MATERIALS AND METHODS

The present study was carried out at M.M Institute of Medical Sciences and Research, Mullana, Ambala in the Department of Biochemistry in collaboration with the Department of Medicine. The study was undertaken after approval by Institutional Ethics Committee.

SELECTION OF PATIENTS

A total of 400 subjects in the age range of 30-70 years, irrespective of sex, constituted the material of the study. Two hundred patients of type 2 diabetes mellitus attending the OPD and/or admitted in wards of Medicine Department of M.M Institute of Medical Sciences and Research, Mullana, Ambala and two hundred of normal subjects in the same age range from amongst the attendants of patients and volunteers were included as controls. The patients were diagnosed as diabetic and obese by the Department of Medicine on the basis of following criteria:

CRITERIA FOR DIAGNOSIS OF DIABETES MELLITUS

Patients were diagnosed as diabetic according to the diagnostic criteria for diabetes mellitus issued by American Diabetes Association\(^{194}\):
<table>
<thead>
<tr>
<th>Condition</th>
<th>Fasting glucose (mg/dl)</th>
<th>2 hours after glucose (mg/dl)</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;100</td>
<td>&lt;140</td>
<td>&lt;5.7</td>
</tr>
<tr>
<td>Impaired Fasting Glycemia</td>
<td>100-125</td>
<td>-</td>
<td>5.7-6.4</td>
</tr>
<tr>
<td>Impaired Glucose Tolerance</td>
<td>-</td>
<td>140-199</td>
<td>5.7-6.4</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>≥126</td>
<td>≥200</td>
<td>≥6.5</td>
</tr>
</tbody>
</table>

Diabetes mellitus is diagnosed by demonstrating any one of the following:

- HbA1c level ≥6.5%
- Fasting plasma glucose level ≥ 126 mg/dl
- Plasma glucose ≥ 200 mg/dl two hours after a 75 g oral glucose load as in glucose tolerance test.
- Symptoms of hyperglycemia or hyperglycemic crisis and random plasma glucose ≥200 mg/dl.
CLASSIFICATION OF WEIGHT STATUS AS PER BMI

<table>
<thead>
<tr>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNDERWEIGHT</td>
</tr>
<tr>
<td>&lt; 18.5</td>
</tr>
<tr>
<td>HEALTHY WEIGHT</td>
</tr>
<tr>
<td>18.5-24.9</td>
</tr>
<tr>
<td>OVERWEIGHT</td>
</tr>
<tr>
<td>25.0-29.9</td>
</tr>
<tr>
<td>OBESITY CLASS I</td>
</tr>
<tr>
<td>30.0-34.9</td>
</tr>
<tr>
<td>OBESITY CLASS II</td>
</tr>
<tr>
<td>35.0-39.9</td>
</tr>
<tr>
<td>OBESITY CLASS III (EXTREME OBESITY)</td>
</tr>
<tr>
<td>≥40</td>
</tr>
</tbody>
</table>

Two hundred patients diagnosed as diabetic by the Department of Medicine were divided into 2 groups in the following manner:

Group I: 100 patients having diabetes mellitus (T2DM) and obesity.

Group II: 100 patients having diabetes mellitus (T2DM) and No obesity.

Two hundred age and sex matched subjects without diabetes selected to serve as controls were also divided into 2 groups as under:

Group III: 100 subjects having only obesity and no diabetes.
Group IV: 100 subjects having no obesity and no diabetes.

A detailed history of the present as well as past illness was recorded and the clinical examination was done as per the proforma attached. Anthropometric indices including height, weight, hip and waist circumference were measured while subjects were in the standing position and wearing light clothing without shoes. Body Mass Index (BMI) was calculated by dividing body weight (in kilograms) by squared height (in square metres) [Figure 14] according to WHO procedure.\[196\] Waist-to-Hip Ratio (WHR) was calculated as waist circumference divided by hip circumference.

Informed consent, both in English as well as vernacular language, was taken from all the participants included in the study.

**INCLUSION CRITERIA**

1. Patients suffering from Type 2 diabetes mellitus and under treatment.
2. Patients of T2DM having documented obesity.
3. Patients of T2DM having no obesity.
4. Subjects having no diabetes but having documented obesity
5. Subjects having no diabetes and no obesity
6. Age 30-70 years of either sex.
EXCLUSION CRITERIA

1. The patients suffering from type 1 diabetes mellitus, rheumatoid arthritis and coronary artery disease.
2. The patients taking antioxidant drugs, corticosteroids, insulin.
3. Pregnancy
4. Hyper or Hypothyroidism, Cushing syndrome or any other endocrinopathy besides diabetes mellitus.

COLLECTION AND PROCESSING OF BLOOD SAMPLE

Seven milliliters (7 ml) of venous blood sample was collected in dry disposable syringe under aseptic conditions from ante-cubital vein of the subjects after an overnight fasting of 10-12 hours. Four milliliters (4 ml) of blood was transferred to a sterile, dry and acid washed vial, allowed to stand for half an hour and after the clot formation, the supernatant fluid was centrifuged to perform the following biochemical investigations.

BIOCHEMICAL INVESTIGATIONS

1. Fasting serum C-peptide level
2. Serum Adiponectin level
3. Serum Leptin level
4. Fasting lipid profile (Total Cholesterol, Triglycerides, HDL-cholesterol and LDL-cholesterol levels)
5. Qualitative C-reactive protein (CRP) test in serum

The remaining 3 ml of blood was transferred to sodium fluoride and potassium oxalate containing vial, and centrifuged to separate plasma which was then used for estimation of fasting plasma glucose, insulin and insulin resistance.

DETAILS OF INVESTIGATIONS:

ESTIMATION OF PLASMA INSULIN

Fasting plasma insulin levels were measured by Chemiluminescence immunoassay (CLIA) using CLIA VAST enabled kit (Monobind Inc. USA) supplied by Lilac Medicare Private Limited.

Principle
The essential reagents required for an immunoenzymometric assay include high affinity and specific antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of an opaque chemiluminescent reaction cell through the interaction of streptavidin coated on the opaque reaction cell and exogenously added biotinylated monoclonal antibody coupled to insulin.
Upon mixing monoclonal biotinylated antibody, the enzyme labeled antibody (tracer component) and test serum containing the native antigen, reaction results between the native antigen and the antibodies without competition or stearic hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equations:

\[
\begin{align*}
&\text{Enz Ab (P)} + \text{Ag ins} + \text{Bn Ab (M)} \\
\xrightarrow[k_a]{k_{-a}} &\text{Enz Ab (P) - Ag ins - Bn Ab (M)}
\end{align*}
\]

Where $\text{Enz Ab (P)}$ = enzyme labeled polyclonal antibody (excess quantity)

$\text{Ag ins} = $ native antigen (variable quantity)

$\text{Bn Ab (M)} = $ biotinylated monoclonal antibody (excess quantity)

$\text{Enz Ab (P) - Ag ins - Bn Ab (M)} = $ antigen-antibodies sandwich complex

$k_a = $ rate constant of association

$k_{-a} = $ rate constant of dissociation

Simultaneously, the complex is deposited in the reaction cell through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[
\begin{align*}
&\text{Enz Ab (P) - Ag ins - Bn Ab (M)} + \text{Streptavidin CW} \\
\rightarrow &\text{Immobilized complex}
\end{align*}
\]

Where $\text{Streptavidin CW} = $ Streptavidin immobilized on well
Immobilized complex = Sandwich complex bound to solid surface

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity, determined by reaction with a substrate that generates light, in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown analyte can be ascertained.

**Materials Required and Used**

1. Micropipettes (5-100 µl and 200-1000 µl).
2. Lumax-A semi automated Chemiluminescence (CLIA) analyzer and Autoplex A processor for CLIA.
3. Test tubes for mixing of reagents.
4. Absorbent paper for blotting CLIA reaction cells.
5. Reaction cell cover for incubation steps.
6. Timer
7. Storage container for storage of wash buffer.
8. Distilled water.
9. Master CLIA VAST enabled kit (Monobind Inc. USA)
Reagents

1. Insulin Calibrators (6 x 2 ml): Six vials of calibrators (marked as A,B,C,D,E and F) containing insulin antigen at levels of 0(A), 5(B), 25(C), 50(D), 100(E) and 300(F) µIU/ml were reconstituted independently with 2 ml of distilled water. The reconstituted calibrators were stable for sixty days at 2-8 ºC.

2. Insulin Tracer (1 x 13 ml): One vial containing enzyme labeled, affinity purified, biotinylated monoclonal mouse anti-insulin IgG antibody in buffer, dye and preservative.

3. Opaque streptavidin coated CLIA light reaction cells (pack size = 96 wells): There are 96 CLIA light reaction cells coated with streptavidin and packaged in an aluminium bag with a drying agent.

4. Wash solution Concentrate (2 x 60 ml): contains a surfactant in buffered saline and a preservative.

5. CLIA Signal Reagent A (1 x 30 ml): contains luminol in buffer.

6. CLIA Signal Reagent B (1 x 30 ml): contains hydrogen peroxide in buffer.

All reagents were brought to room temperature (20-27 ºC) prior to use.
Reagent Preparation

1. **Wash Buffer**: It was prepared by diluting 60 ml of wash concentrate to 3000ml with distilled water in a suitable storage container and stored at room temperature of 20-27 °C.

2. **Working Signal Reagent solution**: It was prepared according to the amount of reagent needed by mixing equal portions of signal reagent A and signal reagent B in a clean container. For example: 1ml of A and 1 ml of B were added per two (2) eight well strips. The unused portion was discarded, else it was stored at 2-8 °C for 36 hours.

Sample Preparation

The samples were collected and stored at a temperature of -20 °C. The stored samples were thawed and mixed thoroughly just prior to assay.

Test Procedure

1. 25 µl of calibrators and samples were pipetted into the appropriate reaction cells.

2. 50 µl of insulin tracer was added into each reaction cell.

3. The plate was swirled for 60 seconds and incubated at room temperature (22 – 26 °C) for 60 minutes.
4. Reaction cells were then washed five times with 350 µl of wash buffer (0.35 ml per cell).
5. 50 µl of working signal reagent solution was added into each reaction cell.
6. The microplate was incubated for five (5) minutes at room temperature.
7. The relative light units (RLUs) were read in each well in a microplate strip luminometer for at least 0.2 seconds/well.

Calculation of Results

A dose response curve was constructed by plotting the mean relative light units or RLU's (Y) of calibrators against the known concentration (X) of standards with units on a linear graph paper. The best-fit curve was drawn through plotted points (Figure 15). The computer data reduction software designed for chemiluminescence assays was used for data reduction. The results were reported as the concentration of insulin (µIU/ml) in samples.

Reference Range

In fasting state, Adult (normal) = 0.7-9.0 µIU/ml

Diabetic (Type 2) = 0.7-25.0 µIU/ml

In well-fed state, normal insulin levels = up to 40 µIU/ml
ESTIMATION OF SERUM C-PEPTIDE

Fasting serum C-peptide levels were measured by **Chemiluminescence immunoassay (CLIA)** using CLIA VAST enabled kit (Monobind Inc. USA) supplied by Lilac Medicare Private Limited.

**Principle**

The essential reagents required for an immunoenzymometric assay include high affinity and specific antibodies (enzyme conjugated and immobilized), with different and distinct epitope recognition, in excess, and native antigen. In this procedure, the immobilization takes place during the assay at the surface of an opaque chemiluminescent reaction cell through the interaction of streptavidin coated on the opaque reaction cell and exogenously added biotinylated monoclonal antibody coupled to C-peptide.

Upon mixing monoclonal biotinylated antibody, the enzyme labeled antibody (tracer component) and test serum containing the native antigen, reaction results between the native antigen and the antibodies without competition or stearic hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equations:

\[
\begin{align*}
\text{Enz Ab}_{(P)} + \text{Ag}_{\text{cpep}} + \text{Btn Ab}_{(M)} & \xrightarrow{k_a} \text{Enz Ab}_{(P)} - \text{Ag}_{\text{cpep}} - \text{Btn Ab}_{(M)} \\
\end{align*}
\]

Where $\text{Enz Ab}_{(P)} = $ enzyme labeled polyclonal antibody (excess quantity)

$\text{Ag}_{\text{cpep}} = $ native antigen (variable quantity)
\[ \text{Enz Ab}_P - \text{Ag}_{\text{cpep}} - \text{Bn Ab}_M = \text{antigen-antibodies sandwich complex} \]

\[ k_a = \text{rate constant of association} \]
\[ k_d = \text{rate constant of dissociation} \]

Simultaneously, the complex is deposited in the reaction cell through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[ \text{Enz Ab}_P - \text{Ag}_{\text{cpep}} - \text{Bn Ab}_M + \text{Streptavidin}_{\text{CW}} \rightarrow \text{Immobilized complex} \]

Where \( \text{Streptavidin}_{\text{CW}} = \text{Streptavidin immobilized on well} \)

Immobilized complex = Sandwich complex bound to solid surface

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity, determined by reaction with a substrate that generates light, in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown analyte can be ascertained.

Materials Required and Used

1. Micropipettes (5-100 µl and 200-1000 µl).
2. Lumax-A semi automated Chemiluminescence (CLIA) analyzer and Autoplex A processor for CLIA.
3. Test tubes for mixing of reagents.

4. Absorbent paper for blotting CLIA reaction cells.

5. Reaction cell cover for incubation steps.

6. Timer

7. Storage container for storage of wash buffer.

8. Distilled water.

9. Master CLIA VAST enabled kit (Monobind Inc. USA)

Reagents

1. **C-peptide Calibrators (6 x 2 ml):** Six vials of calibrators (marked as A,B,C,D,E and F) containing C-peptide antigen at levels of 0(A), 0.2(B), 1.0(C), 2.0(D), 5.0(E) and 10.0(F) ng/ml were reconstituted independently with 2 ml of distilled water. The reconstituted calibrators were stable for sixty days at 2-8 ºC.

2. **C-peptide Tracer (1 x 13 ml):** One vial containing enzyme labeled, affinity purified, biotinylated monoclonal mouse anti-C-peptide IgG antibody in buffer, dye and preservative.

3. **Opaque streptavidin coated CLIA light reaction cells (pack size = 96 wells):** There are 96 CLIA light reaction cells coated with streptavidin and packaged in an aluminium bag with a drying agent.

4. **Wash solution Concentrate (2 x 60 ml):** contains a surfactant in buffered saline and a preservative.
5. **CLIA Signal Reagent A (1 x 30 ml):** contains luminol in buffer.

6. **CLIA Signal Reagent B (1 x 30 ml):** contains hydrogen peroxide in buffer.

**All reagents were brought to room temperature (20-27 °C) prior to use.**

**Reagent Preparation**

1. **Wash Buffer:** It was prepared by diluting 60 ml of wash concentrate to 3000ml with distilled water in a suitable storage container and stored at room temperature of 20-27 °C.

2. **Working Signal Reagent solution:** It was prepared according to the amount of reagent needed by mixing equal portions of signal reagent A and signal reagent B in a clean container. For example: 1ml of A and 1 ml of B were added per two (2) eight well strips. The unused portion was discarded; else it was stored at 2-8 °C for 36 hours.

**Sample Preparation**

The samples were collected and stored at a temperature of -20 °C. The stored samples were thawed and mixed thoroughly just prior to assay.
Test Procedure

1. 25 µl of calibrators and samples were pipetted into the appropriate reaction cells.
2. 50 µl of insulin tracer was added into each reaction cell.
3. The plate was swirled for 60 seconds and incubated at room temperature (22 – 26 ºC) for 60 minutes.
4. Reaction cells were then washed five times with 350 µl of wash buffer (0.35 ml per cell).
5. 50 µl of working signal reagent solution was added into each reaction cell.
6. The microplate was incubated for five (5) minutes at room temperature.
7. The relative light units (RLUs) were read in each well in a microplate strip luminometer for at least 0.2 seconds/well.

Calculation of Results

A dose response curve was constructed by plotting the mean relative light units or RLUs (Y) of calibrators against the known concentration (X) of standards with units on a linear graph paper. The best-fit curve was drawn through plotted points (Figure 16). The computer data reduction software designed for chemiluminescence assays was used for data reduction. The results were reported as the concentration of C-peptide (ng/ml) in samples.
Reference Range

Adult (normal) = 0.7-1.9 ng/ml

ESTIMATION OF SERUM ADIPONECTIN

Serum adiponectin was measured by competitive enzyme immunoassay (ELISA) using kit supplied by BioVendor Research and Diagnostic Products (European Union).\(^\text{199}\)

Principle

The standards, quality controls and samples are incubated in microplate wells pre-coated with recombinant human adiponectin together with polyclonal anti-human adiponectin antibody conjugated to horseradish peroxidase (HRP). Recombinant human adiponectin is produced in HEK293 cell line and contains 225 amino acid residues of human adiponectin and 8 extra amino acids. After washing, the HRP conjugate bound to adiponectin immobilized on the wells is allowed to react with the substrate solution containing tetramethylbenzidine (TMB). The reaction is stopped by the addition of acidic solution and the absorbance of the resulting yellow product is measured. The absorbance is inversely proportional to the adiponectin concentration. A standard curve is constructed by plotting absorbance
values against adiponectin of standards, and concentration of unknown samples is determined using this standard curve.

**Materials Required and Used**

1. Micropipettes (5-100 µl, 200-1000 µl)
2. Microplate Washer (Model: MR-12A of Mindray)
3. Microplate Reader (Model: MR-96A of Mindray)
4. Test tubes for diluting samples
5. Distilled Water
6. Absorbent paper for blotting microtiter plate after washing
7. Timer
8. Glassware for preparing wash solution.

**Reagents Provided In The Kit**

<table>
<thead>
<tr>
<th>S.NO.</th>
<th>KIT COMPONENTS</th>
<th>STATE</th>
<th>QUANTITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Antigen coated microtiter strips</td>
<td>Ready to use</td>
<td>96 wells</td>
</tr>
<tr>
<td>2</td>
<td>Conjugate solution</td>
<td>Ready to use</td>
<td>07 ml</td>
</tr>
<tr>
<td>3</td>
<td>Set of standards</td>
<td>Concentrated</td>
<td>7 x 0.22 ml</td>
</tr>
<tr>
<td>4</td>
<td>Quality control HIGH</td>
<td>Ready to use</td>
<td>0.4 ml</td>
</tr>
<tr>
<td></td>
<td>Quality control LOW</td>
<td></td>
<td>Ready to use</td>
</tr>
<tr>
<td>---</td>
<td>---------------------</td>
<td>---------</td>
<td>--------------</td>
</tr>
<tr>
<td>6</td>
<td>Dilution Buffer</td>
<td></td>
<td>Ready to use</td>
</tr>
<tr>
<td>7</td>
<td>Wash solution (10x)</td>
<td>Concentrated</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Substrate Solution</td>
<td></td>
<td>Ready to use</td>
</tr>
<tr>
<td>9</td>
<td>Stop Solution</td>
<td></td>
<td>Ready to use</td>
</tr>
</tbody>
</table>

All reagents were brought to room temperature prior to use.

I. **Assay Reagents Supplied Ready To Use**

1. Antigen coated microtiter strips in aluminium bag with dessicant
2. Conjugate Solution
3. Dilution Buffer
4. Substrate Solution
5. Stop solution
6. Quality Controls (HIGH and LOW)

   Expected range of concentration for HIGH = 16.38 (12.3 – 20.5 µg/ml)

   LOW = 7.09 (5.3 – 8.9 µg/ml)
II. Assay Reagents Supplied Concentrated

1. Human adiponectin standards: Seven standards of reference for adiponectin concentrations of 10(A), 5(B), 2(C), 1(D), 0.5(E), 0.2(F) and 0.1(G) µg/ml were diluted 3x with dilution buffer just prior to assay. For example: 50 µl of standard was diluted with 100µl of dilution buffer. Mixed well (not to foam). The diluted set of standards was not stored.

2. Wash solution (10x): Wash solution concentrate was diluted (10x) ten-fold in distilled water to prepare a 1x working solution. For example: 100ml of wash solution concentrate (10x) was added to 900ml of distilled water for use in all 96 wells. The diluted wash solution was stored at 2-8 ºC.

Preparation of Samples

The samples were collected and stored at -20 ºC. Samples were thawed and mixed thoroughly just prior to assay. Serum sample was diluted 30x with dilution buffer before proceeding with the assay. For example: 10µl of sample was diluted in 290µl of dilution buffer and mixed well (not to foam).

Procedure

1. 50µl diluted standards, samples, quality controls and dilution buffer (blank) were pipetted into the appropriate wells.

2. 50µl of conjugate solution was added into each well.
3. The plate was shaken and incubated at room temperature for 2 hours.
4. The wells were washed 3 times with 350µl of wash solution (0.35ml per well). After final wash, the plate was inverted and tapped strongly against absorbent paper.
5. 200µl of substrate solution was added into each well avoiding exposure of plate to direct sunlight. The plate was covered with aluminium foil.
6. The plate was again incubated for 10-15 minutes at room temperature without shaking.
7. The colour development was stopped by adding 50µl of stop solution.
8. The absorbance of each well was determined using a microplate reader set to 450nm with reference wavelength set to 630nm (acceptable range: 550-650nm). It was read within five (5) minutes after addition of stop solution.

**Calculation of Results**

The standard curve was constructed by plotting the mean absorbance(Y) of standards against the known concentration(X) of standards in logarithmic scale, using the four-parameter algorithm (Figure 17). Results were reported as concentration of adiponectin (µg/ml) in samples.
The measured concentration of samples and quality controls calculated from the standard curve was multiplied by a dilution factor of 10, because standards were diluted 3x and samples and quality controls were diluted 30x. For example: 1.05µg/ml (from standard curve) x 10 (dilution factor) = 10.5µg/ml.

**Reference Range**

Males: 8.3 - 10.9 µg/ml

Females: 11.4 - 13.6 µg/ml

**ESTIMATION OF SERUM LEPTIN**

Serum Leptin levels were estimated using **Sandwich Enzyme Immunoassay (ELISA)** technique using kit supplied by DRG Company (USA).²⁰⁰

**Principle**

This test is a solid phase enzyme-linked immunosorbent assay (ELISA) based on sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on a leptin molecule. An aliquot of patient sample containing endogenous leptin is incubated in the coated well with a specific biotinylated monoclonal anti leptin antibody. A sandwich complex is formed. After incubation, the unbound material is washed off and a streptavidin peroxidase enzyme complex is added for detection of the unbound leptin. Having
added the substrate solution, the intensity of colour developed is proportional to
the concentration of leptin in patient sample.

**Materials Required and Used**

1. Micropipettes (5-100µl, 200-1000µl)
2. Microplate Washer (Model: MR-12A of Mindray)
3. Microplate Reader (Model: MR-96A of Mindray)
4. Test tubes for diluting sample
5. Distilled Water
6. Absorbent paper for blotting microtiter plate after washing
7. Timer

**Reagents Provided In The Kit**

<table>
<thead>
<tr>
<th>S.NO</th>
<th>KIT COMPONENTS</th>
<th>STATE</th>
<th>QUANTITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anti-leptin antibody coated microtiter wells</td>
<td>Ready to use</td>
<td>96 wells</td>
</tr>
<tr>
<td>2</td>
<td>Set of standards</td>
<td>Lyophilized</td>
<td>6 x 0.5 ml</td>
</tr>
<tr>
<td>3</td>
<td>Quality control HIGH</td>
<td>Lyophilized</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>4</td>
<td>Quality control LOW</td>
<td>Lyophilized</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>5</td>
<td>Assay buffer</td>
<td>Ready to use</td>
<td>11 ml</td>
</tr>
<tr>
<td>6</td>
<td>Antiserum</td>
<td>Ready to use</td>
<td>11 ml</td>
</tr>
</tbody>
</table>
All the reagents and required number of strips were allowed to reach room temperature prior to use.

Reagent Preparation

1. **Standards**: Six standards of reference for leptin concentrations of 0(A), 2(B), 5(C), 25(D), 50(E) and 100(F) ng/ml were reconstituted with 0.5ml of distilled water. They were allowed to stand for 10 minutes in minimum. The vials were mixed several times before use. The reconstituted standards were stable for at least six (6) weeks at 2-8°C.

2. **Quality Controls**: Both quality control HIGH and LOW were reconstituted with 0.5ml of distilled water. They were allowed to stand for at least 10 minutes. The vials were mixed several times before use. The reconstituted standards were stable for at least six (6) weeks at 2-8°C.

   Expected range of concentration of HIGH = 36.88 (25.82 – 47.94) ng/ml

   LOW = 3.42 (1.88 – 4.96) ng/ml
3. **Wash Solution (40x):** It was diluted by adding 30 ml of concentrated wash solution to 1170ml distilled water resulting in a final volume of 1200ml. The diluted wash solution was stable for two (2) weeks at room temperature.

**Procedure**

1. 15µl of standards, samples and controls were dispensed into the appropriate wells.

2. 100µl of assay buffer was pipetted into each well and thoroughly mixed for 10 seconds.

3. The plate was incubated for 120 minutes at room temperature.

4. The contents of the wells were briskly shaken out and the wells were rinsed 3 times with diluted wash solution (300µl per well). After final wash, the wells were stroke sharply on absorbent paper to remove residual droplets.

5. 100µl of antiserum was added to each well.

6. The plate was incubated for 30 minutes at room temperature.

7. Again, the contents of the wells were briskly shaken out and the wells were rinsed 3 times with diluted wash solution (300µl per well). After final wash, the wells were stroke sharply on absorbent paper to remove residual droplets.
8. 100µl of enzyme complex was dispensed into each well.

9. Again the microtiter plate was incubated for 30 minutes at room temperature.

10. The contents of the wells were briskly shaken out and the wells were rinsed 3 times with diluted wash solution (300µl per well). After final wash, the wells were stroke sharply on absorbent paper to remove residual droplets.

11. 100µl of substrate solution was added to each well.

12. The plate was incubated again for 15 minutes at room temperature.

13. The enzymatic reaction was stopped by adding 50µl of stop solution to each well.

14. The absorbance (OD) of each well was determined at 450 ± 10nm with a microplate reader preferably within 10 minutes after addition of stop solution.

**Calculation of Results**

The standard curve was constructed by plotting the mean absorbance (Y) of standards against the known concentration (X) in semi-logarithmic scale, using four-parameter algorithm (Figure 18). The results were reported as concentration of leptin (ng/ml) in samples.
Reference Range
Males = 2.05 - 5.63 ng/ml
Females = 3.63 – 11.09 ng/ml

ESTIMATION OF SERUM TOTAL CHOLESTEROL

Serum total cholesterol (TC) was determined by Cholesterol Oxidase-Peroxidase in Aminoantipyrine (CHOD-PAP) method using ERBA diagnostics fully automated analyzer kit.

Principle

The estimation of cholesterol by CHOD-PAP method involves the following enzymatic reactions:

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

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

\[
2\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine + Phenol} \xrightarrow{\text{Peroxidase}} 4\text{H}_2\text{O} + \text{Quinoeimine}
\]

The absorbance of quinoeimine thus formed is read which is directly proportional to the concentration of cholesterol in the sample.

Reagents

1. Cholesterol reagent consisted of the following:
a. Cholesterol esterase (pancreatic) in concentration of more than 200 IU/L.
b. Cholesterol oxidase (microbial) in concentration of more than 150 IU/L.
c. Horseradish peroxidase in concentration of more than 2000 IU/L.
d. Sodium phenolate = 20 mmol/L
e. 4-aminoantipyrine = 0.5 mmol/L
f. Phosphate buffer (pH = 6.5±0.1) = 68 mmol/L
g. Lipid clearing agent

2. Cholesterol standard (200 mg/dl or 5.14 mmol/L)
3. Aqua-4: double deionized, 0.2 micron, membrane filtered, particle-free water for reconstitution of cholesterol reagent.

**Reagent Preparation**

The cholesterol and aqua-4 were allowed to attain room temperature. Working cholesterol reagent was made by adding 20 ml of aqua-4 to vial 1 of cholesterol reagent. The contents were dissolved by swirling and not by shaking. This working reagent was stable at 2-8°C.

**Procedure**

A set of 3 test tubes were taken and marked as T, S and B for test, standard and blank respectively.
<table>
<thead>
<tr>
<th>S.No</th>
<th>REAGENT</th>
<th>T (µl)</th>
<th>S (µl)</th>
<th>B (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Working reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>Distilled Water</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Standard (200 mg/dl)</td>
<td>-</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Sample (serum)</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mixed well and incubated at 37°C for 10 minutes. The absorbance of all three test tubes was read at 505nm.

**Calculation of Results**

Concentration of cholesterol (mg/dl) =

\[
\text{Concentration of cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}
\]

**Reference Range**

Fasting levels = 140-250 mg/dl
ESTIMATION OF SERUM TRIGLYCERIDES

Serum triglycerides (TG) were measured by Glycerol Phosphatase Oxidase (GPO)-Trinder method using ERBA diagnostic kit for fully automated analyzer.

Principle

The determination of triglycerides involves the following enzymatic reactions:

\[
\text{Triglycerides} + \text{H}_2\text{O} \xrightarrow{\text{lipoprotein lipase}} \text{Glycerol} + \text{free fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{Glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-phosphate} + \text{O}_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{4-aminoantipyrine} + 3, 5-\text{dichloro 2-hydroxybenzene sulphonate (DHBS)} \xrightarrow{\text{Peroxidase}} \text{Quinoeimine} + 2\text{H}_2\text{O}
\]

1. Triglycerides are enzymatically hydrolyzed by lipase to free fatty acids and glycerol.
2. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate (ADP).

3. Glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO) producing $\text{H}_2\text{O}_2$.

4. In Trinder type of colour reaction catalyzed by peroxidase, the $\text{H}_2\text{O}_2$ reacts with 4-aminoantipyrine (4 AAP) and 3,5-dichloro 2-hydroxybenzene sulphonate (DHBS) to produce a red coloured quinoeimine dye.

The intensity of quinoeimine formed is proportional to triglycerides concentration in the sample when measured at 505 nm (500-540nm).

**Reagents**

1. Triglycerides (dynamic extended stability) reagent composed of the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>2.5 mmol/L</td>
</tr>
<tr>
<td>$\text{Mg}^{2+}$</td>
<td>2.5 mmol/L</td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>0.8 mmol/L</td>
</tr>
<tr>
<td>3,5 DHBS</td>
<td>1 mmol/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>More than 2000 U/L</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>More than 550 U/L</td>
</tr>
<tr>
<td>Glycerol phosphate oxidase</td>
<td>More than 8000 U/L</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>More than 3500 U/L</td>
</tr>
<tr>
<td>Buffer (pH 7.0 ± 0.1 at 20°C)</td>
<td>53 mmol/L</td>
</tr>
</tbody>
</table>
In addition, it also contains non-reactive fillers, stabilizers and surfactants.

2. Triglycerides standard (200 mg/dl or 2.3mmol/L)

3. Aqua-4 is double deionized, 0.2 micron, membrane filtered, particle-free water for reconstitution of triglycerides reagent.

**Reagent Preparation**

The reagent bottle and aqua-4 were allowed to attain room temperature. Then 20 ml of aqua-4 was added into contents of reagent vial. The contents were dissolved by swirling and then allowed to stand for 10 minutes at room temperature.

**Procedure**

A set of 3 test tubes was taken and marked as T, S and B for test, standard and blank respectively and proceeded as follows:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>REAGENT</th>
<th>T(µL)</th>
<th>S(µL)</th>
<th>B(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Working triglyceride reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>2.</td>
<td>Distilled Water</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>3.</td>
<td>Standard (200 mg/dl)</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Serum</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Mixed all the tubes and incubated for 10 minutes at 37°C. The absorbance was read at 505nm (500-540nm).
Calculation of Results

Concentration of triglycerides (mg/dl) =

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}
\]

Reference Range

Normal fasting levels = 25-160 mg/dl

ESTIMATION OF HDL-CHOLESTEROL (HDL-C)

Serum high density lipoprotein-cholesterol (HDL-C) levels were determined using Precipitation method\textsuperscript{203} using fully automated analyzer based ERBA diagnostics kit.

Principle

The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol-methyl ester (PEGME) coupled classic precipitation method with optimum quantities of PVS/PEGME and selected detergents. Low density lipoproteins(LDL), very low density lipoproteins(VLDL) and chylomicrons (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER). The enzymes selectively react with HDL to produce $\text{H}_2\text{O}_2$ which is detected through Trinder reaction.

\[
\text{HDL + LDL + VLDL + CM} \xrightarrow{\text{PVS}} \text{PVS/PEGME} \xrightarrow{\text{PEGME}} \text{HDL + (LDL + VLDL + CM).PVS/PEGME}
\]
The absorbance of quinone is then read at 600nm (primary wavelength) using 700nm as secondary wavelength.

**Reagents**

1. Reagent 1 contains the following:

<table>
<thead>
<tr>
<th>MES buffer (pH 6.5)</th>
<th>6.5 mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{N,N-bis(4-sulfo} \text{butyl)-3-methylaniline (TODB)})</td>
<td>3.0 mmol</td>
</tr>
<tr>
<td>Polyvinyl sulfonic acid</td>
<td>50mg/L</td>
</tr>
<tr>
<td>Polyethylene glycol methyl ester</td>
<td>30 ml/L</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>2 mmol</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
</tr>
<tr>
<td>Detergent</td>
<td></td>
</tr>
</tbody>
</table>

2. Reagent 2 is composed of the following:

<table>
<thead>
<tr>
<th>MES buffer (pH 6.5)</th>
<th>50 mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol esterase</td>
<td>5kU/L</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>20kU/L</td>
</tr>
</tbody>
</table>
3. Calibrator for HDL (12 mg/dl)

Procedure

A set of 3 test tubes was taken and marked as T, S and B for test, standard and blank respectively and proceeded as follows:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>REAGENT</th>
<th>T(µL)</th>
<th>C(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reagent 1</td>
<td>375</td>
<td>375</td>
</tr>
<tr>
<td>2</td>
<td>Calibrator</td>
<td>-</td>
<td>05</td>
</tr>
<tr>
<td>3</td>
<td>Serum</td>
<td>05</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed and incubated at 37°C for 5 minutes.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Reagent 2</td>
<td>125</td>
<td>125</td>
</tr>
</tbody>
</table>

Mixed and incubated at 37°C for 5 minutes. The absorbance was read at 600nm.

Calculation of Results

Concentration of HDL-C (mg/dl) =
\[
\frac{\text{Absorbance of test} \times \text{Concentration of calibrator (mg/dl)}}{\text{Absorbance of standard}}
\]

Reference Range

Males = 35.3 – 79.5 mg/dl ; Females = 42.0 – 88.0 mg/dl
ESTIMATION OF LDL-CHOLESTEROL (LDL-C)

Low Density Lipoprotein-Cholesterol (LDL-C) was calculated using Friedwald formula, based on assumption that very low density lipoprotein (VLDL) is present in serum at a concentration equal to 1/5 th of triglyceride concentration.\(^ {204} \)

\[
\text{Hence, LDL-C} = \text{TC} - \left( \text{HDL-C} + \frac{\text{TG}}{5} \right)
\]

Where TC = total cholesterol

HDL-C = high density lipoprotein cholesterol

TG = triglycerides

The formula is only valid when all concentrations are given in mg/dl and at serum triglyceride concentration of less than 400 mg/dl.\(^ {205} \) For higher triglyceride levels, sample was appropriately diluted.

DETERMINATION OF INSULIN RESISTANCE

Insulin resistance, a hallmark of obesity, diabetes and cardiovascular diseases, was estimated using Homeostasis Model Assessment (HOMA).\(^ {206} \) It was first developed in 1985 by Matthews et al.\(^ {207} \) It is a method used to quantify insulin resistance and beta-cell function from basal (fasting) glucose and insulin concentrations. HOMA is a model of the relationship of glucose and insulin dynamics that predicts fasting steady state glucose and insulin concentration for a wide range of possible combinations of insulin resistance and \( \beta \)-cell function. Insulin resistance is reflected by the diminished suppressive effect of insulin on
hepatic glucose production. The HOMA model has proved to be a robust clinical and epidemiological tool for assessment of insulin resistance. HOMA describes this glucose-insulin homeostasis by means of a set of simple, mathematically-derived non-linear equations. The approximating equation for insulin resistance has been simplified, and uses a fasting blood sample. It is derived from the use of the insulin-glucose product divided by a constant. The product of fasting plasma glucose (FPG) X fasting plasma insulin (FPI) is an index of hepatic insulin resistance.\textsuperscript{208} HOMA-IR was calculated by the following formula:

\[
\text{HOMA-IR} = \frac{\text{Fasting plasma insulin (µIU/ml)} \times \text{Fasting plasma glucose (mg/dl)}}{405}.
\]

The constant 405 is a normalizing factor i.e the product of normal fasting plasma insulin and the normal fasting plasma glucose typical of a normal healthy individual.\textsuperscript{206}

**QUALITATIVE ESTIMATION OF C-REACTIVE PROTEIN (CRP)**

C-reactive protein (CRP) test in serum was done using diagnostic reagent kit for in vitro detection of CRP by \textit{Qualitative and Semi-quantitative rapid Latex Slide Tests}.\textsuperscript{209}

**Principle**

This test is based on the immunologic reaction between CRP and latex particles which have been coated with monospecific anti human CRP and sensitized to detect levels greater than 6 µg/ml of CRP.
Reagents

Reagent 1: CRP latex reagent
Reagent 2: Positive control serum
Reagent 3: Negative control serum

Accessories

1. Glass slide
2. Disposable applicator sticks
3. Disposable plastic droppers
4. Rubber teats

Procedure

All the reagents as well as the samples were allowed to reach room temperature.

1. Using the disposable plastic dropper, one drop of test serum was placed within the circled area on the special slide provided in the kit.

2. Then one drop of latex CRP reagent was added and mixed well with a disposable applicator stick and spread out to the edge of test area.

3. The slide was gently rocked to and fro for 2 minutes and the macroscopic agglutination was examined under direct light source within 2 minutes.

4. Both positive and negative controls were applied and tested in the same way as was test.
Interpretation

The following conclusions were drawn based on the different observations:

<table>
<thead>
<tr>
<th>OBSERVATION</th>
<th>CONCLUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse agglutination</td>
<td>Strongly positive</td>
</tr>
<tr>
<td>Fine agglutination</td>
<td>Weakly positive</td>
</tr>
<tr>
<td>Smooth suspension without any noticeable change</td>
<td>Negative</td>
</tr>
</tbody>
</table>

STATISTICAL ANALYSIS

Statistical analyses were carried out using SPSS version 20.0 software. Descriptive statistics were calculated for different characteristics of the subjects. Student t-test and one way ANOVA (Analysis of Variance) were used to compare the statistical differences between continuous variables.