. of Standard = 0.200, concentration of standard = 300 mg/dL.
Serum bicarbonate (Henry et al., 1974; Tietz et al., 1982)

Principle

Carbon dioxide in the form bicarbonate ions reacts with PEP, in the presence of PEPC, to form oxaloacetate and phosphate. The oxaloacetate is then converted to malate by the action of MDH and reduced NADH. The decrease in absorbence at 340 nm resulting from the oxidation of NADH is proportional to the amount of CO₂ in the sample.

\[
\text{PEP} + \text{HCO}_3^- \xrightarrow{\text{PEPC}} \text{Oxaloacetate} + \text{H}_2\text{PO}_4^- \\
\text{Oxaloacetate} + \text{NADH} + \text{H} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+ 
\]

Reagents

- PEP 1.8 mM
- Magnesium sulphate 10 mM
- NADH 0.40 mM
- MDH (porcine) 1250 U/L
- PEPC microbial ≥ 200 U/L
- Sodium oxamnate 2.5 mM
- Buffer 50 mM
- pH 7.5 ± 0.1
- Sodium Azide 0.1 %
- CO₂ diluent - Reagent dilute with resin to prevent uptake of atmospheric CO₂
Procedure

Label test tubes Blank, Standard, Controls, Patients, etc. Pipette 2.0 ml carbon dioxide reagent into each tube. Pipette 0.01 ml standard, controls and patients into their respective tubes. Mix well. Incubate all tubes at 37°C for five minutes. Zero spectrophotometer at 340 nm with water. Read and record absorbances of all tubes at 340 nm.

Calculations

\[
\frac{\text{Abs. Reagent Blank} - \text{Abs. Sample}}{\text{Abs. Reagent Blank} - \text{Abs. Standard}} \times C_s = \text{Carbon dioxide (mmol/L)}
\]

where \( C_s \) = value of standard in mmol/L.

Haemoglobin: Shali’s method (Baker and Silverton, 1980)

Principle

Haemoglobin in red blood corpuscles is converted into acid haematin. The brown colour so developed is matched against standard brown tinted glass in the comparator by direct vision. Reading is taken directly as Hb/100 ml blood.

Apparatus and Reagents

1. Sahli graduated tube and standard
2. N/10 HCl
3. 0.02 ml pipette
Procedure

Fill the graduated tube to the 20 mark with N/10 HCl. Add 0.02 ml of blood, mix well and leave for 5-10 min. Add N/10 HCl drop by drop, mixing between each addition, until the colour matches the standard. Read the amount of solution in the graduated tube. The calibrations give the haemoglobin concentration as a percentage. The haemoglobin content in g/100 ml of blood or g% is read directly from the tube.

Plasma proteins (Reinhold, 1953)

Principle

Protein in serum forms a blue coloured complex when reacted with cupric ions in an alkaline solution. The intensity of the violet colour is proportional to the amount of protein present which is read at 540 nm against blank.

Protein $+ \text{Cu}^{II} \xrightarrow{\text{alkaline}} \text{Coloured complex}$

Reagents

- Sulphate-sulphate solution 27.8%
- Stock biuret reagent of Weichselbaum

Dissolved 45 grams of Rochelle salt in about 400 ml of 0.2 N sodium hydroxide and added 15 grams of copper sulphate, stirring continuously until solution is complete. Added 5 grams of potassium iodide and made up to a litre with 0.2 N sodium hydroxide.
• Working biuret solution

200 ml of stock reagent is diluted to a litre with 0.2 N sodium hydroxide which contains 5 grams of potassium iodide per litre.

• Tartarate-iodide solution

Dissolved 9 grams of Rochelle salt in 0.2 N sodium hydroxide containing 5 grams of potassium iodide per litre.

• Solvent Ether, analar

• Bovine albumin

Procedure

Pipette 6.0 ml of the sulphate-sulphite solution into a small albumin tube and on to it layer 0.4 ml of serum. Invert to mix. Then remove at once 2 ml of the mixture and add it to 5 ml of the biuret mixture in 6 x 3/4 inch test tube. To the remaining mixture added about 3 ml of solvent ether, stopper and shaken for 40 times, twice each second for 20 seconds, using a movement of the arm of about 15 inches. Centrifuge for 5 min, that is, just long enough for a firm globulin layer to form. Cap the tubes during the centrifuging. After centrifuging, tilt the tube and insert a pipette into the clear solution below the globulin layer. Pipette 2 ml of this solution add to 5 ml of the biuret reagent in a test tube. Shake well and place both the tubes (total protein and albumin) in a water bath at 37°C for 10 min. Allow to cool for 5 min at room temperature, then read at 540 millimicrons against the blank.
Calculation

Grams protein per 100 ml =
\[
\frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{Concentration of standard}
\]

Total protein and albumin are obtained. Globulin can be calculated as

Total proteins - Albumin = Globulin.

Fibrinogen (Reinhold, 1953)

Principle

A fibrin clot of the plasma formed using calcium chloride and collected.

The clot dissolved in biuret reagent and measured at 540 nm.

Reagents

- Calcium chloride (2.5%)
- Distilled water
- Biuret reagent

Procedure

Mix 0.5 ml of plasma, 14 ml of distilled water, and 0.5 ml of 2.5% calcium chloride solution in a small beaker, place a fine glass rod in the liquid, and allow to stand in an incubator at 37°C until a clot is formed. Thirty minutes is usually sufficient for the clot formation. Then rotate the rod to collect the clot on to it. Press the rod against the side of the beaker to squeeze out any solution and to compress the clot. Dry by pressing carefully against a piece of filter paper. Then
transfer the clot to the tube in which the digestion is to be carried out. Dissolve the clot in 3.5 ml of the biuret reagent. The concentration of fibrinogen can be determined by the biuret method (Reinhold, 1953).

Calculation

\[
\text{Mg. fibrinogen per 100 ml} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{100}{0.5} \times 0.1 \times 16.4
\]

\[
= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 328
\]


Principle

If a prick is given in the skin with a needle, bleeding occurs continuously for sometime and then stops. The time elapse between skin puncture and the arrest of bleeding is called the bleeding time.

Procedure

Finger tip is pricked to get a puncture to a depth of about 2 mm with a needle. Start a stop watch at the same time as the finger tip is punctured. Touch the formed drops of blood with the edge of a filter paper every 30 sec. Avoid touching the skin or the result may be affected. Take the time when bleeding stops. The time interval from the appearance of blood to the stoppage of blood is the bleeding time.
Clotting time: Lee-White's capillary tube method (Baker and Silverton, 1980)

**Principle**

If blood is removed from the body and kept in a glass capillary tube or a test tube, it coagulates forming a jelly like mass. Time elapse between the withdrawal of blood and clot formation is the coagulation time.

**Procedure**

Take four test-tubes with an internal diameter of 8 mm and place them in a 37°C water bath. Take venous blood with a clear dry syringe, taking care that no tissue juices enter the syringe. Start a stop watch immediately blood enters the syringe. Deliver 1 ml amounts of the blood into each of the four warmed test-tubes. Examine them at 30 s intervals and observe for clotting by gently tilting the tube. The clotting is reported as the average of the times given by the four tubes.

Prothrombin Time: One stage by Quick's method (Baker and Silverton, 1980)

**Principle**

This test determines the time plasma takes to clot after tissue factor and calcium are added. The normal range varies with the particular tissue factor used. When the result is expressed as “Prothrombin activity” the lower limit of the normal is taken as 60 percent. In addition to sensitivity to prothrombin levels, this
tests detects deficiency of factors V, VII, X, fibrinogen and some inhibitors. This is a sensitive and a reliable test.

Reagents

- Dried brain extract (brain thromboplastin)
- M/40 calcium chloride
- Control and test plasma

Procedure

Using a 0.1 ml pipette with a rubber teat, place 0.1 ml of test plasma into each of three small test-tubes and place in a 37°C water bath. Add 0.1 ml of the brain suspension to each tube and allow the contents to reach 37°C. Add 0.1 ml of calcium chloride solution to the first tube, simultaneously starting a stop watch. Mix and leave the tube in the bath for 9-10s, then remove and examine for clot formation, stopping the watch at the first sign of a clot. Note time taken to clot and repeat procedure with other two tubes. Repeat the whole procedure using the control normal plasma. Normal plasma should clot between 11 and 14s after the addition of the calcium chloride. These can be reported as the time taken in seconds for the test to clot, quoting also the clotting time of the control plasma, or can be expressed as a prothrombin index. This is calculated as follows:

\[
\text{Prothrombin index} = \frac{\text{normal plasma clotting time}}{\text{test plasma clotting time}} \times 100\%
\]
RBC Count (Baker and Silverton, 1980)

By the conventional method of using Neubauer's Chamber using Hayem's fluid.

Reagents

- Red cell diluting fluid (Hayem's fluid)
- Mercuric chloride 0.5 g
- Sodium sulphate 5.0 g
- Sodium chloride 1.0 g
- Distilled water 200.0 ml

Procedure

After cleaning with alcohol finger is pricked to get a sufficient drop of blood and the blood is sucked in the RBC diluting pipette up to 0.5 mark. Suck the dilution fluid (Hayem's fluid) up to 101 mark. This will give a final dilution of 1 in 200. Wipe the outside of the pipette with a piece of clean gauze. Close the tip of the pipette with the thumb and mix well by shaking. Thoroughly clean the counting chamber and the coverglass, place on a flat horizontal surface. Mix the suspension well by shaking the pipette for 3-4 min and discard about a quarter of the mixture. Fill the chamber by holding the pipette at an angle of 45° after putting the coverslip. The charging chamber will be covered by the fluid by capillary attraction. Fluid is not allowed to overflow into the channels. Place the chamber on the microscope stage, allow several minutes for the cells to settle. Then first see the field under low power and count cells under high power. Cells
in the four corner squares and in the central square are counted which will come 80 of the 400 small squares.

**Calculation**

Let \( N \) = number of cells counted in 80 small squares.

The area of each small square is \( 1/400 \) mm\(^2\), and the depth of the chamber is 1/10 mm.

The volume of fluid over small square is therefore

\[
\frac{1}{400} \times \frac{1}{10} = \frac{1}{4000} \text{ mm}^3
\]

If \( N \) cells are counted in \( 80/4000 \) mm\(^3\) of diluted blood.

1 mm\(^3\) of diluted blood contains \( N \times \frac{4000}{80} \) cells.

Since blood is diluted 1 in 200, 1 mm\(^3\) of blood contains \( N \times \frac{4000}{80} \times 200 \) cells

\[
= N \times 10000 \text{ cells.}
\]

**WBC Count (Baker and Silverton, 1980)**

By the conventional method of using Neubauer's Chamber using Turk's fluid.
Reagents

- White cell diluting fluid (Turk’s fluid)
  - Glacial acetic acid 1%
  - Aniline Gentian violet 1 drop
  - Distilled water 100 ml

Procedure

Draw blood to the 0.5 mark on the stem of a white cell pipette and diluting fluid to the 11 mark immediately above the bulk. Mix well. Discard a few drops of blood from the stem of the pipette. Place the coverslip on the charging chamber and charge the Neubauer counting chamber and count the cells in the 4 corner square millimeters, and those in the central square millimetre of the ruled area.

Calculation

Let \( N \) = number of cells counted in 5 mm\(^2\).

Since the depth of the chamber is 1/10 mm,

\( N \) cells are counted in \( 5/10 = 1/2 \) mm\(^3\) of diluted blood.

Therefore 1 mm\(^3\) of diluted blood contains \( N \times 2/1 \) cells. Since blood was diluted 1 in 20, 1 mm\(^3\) of blood contains

\[ N \times 2/1 \times 20 = 40N \text{ cells} \]
Hormonal assay by Elisa method using standard kit

Principle

Monoclonal antibody and enzyme labeled antibodies react with serum containing the native antigen (hormones), results in the formation of soluble sandwich complex. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. This can be quantitated by reaction with a suitable substrate to produce colour.

Luteinising hormone (Kosasa, 1981) & Follicle stimulating hormone (Odell, 1981)

Reagents

a. LH/FSH Calibrators. This contains six reference vials of 0.5, 25, 50, 100 & 200 ml/μl.

b. Enzyme-LH/FSH-antibody conjugate and Biotinylated monoclonal mouse IgG in buffer

c. Streptavidin coated microplates(96 wells).

d. Wash solution concentrate contains surfactant in phosphate buffered saline.

e. Wash Buffer, prepared by diluting 20 ml of wash concentrate to 1000ml. This reagent is stable at room temperature.

f. Substrate A, contains tetramethylbenzidine in the buffer.

g. Substrate B, contains hydrogen peroxide in buffer.
h. Working Substrate, prepared by mixing equal volume of substrate A and B. This reagent is freshly prepared before use.

i. Stop Solution (1N HCl)

Procedure

1. Formatted the microplate's well for each serum references, control or serum specimen.

2. Pipetted 50 µl of the appropriate serum reference, control or serum into the assigned well.

3. Added 100 µl reagent -b to each well.

4. Swirled the microplate gently for 30 seconds.

5. Incubated 60 minutes at room temperature.

6. Discarded the contents of microplate by aspiration.

7. Added 300 µl of reagent-e and aspirated. This was repeated for two times.

8. Added 100 µl of reagent-h to all wells.

9. Incubated at room temperature for 15 minutes.

10. Added 50 µl of reagent-i to all wells.

11. The absorbance was read in each well at 450 nm in a microplate reader.
Estriol (Osterman, 1979)

Reagents

a. Incubation Buffer
b. Estradiol standards
c. Estrol-HRP conjugate
d. Diluted Marker, prepared by diluting 20 μl of reagent-c to 2.0 mL with reagent-a.
e. Microplate
f. Tetramethylbenzidine-substrate
g. Stop solution (1N HCl)

Procedure

1. Formatted the microplate’s well for each estradiol standards, control or serum specimen and blank.
2. Pipetted 20 μl of the appropriate standard, control or serum into the assigned wells except blank.
3. Added 200 μl reagent-c to each well, except blank.
4. Incubated for 60 minutes at 37°C.
5. Discarded the contents of microplate by decantation.
6. Added 300 μl of distilled water and decanted. This was repeated for two times.
7. Added 200 μl of reagent-f to all wells including blank.
8. Incubated at 20-25°C for 15 minutes in the dark.

9. Added 50 µl of reagent-g to all wells and absorbance was recorded at 450 nm.

**Progesterone (Elder, 1987)**

**Reagents**


b. Enzyme conjugated: progesterone conjugated to horseradish peroxidase.

c. Progesterone antibody solution.

d. Reference standard set: prepared 0, 0.5, 1, 5, 10 and 30 ng/ml in the human serum base.

e. Washing buffer concentrate (50 ml) (20X).

f. Working washing buffer: Prepared by adding the concentrate 50 ml into 950 ml of distilled water.

g. Solution A (11 ml): Buffer solution containing hydrogen peroxide.

h. Solution B (11 ml): Tetramethylbenzidine solution.

i. Stop solution (1N H₂SO₄)

**Procedure**

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

2. Dispense 10 µl of standards, controls or the serum samples and blank into appropriate wells.
3. Dispense 50 µl of antibody solution into each well except the blank well.

4. Dispense 50 µl of the enzyme conjugate into each well except the blank well.

5. Incubate for 60 minutes at room temperature.

6. Remove incubation mixture and rinse the wells with working washing buffer 5 times.

7. Dispense 100 µl of the solution A and solution B into each well including the blank well.

8. Incubate for 30 minutes at room temperature.

9. Stop reaction by adding 50 µl of 1N H₂SO₄ to each well and read O.D at 450 nm with microwell reader.

10. Record absorbencies.

**Calculation**

A standard dose response curve was prepared by plotting activity of standard references against concentration. From comparison to the dose response curve, the concentration of hormones was found out.

**Statistical analysis**

Statistical analyses of results were carried out (Gomez and Gomez, 1984) using unpaired 't' test and correlation coefficient to find the comparison and correlation of respiratory, biochemical, haematological and hormonal parameters during different phases of menstrual cycle.
Unpaired 't' test

Data on respiratory biochemical, haematological and hormonal functions, during different phases of menstrual cycle were compared by applying unpaired 't' test.

\[
t = \frac{\bar{x}_1 - \bar{x}_2}{\text{S.E.}[\bar{x}_1 - \bar{x}_2]}
\]

\[
= \frac{\bar{x}_1 - \bar{x}_2}{\text{S.E.}[\bar{x}_1 - \bar{x}_2]}
\]

**Calculation of standard error of** \((\bar{x}_1 - \bar{x}_2)\)

\[
\text{S.E.} (\bar{x}_1 - \bar{x}_2) = \text{S.D.} \sqrt{\left(\frac{1}{n_1} - \frac{1}{n_2}\right)}
\]

where \(\text{S.D.} = \sqrt{\text{S.D.}^2}
\]

\[
= \sqrt{\frac{(n_1 - 1)\text{S.D.}^2_1 + (n_2 - 1)\text{S.D.}^2_2}{n_1 + n_2 - 2}}
\]

- \(n_1\) = sample size of group I
- \(n_2\) = sample size of group II
- \(\bar{x}_1\) = mean of first sample
- \(\bar{x}_2\) = mean of second sample
- \(\text{S.D.}^2_1\) = variance of the first sample
- \(\text{S.D.}^2_2\) = variance of the second sample
- \(\text{S.D.}^2\) = combined or pooled variance

Degree of freedom \((df) = n_1 + n_2 - 2\)
Correlation Analysis

When the degree of linear association between two variables is required, Karl Pearson's correlation coefficient was calculated.

\[ r = \frac{\text{Cov}(X, Y)}{\sqrt{\text{Var}(X) \cdot \text{Var}(Y)}} \]

where,

\[ r = \text{correlation coefficient} \]

\[ \text{Covariance} = \text{Cov}(X, Y) = \frac{\Sigma xy}{n} - \bar{x} \bar{y} \]

\[ \text{Variance} = \text{Var}(X) = \frac{\Sigma x^2}{n} - (\bar{x}^2) \]

\[ \text{Var}(Y) = \frac{\Sigma y^2}{n} - (\bar{y}^2) \]

and it is tested for significance using 't' test.

where,

\[ t = \frac{\sqrt{n-2}}{\sqrt{1 - r^2}} \]

follows 't' distribution with n-2 degrees of freedom.