CHAPTER 4
MATERIALS AND METHODS
Diabetes is a major medical concern owing to its high prevalence and potential deleterious effects on a patient’s physical and psychological state. The present study was designed to analyze different biochemical markers and their association with HOMA-IR in diabetic and healthy control individuals. The present study also analyzed expression of genes involved in lipid metabolism from peripheral blood mononuclear cells (PBMCs) of T2DM and healthy controls. The study design and detailed methodology used for the analysis of above parameters is mentioned below.

Since dyslipidemia is one of the most frequent co-morbidities in T2DM, the effects of co-administration of omega-3 fatty acids with metformin, a frequently used OHA, were also examined. The animal study was designed to examine the comparative effects of metformin and omega-3 fatty acids on serum biochemical markers, expression of transcription factors and genes involved in lipid metabolism and inflammation in nicotinamide (NIC)-streptozotocin (STZ) induced diabetic rats. The study design and the methodology used for the analyses are explained in this chapter.

I. HUMAN STUDY:

4.1 Study Design:

The present study was an observational, cross-section study.

4.1.1 Ethical Approval:

The study was approved by Institutional Human Ethics Committee of Bharati Vidyapeeth Medical College, Bharati Vidyapeeth Deemed University, Pune, Maharashtra, India. (Ref. No.: BVDU/MC/2/2012-2013).

4.1.2 Sample Size:

In the present study, 100 (45 males and 55 females) type 2 diabetics, registered and undergoing treatment at Bharati Hospital and Research Centre, Pune and 100 (43 males and 57 females) healthy individuals were recruited.
4.1.3 Case Definition:

Diagnosis of type 2 diabetes was based on the American Diabetes Association (ADA) criteria i.e. fasting plasma glucose ≥126 mg/dl (American Diabetes Association, 2012).

4.1.4 Inclusion and Exclusion Criteria:

The clinically diagnosed T2DM participants as per ADA criteria, aged between 40 to 60 years were recruited for the study.

Participants with body injuries, history of recent surgery and those who were underweight (body mass index ≤ 18.5) and alcoholics were excluded.

4.1.5 Data Recording:

Informed written consent was obtained from all the participants (Annexure IV). All the participants included in the study were delivered a questionnaire (Annexure IV) to record relevant clinical history (age, blood pressure, medications etc.) and physical examination (anthropometric measurements). The detailed information about antidiabetic medications consumed by diabetic participants was noted.

4.2 Metabolic Measures:

4.2.1 Anthropometric Measurements:

Informed consent was taken and anthropometric measurements were taken as per the WHO guidelines. Anthropometric measurements include height, weight, waist circumference and hip circumference. Waist circumference was measured halfway between the last rib and the iliac crest with full abdominal relaxation. Height was measured to the nearest 0.1 cm with the individual standing barefoot. Body weight was measured to the nearest 0.1 kg on a balanced scale. The body mass index (BMI) was calculated by using the formula: BMI = weight in kilograms / (height in meters)^2 and waist to hip ratio (WHR) was calculated by using formula: WHR = waist circumference (cm) / hip circumference (cm).
4.2.2 Blood Pressure:

Blood pressure was measured in sitting position using standard mercury sphygmomanometer after patient had rested for at least 10 minutes.

4.3 Blood Sample Collection and Separation:

4.3.1 Sample Collection:

After overnight fast, venous fasting blood samples were collected in the plain and ethylenediaminetetraacetic acid (EDTA) vacutainers.

4.3.2 Blood Separation:

Serum was separated from plain vacutainer by centrifugation at 2000 rpm for 15 minutes and stored at -80°C for further analysis. Blood samples in EDTA vacutainers were subjected to separation of plasma, erythrocytes and PBMCs by density gradient centrifugation.

4.3.3 Isolation of PBMCs from Whole Blood:

Whole blood (3 ml) was mixed gently with equal volume of 10mM sterile phosphate buffered saline (PBS) and the mixture was layered carefully on 3 ml Histopaque-1077 (Sigma-Aldrich, Inc., USA), in a 15 ml conical centrifuge tube. The contents were centrifuged at 15,500 rpm for 30 minutes at room temperature. After centrifugation, the middle opaque interface, containing PBMCs, was aspirated with a pipette into sterile 15 ml centrifuge tube. Plasma supernatant and erythrocyte sediment were pipetted into separate tubes and stored at -80°C for further analysis. The PBMCs were washed with 3 ml PBS (10 mM) and centrifuged at 12,500 rpm for 10 minutes and the upper layer was discarded. A pellet of PBMCs was immediately suspended in 1 ml TRIzol reagent (Sigma-Aldrich, Inc., USA) and stored at -80°C until processed for RNA isolation.

4.4 Estimation of Biochemical Markers:

Levels of glucose, total cholesterol, high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), serum glutamic oxaloacetic
transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP) and bilirubin (Coral Clinical System, Goa, India) were estimated from serum/plasma using commercial available kits. Superoxide dismutase and catalase activity (Sigma Life Science, USA) were estimated from erythrocyte using commercial available kits. Adiponectin, leptin, interleukin 8, insulin (Invitrogen, USA) was analyzed from serum/plasma using enzyme linked immunosorbent assay (ELISA) kits. Very high density lipoprotein cholesterol (VLDL) was estimated by using the formula: VLDL= Triglycerides/5. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by using the formula: HOMA-IR = [FPG (mmol/L) x FPI (mU/mL)/22.5] (Fasting plasma insulin (FPI), fasting plasma glucose (FPG), 22.5 is constant) (Wallace et al. 2004).


Glucose levels were estimated using commercially available kits (Coral Clinical Systems; Catalog No: GLU011). The GOD/POD method (glucose oxidase-peroxidase) method was employed for estimation of glucose levels (Trinder, 1969).

Principle:

Four types of enzymes oxidize glucose as a principal substrate, viz. glucose dehydrogenase, quinoprotein glucose dehydrogenase, glucose 1-oxidase, glucose 2-oxidase. GOD/POD method uses glucose-2-oxidase (GOD) enzyme for estimation of glucose. Glucose is oxidised to gluconic acid and hydrogen peroxide in the presence of glucose-2-oxidase. Hydrogen peroxide further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the color formed is directly proportional to the amount of glucose present in the sample. After completion of reaction, absorbance was read at 490 nm.

Reaction:

\[
\text{Glucose oxidase} \\
\text{Glucose + O}_2 + \text{H}_2\text{O}_2 \rightarrow \text{Gluconate + H}_2\text{O}_2
\]

\[
\text{Peroxidase} \\
\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine + Phenol} \rightarrow \text{Red Quinoneimine Dye + H}_2\text{O}_2
\]
Normal Value:

Fasting serum glucose level: 70-110 mg/dl
Postprandial glucose level: Upto 150 mg/dl.

Reagents:

L1: Glucose Reagent
S: Glucose Standard (100 mg/dl)

Assay:

The tubes were labeled properly as blank (B), standard (S) and test (T). In each tube 1 ml glucose reagent was added. Distilled water (0.01 ml) was added in the blank tube while 0.01 ml standard glucose (S) was added in standard reaction. The test reaction was set with addition of 0.01 ml serum sample. Contents were mixed by inverting and the tubes were incubated at 37°C for 10 minutes. Absorbance of the standard (Abs. S) and test Sample (Abs. T) was measured within 60 minutes, at 490 nm and glucose was estimated by using the following formula:

\[
\text{Glucose (mg/dl)} = \frac{\text{Abs. T}}{\text{Abs. S}} \times 100
\]

100 = Concentration of standard

4.4.2 Estimation of Insulin: (ELISA Method) (Instruction manual of Human insulin kit, Catalog No. KAQ1251) (Invitrogen USA).

The Invitrogen Human Insulin ELISA kit (Invitrogen, USA; Catalog No. KAQ1251) was used for insulin analysis.

Principle:

The assay uses monoclonal antibodies directed against distinct epitopes of insulin. Samples including standards of known insulin content, control specimens, and unknowns are pipetted into these wells. A detector monoclonal antibody labeled with horseradish peroxidase (HRP) is added. Reaction of secondary antibody with substrate solution [tetramethylbenzidine (TMB) - H₂O₂] produces a color which can
be measured spectrophotometrically. The intensity of color is directly proportional to the concentration of insulin in the sample.

**Reagents and Plasticware:**

i) Anti-insulin HRP Conjugate  
ii) Wash Buffer Concentrate (200X)  
iii) Chromogen  
v) TMB (Tetramethylbenzidine)  
vi) Insulin Antibody-coated Microtiter Plate

**Assay:**

The wells of microtitre plate were labeled and 50 μl of each standard, control or samples were added to appropriate wells. Anti-insulin-HRP conjugate (50 μl) was added to these wells, the plate was covered and incubated for 30 minutes at room temperature. After incubation, the contents were decanted thoroughly from well and discarded. The wells were washed 3 times with washing buffer. Chromogen solution (100 μl) was added to each well, within 15 minutes of washing. The plate was incubated in dark, at room temperature for 15 minutes and 100 μl of stop solution was added to each well. The solution was mixed gently by tapping on the side of the plate. The absorbance of yellow color was measured at 450 nm. Standard curve was prepared using absorbance of different concentrations of the standard insulin. Insulin concentration from unknown samples was calculated from the standard curve and expressed as U/ml.

**4.4.3 Lipid Profile:**

**4.4.3.1 Estimation of Total Cholesterol: (CHOD/PAP Method)** (Allain et al. 1974; Instruction manual of total cholesterol kit, Catalog No. CHO 010) (Coral Clinical Systems, Goa, India).

Total Cholesterol was estimated using commercially available kit (Coral Clinical Systems; Catalog No. CHO(SR):01, using CHOD/PAP Method (Allain et al. 1974).
Principle:

Cholesterol esterase hydrolyses esterified cholesterol to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the color formed is directly proportional to the amount of cholesterol present in the sample.

Reaction:

\[
\text{Cholesterol Esterase} \quad \text{Cholesterol Esters} + \text{H}_2\text{O} \quad \rightarrow \quad \text{Cholesterol} + \text{Fatty Acids}
\]

\[
\text{Cholesterol Oxidase} \quad \text{Cholesterol} + \text{O}_2 \quad \rightarrow \quad \text{Cholestenone} + \text{H}_2\text{O}_2
\]

\[
\text{Peroxidase} \quad \text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{Phenol} \quad \rightarrow \quad \text{Red Quinoneimine Dye} + \text{H}_2\text{O}
\]

Normal Value: As per the National Cholesterol Education Program (NCEP), Adult Treatment Panel (ATP) III guidelines, the range for total cholesterol is:

- Desirable: < 200 mg/dl
- Borderline high: 200-239 mg/dl
- High: > 240

Reagents:

- L1: Cholesterol Reagent I
- S: Cholesterol Standard (200 mg/dl)

Assay:

The tubes were labeled properly as blank (B), standard (S) and test (T). Cholesterol reagent (1 ml) was added to each tube. Distilled water (0.01 ml) was added in blank tube (B). Cholesterol standard (0.01 ml) was added to standard (S) tube. The test (T) reaction was set by adding 0.01 ml serum in a tube labeled as test (T). Contents in the tubes were mixed well and the tubes were incubated at room
temperature for 15 minutes. The absorbance of the reaction mixture was read at 505 nm. The total cholesterol (mg/dl) was calculated by using the following formula:

\[
\text{Abs. } T = \frac{\text{Cholesterol (mg/dl)}}{\text{Abs. } S} \times 200
\]

200 = Concentration of standard

4.4.3.2 Estimation of Triglycerides: (GPO/PAP Method) (Bucolo and David, 1973; Fossati and Prencipe, 1982; Instruction manual of triglyceride kit, Catalog No. TGL 010) (Coral Clinical Systems, Goa, India).

Triglyceride levels were estimated by using commercially available kit (Coral Clinical Systems; Catalog No. TGL (SR):01(P), using GPO/PAP Method (Bucolo and David, 1973; Fossati and Prencipe, 1982).

**Principle:**

Lipoprotein lipase hydrolyses triglycerides to glycerol and free fatty acids. The glycerol is phosphorylated by glycerol kinase to form glycerol 3 phosphate, which is oxidised by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Intensity of the color formed is directly proportional to the amount of triglycerides present in the sample.

**Reaction:**

\[
\text{Triglyceride} \xrightarrow{\text{Lipoprotein Lipase}} \text{Glycerol + Free Fatty Acids}
\]

\[
\text{Glycerol + ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol 3 Phosphate + ADP}
\]

\[
\text{Glycerol 3 Phosphate + O}_2 \xrightarrow{\text{Peroxidase}} \text{Dihydroxyacetone phosphate + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine + Phenol} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine Dye} + \text{H}_2\text{O}
\]
Normal Value: As per the National Cholesterol Education Program (NCEP), Adult Treatment Panel (ATP) III guidelines, the range for triglyceride is:

- Normal: < 150 mg/dl
- Borderline: 150-190 mg/dl
- High: 200-499 mg/dl
- Very high: > 500 mg/dl

Reagents:

- L1: Triglycerides Reagent
- L3: Triglycerides Standard (200 mg/dl)

Assay:

The tubes were labeled properly as blank (B), standard (S) and test (T). In each tube, 1ml of triglycerides reagent was added. In blank (B) tube, 0.01 ml of distilled water was added, in standard (S) tube 0.01 ml of triglyceride standard was added and 0.01 ml of serum was added in test (T). Contents of the tubes were mixed well and the tubes were incubated at room temperature for 15 minutes. The absorbance of the reaction mixture was read at 505 nm. The total triglyceride (mg/dl) content was calculated by using following formula:

\[
\text{Triglycerides (mg/dl)} = \frac{\text{Abs. } T}{\text{Abs. } S} \times 200
\]

200 = concentration of standard

Very High Density Lipoprotein Cholesterol (VLDL) was estimated by using the formula: Triglycerides/5 (Warnick et al. 1990).

4.4.3.3 Estimation of HDL-D Cholesterol: (Direct Enzymatic Method) (Grillo et al. 1981; Instruction manual of HDL-D cholesterol kit, Catalog No. HDL 030) (Coral Clinical Systems, Goa, India).

HDL-D cholesterol levels in the samples were estimated by commercially available kit (Coral Clinical Systems; Catalog No. HDL 030), using Direct Enzymatic Method (Grillo et al. 1981).
**Principle:**

The method employed for estimation of HDLc depends on the properties of a detergent which selectively solubilises HDL so that the HDLc is released to react with the cholesterol esterase, cholesterol oxidase and chromogens to give color. The non HDL lipoproteins, LDL, VLDL and chylomicrons are inhibited from reacting with the enzymes due to adsorption of the detergent on their surfaces. The intensity of the color formed is proportional to the HDLc concentration in the sample.

**Normal Value:** As per the National Cholesterol Education Program (NCEP), Adult Treatment Panel (ATP) III guidelines for HDL, the range of HDL is:

- Low: < 40mg/dl
- High: ≤ 60mg/dl

**Reagents:**

L1: HDL-D Reagent I
L2: HDL-D Reagent II
C: Calibrator

**Assay:**

The tubes were labeled properly as blank (B), calibrator (C) and test (T). In each tube, 375 μl L1 was added. In calibrator (C) tube, 0.05 ml calibrator was added and 0.05 ml serum was added in test (T). Contents of the tubes were mixed well and the tubes were incubated at 37°C for 5 minutes. Absorbance ($A_1$) of this mixture was read at 570 nm. L2 (125 μl) was added in all the tubes. Contents of the tubes were mixed well and tubes were incubated at 37°C for 5 minutes. Absorbance ($A_2$) of the reaction mixture was read at 570 nm. The HDLc (mg/dl) concentration was calculated by using following formula:

\[
\begin{align*}
\Delta AC &= A_2 C - A_1 C \\
\Delta AT &= A_2 T - A_1 T \\
\text{HDLc (mg/dl)} &= \frac{\Delta AT}{\Delta AC} \times \text{Concentration of calibrator}
\end{align*}
\]
4.4.3.4 Estimation of LDL-D Cholesterol: (Direct Enzymatic Method) (Okada et al. 1998; Instruction manual of LDL-D cholesterol kit; Catalog No. LDL 040) (Coral Clinical Systems, Goa, India).

LDL-D cholesterol levels were estimated by commercially available kit (Coral Clinical Systems; Catalog No. LDL 040), using direct enzymatic method (Okada et al. 1998).

Principle:

The assay takes place in two steps. First by the elimination of lipoprotein non-LDL-D cholesterol and then the measurement of LDLc. The intensity of the color formed is proportional to the LDLc concentration in the sample.

Reaction:

Elimination of non-LDL Cholesterol:

\[
\text{Cholesterol Esterase} \quad \text{Cholesterol Esters} + \text{H}_2\text{O} \rightarrow \text{Cholesterol} + \text{Fatty Acids}
\]

\[
\text{Cholesterol Oxidase} \quad \text{Cholesterol} + \text{O}_2 \rightarrow 4\text{-Cholestenone} + \text{H}_2\text{O}_2
\]

\[
\text{Catalase} \quad 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

Estimation of LDL Cholesterol:

\[
\text{Cholesterol Esterase} \quad \text{Cholesterol Esters} \rightarrow \text{Cholesterol} + \text{Fatty Acids}
\]

\[
\text{Cholesterol Oxidase} \quad \text{Cholesterol} + \text{O}_2 \rightarrow 4\text{-Cholestenone} + \text{H}_2\text{O}_2
\]

\[
\text{Peroxidase} \quad 2\text{H}_2\text{O}_2 + \text{TODS} + 4\text{-Aminoantipyrine} \rightarrow \text{Quinonimine} + 4\text{H}_2\text{O}
\]

Normal Value: As per the National Cholesterol Education Program (NCEP), Adult Treatment Panel (ATP) III guidelines for LDL, the range of LDL is:

Optimal: < 100 mg/dl
Above optimal: 100-129 mg/dl
Borderline: 130-159 mg/dl
High: 160-189 mg/dl
Very high: > 190 mg/dl

Reagents:

L1: LDL-D Reagent I
L2: LDL-D Reagent II
C: Calibrator

Assay:

The tubes were labeled properly as blank (B), calibrator (C) and test (T). In each tube, 375 µl of LDL-D reagent I (L1) was added. In calibrator (C) tube, 0.05 ml of calibrator was added and 0.05 ml of serum was added in test (T). Contents of the tubes were mixed well and the tubes were incubated at 37°C for 5 minutes. Absorbance of this mixture was read at 570 nm. In all the tubes, 125 µl LDL-D reagent II (L2) was added. All the tubes were mixed well and incubated at 37°C for 5 minutes. Absorbance of the reaction mixture was read at 570 nm. The LDLc (mg/dl) concentration was calculated by using following formula:

\[
\text{LDLc (mg/dl)} = \frac{\text{Abs. T}}{\text{Abs. C}} \times \text{Concentration of calibrator}
\]

4.4.4 Liver Function Tests:


SGOT activity was estimated by using commercially available kit (Coral Clinical Systems; Catalog No. GOT 010), using Reitman & Frankel's Method; (Reitman and Frankel, 1957; Tietz, 1970).
Principle:

SGOT converts L-Aspartate and α-Ketoglutarate to oxaloacetate and glutamate. The oxaloacetate formed reacts with 2, 4-Dinitrophenyl hydrazine (2, 4, DNPH) to produce a hydrazone derivative, which in an alkaline medium produces a brown colored complex intensity of which can be measured spectrophotometrically. A calibration curve is plotted using a pyruvate standard. The activity of SGOT (AST) is read off this calibration curve.

Reaction:

\[
\text{L- Aspartate + α-Ketoglutarate} \xrightarrow{\text{SGOT}} \text{Oxaloacetate + L-Glutamate} \quad \text{pH 7.4}
\]

\[
\text{Oxaloacetate + 2, 4, DNPH} \xrightarrow{\text{Alkaline}} \text{2, 4, Dinitrophenyl Hydrazone (Brown Colored Complex)}
\]

Normal Value:

Serum: 8-40 units/ml

Reagents:

L1: Substrate Reagent
L2: DNPH Reagent
L3: NaOH Reagent (4 N)
S: Pyruvate Standard (2 mM)

Working NaOH Solution I: NaOH reagent (1 ml) was diluted up to 10 ml with distilled water

Assay:

The tubes were labelled properly as blank (B), standard (S1, S2, S3, S4 and S5), and test (T). L1 reagent (0.50, 0.45, 0.40, 0.35, 0.30 ml) was added in all the tubes of standard. pyruvate standard (0.05, 0.1, 0.15, and 0.20 ml) was added in tubes S1, S2, S3, S4 and S5 respectively. Distilled water (0.10 ml) and L2 (0.5 ml) was added in all tubes of standard. The Contents of the tubes were mixed well and incubated at room temperature for 20 minutes. Working NaOH solution I (5 ml) was added to all the tubes. Contents of the tubes were mixed well and incubated at room
temperature for 10 minutes. The absorbance of standard tubes S1 to S5 was measured against blank S1.

L1 reagent (0.50 ml) was added in B and T tubes. The tubes were incubated at 37ºC for 3 minutes. Serum (0.10 ml) was added in the test (T). Contents of the tubes were mixed well and incubated at 37ºC for 60 minutes. L2 reagent (0.50 ml) was added in tubes B and T. Contents of the tubes were mixed well and incubated at room temperature for 20 minutes. Distilled water (0.10 ml) was added in blank (B) tube. Working NaOH solution I (5 ml) was added to B and T tubes. Contents of the tubes were mixed well and incubated at room temperature for 10 minutes. The absorbance of blank, standard and test were read at 505 nm. SGOT activity was determined by using standard graph and expressed as U/ml.


SGPT activity was estimated using commercially available kit (Coral Clinical Systems; Catalog No. GPT 010), using Reitman & Frankel's Method; (Reitman and Frankel, 1957; Tietz, 1970).

Principle:

SGPT converts L-Alanine and α-Ketoglutarate to pyruvate and glutamate. The pyruvate formed reacts with 2, 4, Dinitrophenyl hydrazine (2, 4, DNPH) to produce a hydrazone derivative, which in an alkaline medium produces a brown colored complex whose intensity is measured spectrophotometrically. A calibration curve is plotted using a pyruvate standard. The activity of SGPT (ALT) is read off this calibration curve.

Reaction:

\[
\begin{align*}
\text{L-Alanine} + \alpha\text{-Ketoglutarate} & \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{L-Glutamate} \\
\text{pH 7.4} & \\
\text{Alkaline Medium} & \xrightarrow{2, 4\text{-DNPH}} 2, 4\text{-Dinitrophenyl Hydrazine (Brown Colored Complex)}
\end{align*}
\]
**Normal Value:**

Serum: 5-35 units/ml

**Reagents:**

L1: Substrate Reagent  
L2: DNPH Reagent  
L3: NaOH Reagent (4 N)  
S: Pyruvate Standard (2 mM)  

Working NaOH Solution I: NaOH reagent (1 ml) was diluted up to 10 ml with distilled water

**Assay:**

The tubes were labelled properly as blank (B), standard (S1, S2, S3, S4 and S5), and test (T). L1 reagent (0.50, 0.45, 0.40, 0.35, 0.30 ml) was added in all the tubes of standard. Pyruvate standard (0.05, 0.1, 0.15, and 0.20 ml) was added in tubes S1, S2, S3, S4 and S5 respectively. Distilled water (0.10 ml) and L2 (0.5 ml) was added in all tubes of standard. Contents of the tubes were mixed well and incubated at room temperature for 20 minutes. Working NaOH solution I (5 ml) was added to all the tubes. Contents of the tubes were mixed well and incubated at room temperature for 10 minutes. The absorbance of standard tubes S1 to S5 was measured against blank S1.

L1 reagent (0.50 ml) was added in B and T tubes. The tubes were incubated at 37°C for 3 minutes. Serum (0.10 ml) was added in the test (T). Contents of the tubes were mixed well and incubated at 37°C for 30 minutes. L2 reagent (0.50 ml) was added in tubes B and T. Contents of the tubes were mixed well and incubated at room temperature for 20 minutes. Distilled water (0.10 ml) was added in blank (B) tube. Working NaOH solution I (5 ml) was added to B and T tubes. Contents of the tubes were mixed well and incubated at room temperature for 10 minutes. The absorbance of blank, standard and test were read at 505 nm. SGOT activity was determined by using standard graph and expressed as U/ml.
4.4.4.3 Estimation of Total Bilirubin: (Modified Jendrassik and Grof’s Method)  
(Jendrassik and Grof, 1938; Instruction manual of total bilirubin kit, Catalog No. BIL 010) (Coral Clinical Systems, Goa, India).

Total bilirubin levels were estimated using commercially available kit (Coral Clinical Systems; Catalog No. BIL 010), using modified Jendrassik and Grof’s Method (Jendrassik and Grof, 1938).

**Principle:**

Bilirubin reacts with diazotised sulphanilic acid to form a colored azobilirubin compound. The unconjugated bilirubin couples with the sulphanilic acid in the presence of a caffeine-benzoate accelerator. The intensity of the color formed is directly proportional to the amount of bilirubin present in the sample.

**Reaction:**

\[
\text{Bilirubin + Diazotized Sulphanilic Acid } \rightarrow \text{ Azobilirubin Compound}
\]

**Normal Value:**

Serum: 0.3-1.23 mg/dl

**Reagents:**

L1: Total Bilirubin Reagent  
L2: Total Nitrite Reagent

**Assay:**

The tubes were labelled properly as blank (B), and test (T). In each tube, 1 ml L1 was added. In test (T) tube, 0.05 ml of L2 was added. In each tube, 0.1 ml of serum was added. Contents were mixed by inverting and the tubes were incubated at room temperature for 10 minutes. Absorbance of blank and test sample was measured at 546 nm. Total bilirubin (mg/dl) was calculated by using following formula:

\[
\text{Total bilirubin (mg/dl)} = \text{Abs. T} \times 13
\]
4.4.4.4 Estimation of Alkaline Phosphatase (ALP): (Modified Kind & King's Method) (Kind and King, 1954; Instruction manual of ALP kit, Catalog No. ALP 010) (Coral Clinical Systems, Goa, India).

ALP activity was estimated using commercially available kit (Coral Clinical Systems; Catalog No. ALP 010), using modified Kind & King's Method (Kind and King, 1954).

**Principle:**

ALP at an alkaline pH hydrolyses disodium phenylphosphate to form phenol. The phenol formed reacts with 4-Aminoantipyrine in the presence of potassium ferricyanide, as an oxidising agent, to form a red colored complex. The intensity of the color formed is directly proportional to the activity of ALP present in the sample.

**Reaction:**

\[
\text{Disodium Phenylphosphate} \xrightarrow{\text{ALP}} \text{Phenol} + \text{Disodium Hydrogen Phosphate} + H_2O
\]

\[
\text{pH 10.0} \quad \text{Alkaline Medium} \xrightarrow{K_3\text{Fe(CN)}_6} \text{Red Colored Complex}
\]

**Normal Value:**

Serum: 3.0-13.0 KA Units

**Reagents:**

L1: Buffer Reagent
L2: Substrate Reagent
L3: Color Reagent
L4: Phenol Standard (10 mg/dl)

**Assay:**

The tubes were marked properly as blank (B), standard (S), control (C), and test (T). In blank tube, 1.05 ml distilled water was added while in other tubes 1 ml distilled water was added. In each tube, 1 ml L1 and 0.10 ml L2 was added. Contents
of the tubes were mixed well and the tubes were incubated at 37°C for 3 minutes. In test (T) tube, 0.05 ml serum was added and 0.05 ml L4 was added in standard (S) tube. Contents of the tubes were mixed well and the tubes were incubated at 37°C for 15 minutes. L3 (1 ml) was added in all tubes and 0.05 ml of serum sample was added in control (C) tube. Contents of the tubes were mixed well and absorbance was read at 510 nm. Serum alkaline phosphatase activity (KA units) was calculated by using following formula:

$$\text{Total ALP activity (KA Units) = } \frac{\text{Abs. } T - \text{Abs. C}}{\text{Abs. S - Abs. B}} \times 10$$

### 4.4.5 Antioxidant Enzymes:

#### 4.4.5.1 Estimation of Catalase: (Colorimetric Method) (Fossati et al. 1980; Zamocky and Koller, 1999; Ding et al. 2000; Instruction manual of catalase kit, Catalog No. CAT100) (Sigma-Aldrich kit, USA).

Catalase activity was determined using commercially available kit (Sigma-Aldrich kit; Catalog No. CAT100), by colorimetric method (Fossati et al. 1980; Zamocky and Koller, 1999; Ding et al. 2000).

**Principle:**

Catalase mediated removal of H$_2$O$_2$ protects the cell from oxidative damage. This assay is based on measurement of hydrogen peroxide remaining after the action of catalase. First, the catalase converts hydrogen peroxide to water and oxygen (catalytic pathway) and then this enzymatic reaction is stopped with sodium azide. An aliquot of the reaction mix is then assayed for amount of hydrogen peroxide remaining by a colorimetric method. The colorimetric method uses a substituted phenol (3,5-dichloro-2-hydroxybenzenesulfonic acid), which couples oxidatively to 4-aminoantipyrine in the presence of hydrogen peroxide and horseradish peroxidase (HRP) to give a red quinoneimine dye (N-(4-antipyryl)-3-chloro-5-sulfonatep-benzoquinone-monoimine) that absorbs at 520 nm.

*Reaction:*

$$2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2$$
Reagents:

i) Assay Buffer (10X)
ii) Chromogen Reagent
iii) Stop Solution
iv) 3% (w/w) Hydrogen Peroxide Solution

Assay:

Erythrocytes (0.1 ml) from the blood samples were mixed with 0.2 ml ice cold PBS (10mM). Contents were vigorously vortexed for 2-4 minutes followed by centrifugation at 10,000 rpm for 5 minutes. The resulting supernatant was used for catalase assay. The tubes were labeled properly as blank (B) and sample (S). In blank (B) tube, 75 μl 1X assay buffer and 25 μl H₂O₂ (200mM) solution was added. In sample (S) tube, 1 μl sample, 74 μl 1X assay buffer and 25 μl H₂O₂ (200mM) solution was added. Contents were mixed well and the tubes were incubated at room temperature for 5 minutes. In each tube, 900 μl of the stop solution was added and contents were mixed. An aliquot of 10 μl was pipetted into separate tubes for catalase enzymatic reaction mixture. Color reagent (1 ml) was added and the contents were mixed. All tubes were incubated at room temperature for 15 minutes. The absorbance of red color was measured at 520 nm. Catalase activity (μmoles/minutes/ml) was calculated by using the following formula:

\[
\text{Catalase activity (μmoles/minutes/ml) = } \frac{\Delta \mu \text{ moles (H}_2\text{O}_2) \times d \times 100}{v \times t}
\]

Where,

\[\Delta \mu \text{ moles (H}_2\text{O}_2) = \mu \text{ moles (H}_2\text{O}_2) \text{ Blank} - \mu \text{ moles (H}_2\text{O}_2) \text{ Sample},\]

\[\mu \text{ moles (H}_2\text{O}_2) \text{ Blank} = A_{540} \text{ Blank},\]

\[\mu \text{ moles (H}_2\text{O}_2) \text{ Sample} = A_{540} \text{ Sample},\]

\[d = \text{ Dilution factor},\]

\[v = \text{ Volume of catalase in the reaction},\]

\[t = \text{ Reaction duration in minutes},\]

\[100 = \text{ Dilution of aliquot from catalase reaction}\]
4.4.5.2 Estimation of Superoxide Dismutase (SOD): (Colorimetric Method)
(Instruction manual of SOD kit, Catalog No. Catalog No. 19160 SOD) (Sigma-Aldrich kit, USA).

SOD activity was determined using commercially available kit (Sigma-Aldrich, Catalog No. 19160 SOD), by colorimetric method.

Principle:

Superoxide dismutase (SOD), which catalyses dismutation of superoxide anion \((O_2^-)\) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. An indirect method using nitroblue tetrazolium (NBT) is commonly used for SOD analysis. This method allows convenient SOD assaying by utilizing Dojindo’s highly water-soluble tetrazolium salt, WST-1 \((2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, \) monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with \(O_2\) is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the IC50 (50% inhibition activity of SOD) can be determined by a colorimetric method.

Reaction:
Reagents:

i) Phosphate Buffered Saline (PBS) (10 mM)

ii) WST Solution: Working WST solution reagent was prepared by mixing together 1 part of WST solution and 19 parts of buffer solution

iii) Enzyme Solution: Working enzyme solution was prepared by mixing together 15 μl of enzyme solution and 2.5 ml of dilution buffer

iv) Buffer Solution

Assay:

Erythrocytes (0.2 ml) from the blood samples were mixed with 0.5 ml ice cold PBS (10 mM). The tubes were centrifuged at 10,000 rpm for 5 minutes. The resulting pellet was re-dissolved in 2 volumes ice cold PBS (10 mM) and contents were vigorously vortexed for 2-4 minutes followed by centrifugation at 10,000 rpm for 5 minutes. The resulting supernatant was used for SOD assay. The wells were labeled as blank 1, blank 2, blank 3 and sample (S). Sample solution (20 μl) was added in each sample and blank 2 well. Double distilled water (20 μl) was added in each blank 1 and blank 3 well. WST working solution (200 μl) was added in each well and contents were mixed. Dilution buffer (20 μl) was added in each blank 2 and blank 3 wells. Enzyme working solution (20 μl) was added in each sample and blank 1 and contents were mixed thoroughly. Plate was incubated at 37°C for 20 minutes and absorbance was read at 450 nm in ELISA plate reader (Make: Bio-Rad). SOD activity (% inhibition rate) was calculated by using the following formula:

\[
\text{SOD} \text{ (%) } = \frac{[\text{A blank 1 - A blank 3) - (A sample - A blank 2)]}{\text{A blank 1 - A blank 3)}} \times 100
\]

4.4.6 Inflammatory Marker:

Estimation of Interleukin 8 (IL8): (ELISA Method) (Instruction manual of Human IL8 kit, Catalog No. KHC0081) (Invitrogen, USA).

The Invitrogen human IL8 kit, a solid phase sandwich Enzyme Linked Immunosorbent Assay (ELISA) (Invitrogen, USA; Catalog No. KHC0081) was used for estimation of IL8 content.
**Principle:**

The assay uses monoclonal antibodies specific for human IL8 coated onto the wells of the microtiter strips. Samples including standards of known human IL8 content, control specimens, and unknowns, are pipetted into these wells followed by the addition of a secondary biotinylated monoclonal antibody. During the first incubation, the human IL8 antigen binds to the immobilized (capture) antibody on one site and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, streptavidin-peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove the entire unbound enzyme, a substrate solution is added, which is converted to a colored product by bound enzyme. The intensity of this colored product is measured at 450 nm and it is directly proportional to the concentration of human IL8 in the specimen.

**Reagents and Plasticware:**

i) Hu IL8 Biotin Conjugate (Biotin-labeled Anti-IL8)

ii) Streptavidin-Peroxidase (HRP) (100X)

iii) Streptavidin-Peroxidase (HRP) Diluent

iv) Wash Buffer Concentrate (25X)

v) Stabilized Chromogen, Tetramethylbenzidine (TMB)

vi) Stop Solution

vii) Hu IL8 Antibody Coated 96-well Plate (12 x 8-well strips)

**Assay:**

The wells of microtitre plate were labeled and 50 μl of each standard, samples or controls were added to appropriate wells. Chromogen blank was prepared by adding 50 μl of the standard diluent buffer. Biotin conjugate solution (50 μl) was added to these wells except the chromogen blank. The solution was mixed by gently tapping on the side of the plate. The plate was covered and incubated at room temperature for 1 hour and 30 minutes. After incubation, the contents were decanted thoroughly from wells and discarded. The wells were washed 4 times with washing buffer. Streptavidin-HRP (100 μl) working solution was added to each well except the chromogen blank. The plate was covered and incubated at room temperature for 30
minutes. The wells were washed 4 times with washing buffer. Stabilized chromogen (100 μl) was added to each well. The plate was incubated in dark, at room temperature for 30 minutes and 100 μl of stop solution was added to each well. The solution was mixed gently by tapping on the side of the plate. The absorbance of blue color was measured at 450 nm. Standard curve was prepared using absorbance of different concentrations of the standard IL8. IL8 concentration of unknown sample was calculated from the standard curve.

4.4.7 Adipocytokines:

4.4.7.1 Estimation of Adiponectin: (ELISA Method) (Instruction manual of Human adiponectin kit, Catalog No. KHP0041) (Invitrogen, USA).

The Invitrogen human adiponectin kit, a solid phase sandwich-ELISA (Invitrogen, USA; Catalog No. KHP0041), was used for estimation of adiponectin from serum samples.

Principle:

This assay utilizes a biotin-conjugated polyclonal antibody preparation specific for adiponectin and streptavidin conjugated to horseradish peroxidase (HRP). TMB (3,3',5,5' tetramethyl-benzidine) is used as a substrate solution. The enzyme-substrate reaction is terminated by addition of sulphuric acid and the color change is measured spectrophotometrically at 450 nm which is directly proportional to the concentration of adiponectin in the specimen.

Reagents and Plasticware:

i) Wash Concentrate (5X)
ii) Diluent (5X)
iii) Secondary Antibody
iv) Detector (100X)
v) Standard, Recombinant Human Adiponectin
vi) Substrate I
vii) Substrate II
viii) Stop Solution
ix) Antibody Coated 96-well Plate (12 x 8-well strips)
Assay:

Samples were diluted 1:100 with 1X diluent. Diluted samples were again diluted, 1:20 with 1X diluent. The samples are thus diluted 1:2000. The wells of antibody-coated plate were labeled and 100 μl of standards 0 to 7, the reconstituted QC sample and pre-treated plasma sample were added to these wells. Plate was incubated at 37°C for 1 hour. After incubation, the contents were decanted from the wells and discarded. The wells were washed 3 times with 250 μl wash solution (1X). Secondary antibody (100 μl) was added to each well and incubated at 37°C for 1 hour. After incubation, the contents were decanted thoroughly from wells and discarded. The wells were washed with 250 μl wash solution (1X). Detector 1X (100 μl) was added to each well and incubated at 37°C for 1 hour. After incubation, the contents were decanted thoroughly from well and discarded. The wells were washed 5 times with 250 μl wash solution (1X). Substrate solution (100 μl) was added into each well and incubated in dark, at room temperature for 20 minutes and 100 μl stop solution was added to each well and absorbance was measured at 450 nm. Standard curve was prepared using absorbance of different concentrations of standard adiponectin. Adiponectin concentrations from unknown samples were calculated from the standard curve. The adiponectin concentrations were multiplied by the dilution factor to obtain the concentrations of the undiluted samples.

4.4.7.2 Estimation of Leptin: (ELISA) (Instruction manual of Human leptin kit, Catalog No. KA2281) (Invitrogen, USA).

The Invitrogen human leptin kit, a solid phase sandwich Enzyme Linked Immunosorbent Assay (ELISA) (Invitrogen, USA; Catalog No. KAC2281), was used for estimation of leptin from serum samples.

Principle:

The assay uses monoclonal antibody specific for human leptin coated onto the wells of the microtiter strips. Samples, including standards of known leptin content, control specimens, and unknowns, are pipetted into these wells followed by addition of a secondary biotinylated monoclonal antibody. During the first incubation, the Hu leptin antigen binds to immobilized (captured) antibody on one site and to the solution
phase, biotinylated antibody, on a second site. After removal of excess second antibody, streptavidin-peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After second incubation and washing to remove the entire unbound enzyme, a substrate solution is added, which is converted to a colored product by the bound enzyme. The intensity of this colored product is measured at 450 nm and it is directly proportional to the concentration of leptin present in the specimen.

Reagents and Plasticware:

i) Hu Leptin Biotin Conjugate (Biotin-labeled Anti-human Leptin)
ii) Streptavidin-Peroxidase (HRP) (100X) Concentrate
iii) Streptavidin-Peroxidase (HRP) Diluent
iv) Wash Buffer Concentrate (25X)
vi) Stop Solution
vii) Hu Leptin Antibody-Coated 96-well Plate (12 x 8-well strips)

Assay:

Serum samples were diluted 1:100 with standard diluent buffer. Standard diluent buffer (100 μl) was added into the chromogen blank well. The wells of antibody-coated plate were labeled and 100 μl of standards, samples or control were added to these wells. Hu leptin biotin conjugate (100 μl) was added to each well except the chromogen blank. The contents of tubes were mixed and incubated at room temperature for 2 hours. After incubation, the contents were decanted from wells and discarded. The wells were washed 4 times with 250 μl wash solution (1X). Streptavidin-HRP working solution (100 μl) was added to each well except the chromogen blank and incubated at room temperature for 30 minutes. After incubation, the contents were decanted and discarded. The wells were washed 4 times with 250 μl wash solution (1X). Stabilized chromogen (100 μl) was added to each well. Plate was incubated in dark at room temperature for 30 minutes and 100 μl of stop solution was added to each well. The solution was mixed gently by tapping on the side of the plate. The absorbance of yellow color was measured at 450 nm. Standard curve was prepared using absorbance of different concentrations of standard leptin. Leptin
concentration from unknown samples was calculated from the standard curve. Leptin concentration was determined by considering the appropriate dilution factor to obtain the concentrations of the undiluted samples.

4.5 Molecular Analysis:

4.5.1 Isolation of RNA from PBMCs:

A fraction of peripheral blood mononuclear cells (PBMCs) suspended in TRIzol reagent was thawed and total RNA was extracted by TRIzol method (Chomczynski and Sacchi, 1987). Chloroform (200 µl) (Sigma-Aldrich, Inc., USA) was added to the contents, vortexed vigorously for 15 seconds and the contents were incubated for 2-3 minutes at room temperature. The contents were then centrifuged at 12000 rpm for 15 minutes at 4°C. The resulting aqueous phase was aspirated carefully, without disturbing an interphase, with a pipette, into a sterile 1.5 ml eppendorf tube. Ice-cold isopropyl alcohol (0.5 ml) (Sigma-Aldrich, Inc., USA) was added to the aqueous phase and the tubes were incubated at -20°C overnight for RNA precipitation. After incubation, the tubes were kept on ice for 10 minutes. The contents were centrifuged at 12,000 rpm for 15 minutes at 4°C. The resulting supernatant was discarded and the pellet was washed with 500 µl, 75% ice-cold ethanol. The contents were centrifuged at 7500 rpm for 5 minutes at 4°C. The resulting supernatant was discarded and pellet i.e. RNA was immediately suspended in 20 µl diethylpyrocarbonate-treated water (DEPC, Sigma-Aldrich, Inc., USA). The RNA samples were further processed by the DNA-free™ DNase Treatment Kit (Cat No. AM1906, Ambion, Austin, Texas) to remove the contaminating DNA. DNase I Buffer (1X) 2 µl and rDNase (1 µl) was added. The contents were mixed gently and incubated at 37°C for 20-30 minutes. DNase inactivation reagent 2 µl was added and mixed well. Contents were incubated at room temperature for 2 minutes and mixed occasionally. The contents were then centrifuged at 10000 rpm for 1.5 minutes and supernatant i.e. RNA was transferred to the fresh tube.

4.5.2 RNA Quantification and Quality Check:

Quantification of RNA was performed by spectrophotometric method using NanoDrop spectrophotometer (ND1000, USA). The upper and lower optical surfaces were cleaned with 1 µl deionized water and both surface were wiped off with tissue
paper. The software was turn on by clicking on the icon and in nucleic acid module, RNA analysis was selected. The spectrophotometer was initialized by placing 1 µl deionized water onto the lower optical surface, lever arm was lowered and NanoDrop was initialized. Both optical surfaces were cleaned with tissue paper and DEPC (1 µl) was placed onto the lower optical surface, lever arm was lowered and blank was analyzed on NanoDrop software. Both optical surfaces were cleaned with the tissue paper. Unknown RNA sample (1 µl) was placed on lower optical surface and lever arm was lowered. The concentration of unknown RNA sample was measured by NanoDrop software. RNA was quantified and purity assessed using a NanoDrop at absorbance 260 nm, 230 nm and 280 nm. RNA concentration was determined by the measurement of absorbance at 260 nm, while the ratios 260/280 and 260/230 were recorded to detect contamination by phenols, proteins and other organic compounds. Further, 2 µl RNA samples were run on 0.8% agarose gel electrophoresis to check the integrity of the RNA. Pure RNA was used for cDNA synthesis.

4.5.3 cDNA Synthesis:

cDNA was prepared by using high capacity cDNA Reverse Transcription kit (Applied Biosystems, USA; Catalog No. 4368814). DNA-free RNA (2 µg/µl) was reverse transcribed to cDNA. Briefly, the 1X reaction mixture (20 µl) was prepared in polymerase chain reaction (PCR) tube by mixing, 2 µl RT Buffer (10X), 0.8 µl 100mM dNTP (25X) mixture, 2 µl Random primer (10X), 1 µl MultiScribe™ Reverse Transcriptase, 4.2 µl DEPC water and 10 µl RNA sample. The contents were mixed by gentle tapping of the tubes. The tube was sealed and briefly centrifuged to spin down the contents and to eliminate any air bubbles. These tubes were placed in thermal cycler (Applied Biosystems, Veriti™ Thermal Cycler) and were incubated at 25°C for 10 minutes; then at 37°C for 120 minutes and finally at 85°C for 5 minutes. The contents of the tubes were cooled at 4°C for 5 minutes. The synthesized cDNA was stored at -80°C until processed for quantitative real-time polymerase chain reaction.

4.5.4 Quantitative Real-Time Polymerase Chain Reaction Analysis:

Standard quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the use of SYBR Green master mix (Applied Biosystems, USA) for
the following genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peroxisome proliferator activated receptor γ (PPARγ), sterol regulatory element binding protein 1 (SREBP1), nuclear factor kappa β (NFκβ), fatty acid synthase (FAS), long chain acyl CoA synthetase (ACSL), malonyl-CoA-acyl carrier protein transacylase (MCAT) and tumor necrosis factor α (TNFα). KicqStart Primers (Sigma-Aldrich, USA) were used for gene expression studies and are listed in Table 4. qRT-PCR was performed by using the Applied Biosystems 7300 standard system. The cDNA was diluted 40 times with sterile double distilled water and used for qRT-PCR. The 1X reaction mixture was prepared by using 5 μl SYBR Green master mix, 0.5 μl forward, 0.5 μl reverse primer, 2 μl sterile double distilled water and 2 μl cDNA. The reactions for each gene were performed in duplicate. The qRT-PCR temperature profile used for gene expression analysis is given below:

The run started with a hold at 50°C for 2 minutes, followed by initial denaturation at 95°C for 10 minutes, and 40 cycles, each comprising denaturation at 95°C for 0.15 sec, annealing at 60°C for 1 minute, and dissociation at 95°C for 0.15 sec. The qRT-PCR run was followed by a single final extension at 60°C for 30 sec and denaturation at 95°C for 0.15 sec. Relative expression levels of genes were calculated and expressed as $2^{ΔCT}$ where $ΔCT$ is $C_T$ (GAPDH) – $C_T$ (target gene), a modification of $2^{-ΔΔCT}$ method (Gaines et al. 2010).
Table 4: List of Primers Used for Quantitative Real-Time PCR in Human Study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GAPDH F</td>
<td>CTTTTCGTCGCCAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAPDH R</td>
<td>TTGATGGCAACAATATCCAC</td>
</tr>
<tr>
<td>PPARγ</td>
<td>PPARγ F</td>
<td>AAAGAAGGCCAACACTAAACC</td>
</tr>
<tr>
<td></td>
<td>PPARγ R</td>
<td>TGGTCATTTCGTTAAGGC</td>
</tr>
<tr>
<td>SREBP1</td>
<td>SREBP1 F</td>
<td>AATTCTGGGTTTGTGTTCTTC</td>
</tr>
<tr>
<td></td>
<td>SREBP1 R</td>
<td>AAAAGTTGTGTACCTTGTGG</td>
</tr>
<tr>
<td>NFκβ</td>
<td>NFκβ F</td>
<td>CACAAGGAGACATGAAACAG</td>
</tr>
<tr>
<td></td>
<td>NFκβ R</td>
<td>CCCAGAGACCTCATAGTTG</td>
</tr>
<tr>
<td>ACSL</td>
<td>ACSL F</td>
<td>TGAGTGGGTGATTATGACAC</td>
</tr>
<tr>
<td></td>
<td>ACSL R</td>
<td>GTTGACTATGTACGTGATGG</td>
</tr>
<tr>
<td>MCAT</td>
<td>MCAT F</td>
<td>CTGTCGAGAAACTACATCAC</td>
</tr>
<tr>
<td></td>
<td>MCAT R</td>
<td>GATGAGCCTTCAGCAATTC</td>
</tr>
<tr>
<td>FAS</td>
<td>FAS F</td>
<td>CAATACAGATGCTTCAAGG</td>
</tr>
<tr>
<td></td>
<td>FAS R</td>
<td>GATGATCCAAATGACTCAGGG</td>
</tr>
<tr>
<td>TNFα</td>
<td>TNFα F</td>
<td>AGGCAGTCAGATCATCCTTC</td>
</tr>
<tr>
<td></td>
<td>TNFα R</td>
<td>TTATCTCAGCTCCACG</td>
</tr>
</tbody>
</table>

**GAPDH**: Glyceraldehyde-3-phosphate dehydrogenase; **PPARγ**: Peroxisome proliferator activated receptor γ; **SREBP1**: Sterol regulatory element binding protein 1; **NFκβ**: Nuclear factor kappa β; **FAS**: Fatty acid synthase; **ACSL**: Long chain acyl CoA synthetase; **MCAT**: Malonyl-CoA-acyl carrier protein transacylase; **TNFα**: Tumor necrosis factor α; **F**: Forward primer sequence; **R**: Reverse primer sequence

### 4.6 Data Analyses:

Data were analyzed based on the gender, type of treatment, fasting blood glucose levels and duration of the disease. The data from diabetic individuals were grouped accordingly and analyzed for their correlation with other markers and difference among the means of the groups. Divisions of diabetic individuals into different groups for data analysis are given below:
4.6.1 Depending on the Gender:

In the present study, 43 male and 57 female healthy participants and 45 male and 55 female type 2 diabetics were assessed.

4.6.2 Depending on Type of Treatments:

The diabetic participants (45 males and 55 females) were grouped into following groups depending on type of drugs consumed: 1. Diabetic participants consuming only metformin (Males=17, Females=17). 2. Diabetic participants consuming metformin in combination with other drugs (Males=25, Females=34), and 3. Diabetic participants on insulin treatment in combination with other drugs (Males=3, Females=4).

4.6.3 Depending on Fasting Blood Glucose Levels:

The detailed information about fasting blood glucose (FBG) levels of diabetic participants was noted. The diabetic individuals were grouped into following groups depending on FBG levels: 1. Normal FBG levels (Glucose < 126 mg/dl), (Males=25, Females=36) 2. High FBG levels (Glucose \( \geq \) 126 mg/dl), (Males=20, Females=19).

4.6.4 Depending on Disease Duration:

In the present study, diabetic individuals were grouped, based on disease duration: Group I individuals had diabetes for \( \geq \) 0 years and \( \leq \) 3 years (Males=26, Females=35); Group II individuals were with diabetes for >3 years and \( \leq \) 7 years (Males=10, Females=11) While Group III had the participants which had diabetes for more than 7 years (Males=9, Females=9).

4.7 Statistical Analysis:

Values are presented as mean ± standard error (SE). The data were analyzed by using SPSS/PC+ statistical package (Version 20.0, Chicago, IL). The data were checked for normal distribution by testing for skewness. Skewed variables were transformed to normality using log to the base 10 transformation. Two tailed unpaired t-test was used to compare means of different parameters and mean values of various parameters from the high FBG group were compared with those of the normal FBG
group and considered significant if $P \leq 0.05$. In addition, mean values of various parameters from the disease duration groups were compared using Fischer’s least significance difference test and considered significant if $P \leq 0.05$. Correlation analysis was done using Pearson correlation coefficients to correlate HOMA-IR, FBG levels and duration of disease with biochemical parameters. Correlation was considered significant if $P \leq 0.05$ (2-tailed).

II. ANIMAL STUDY:

As per the literature, altered lipid profile was associated with T2DM. Diabetic dyslipidemia is a one of the risk factor for many diseases which causes 61% mortality in diabetic people. So there is urgent need for alternative/complimentary approaches such as supplementation of omega-3 fatty acids. There is strong scientific evidence demonstrating anti-inflammatory, anti-atherogenic, vasodilatory and lipid lowering properties of omega-3 fatty acids. The present animal study was designed to examine the comparative effects of metformin and omega-3 fatty acids on serum biochemical markers, expression of transcription factors and genes involved in lipid metabolism and inflammation in nicotinamide (NIC)-streptozotocin (STZ) induced diabetic rats.

4.8 Chemicals and Reagents:

Flax oil (Alvel-500) capsules were purchased from Real World Nutritional Laboratory (Pune, India) that contained 50% alpha-linolenic acid (ALA), 20% oleic acid and 12% linoleic acid. Fish oil capsules (MaxEPA) were purchased from Merck Limited (Goa, India) which contained 60% EPA and 40% DHA. Streptozotocin (STZ) and nicotinamide (NIC) were purchased from Sigma Life Sciences, USA and metformin (Glycomet-250 mg; USV Limited) was purchased from local pharmacy.

4.9 Experimental Animals:

The study was carried out as per the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines after the approval of Institutional Animal Ethics Committee (Ref. No: BVDUMC/189/2014-2015). The male albino Wistar rats weighing between 150-200g were procured from the institutional animal house and maintained under standard conditions throughout the
experimentation (temperature 25±2°C, 12-h light: 12-h dark cycle). Animals were fed with standard pellet diet (Nutrivet life science, Pune, M.S., India) and water was supplied ad libitum.

4.10 Study Design:

Animals were randomly divided in five groups (n=6). Nicotinamide (NIC)-streptozotocin (STZ) was used for diabetes induction. After confirmation of stable hyperglycemia, metformin or flax/fish oil intervention was given for 30 days. Blood sample was collected and used for various biochemical estimations. Liver tissues were used for histological analysis. The study protocol is given in Fig. 23.

Fig. 23: Study Protocol

4.11 Induction of Diabetes:

Animals were randomly assigned to five groups (n=6). Diabetes was induced by NIC-STZ. Animals were first treated with NIC (110 mg/kg body weight) in saline through intra-peritoneal (i.p) injection. After 15 minutes of NIC treatment, STZ (65 mg/kg body weight i.p) was administered in ice-cold sodium citrate buffer, pH
4.0. The fasting blood glucose levels of the rats were estimated after 48 hrs and on day 7 of STZ injection. Stable hyperglycemia was confirmed by elevated fasting blood glucose levels on day 7 after STZ injection. Animals with fasting blood glucose levels above 200 mg/dl were considered as diabetic and used for the study. After development of stable hyperglycemia, metformin or flax/fish oil treatment was given for 30 days. The dose of flax and fish oil was based on earlier studies (Devarshi et al. 2013, Jangale et al. 2013, Chavan et al. 2013). Animals were randomly assigned to one of the following groups: Group I: Control group (n=6): received feed and water normally throughout the experiment; Group II: STZ induced diabetic group (n=6): administered NIC (110 mg/kg body weight) and STZ (65 mg/kg body weight) i.p. single dose; Group III: Metformin group (n=6): administered metformin (200 mg/kg, body weight/day p.o); Group IV: Flax oil group (n=6): administered flax oil (500 mg/kg body weight/day, p.o.); Group V: Fish oil group (n=6): administered fish oil (500 mg/kg body weight/day, p.o.). After 15 days of treatment, animals were fasted overnight and blood was collected by retro-orbital puncture. Animals were sacrificed after 30 days of treatment and fasting blood was collected by cardiac puncture in the plain and EDTA vacutainers. Serum was separated from plain vacutainer by centrifugation at 2000 rpm for 15 minutes and stored at -80°C for further analysis. Blood samples in EDTA vacutainers were subjected to separation of PBMCs by density gradient centrifugation. Liver tissues were excised immediately, washed in saline, weighed and stored in 10% neutral buffered formalin for histological analysis.

4.12 Blood Biochemistry:

Serum glucose, total cholesterol, triglycerides, HDL, LDL, SGOT, SGPT, ALP and total bilirubin were estimated using commercial kits (Coral Clinical System, Goa, India). VLDL was estimated by using the formula: (triglyceride/5) (Warnick et al., 1990). Kits and procedures were same as per the human study.

4.13 Molecular Analysis:

4.13.1 Isolation of PBMCs from Whole Blood:

PBMC’s were isolated by density gradient centrifugation by layering the blood sample over Histopaque-1077 (Sigma-Aldrich, Inc., USA) as explained in 4.3.3.
4.13.2 Isolation of RNA from PBMCs:

RNA was isolated by TRIzol (Invitrogen Co., Carlsbad, CA, USA) method. The protocol for RNA extraction by TRIzol method was same as followed in human study.

4.13.3 RNA Quantification and Quality Check:

Quantification of RNA was performed by spectrophotometric method using NanoDrop spectrophotometer (ND1000, USA) as explained earlier. The integrity of the RNA was analyzed by visualizing RNA on agarose gel after electrophoresis.

4.13.4 cDNA Synthesis:

cDNA was prepared by using high-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA; Catalog No. 4368814) as per the manufacturer’s instructions. The stepwise protocol is explained in human study.

4.13.5 Quantitative Real-Time Polymerase Chain Reaction Analysis:

Standard quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the use of SYBR Green master mix (Applied Biosystems, USA) for the following genes: GAPDH, PPARγ, SREBP1, NFκβ, FAS, ACSL, MCAT and TNFα. KicqStart Primers (Sigma-Aldrich, USA) were used for gene expression studies and are listed in Table 5. The qRT-PCR was performed using the Applied Biosystems 7300 standard system as per the protocol in human study.
Table 5: List of Primers Used for Quantitative Real-Time PCR in Animal Study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Name</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GAPDH F</td>
<td>AGTTCAACGGCAGGATCAAG</td>
</tr>
<tr>
<td></td>
<td>GAPDH R</td>
<td>TACTCAGCACCAGCCTCACC</td>
</tr>
<tr>
<td>PPARγ</td>
<td>PPARγ F</td>
<td>AAGACAACAGACAATCACC</td>
</tr>
<tr>
<td></td>
<td>PPARγ R</td>
<td>CAGGGATATTATTTTGCACTGAC</td>
</tr>
<tr>
<td>SREBP1</td>
<td>SREBP1 F</td>
<td>AAACCTGAAGTGGTAGAAAAC</td>
</tr>
<tr>
<td></td>
<td>SREBP1 R</td>
<td>TTACCTCAAAGGCTGGG</td>
</tr>
<tr>
<td>NFκβ</td>
<td>NFκβ F</td>
<td>AAAAAACGGACTAGAGATTG</td>
</tr>
<tr>
<td></td>
<td>NFκβ R</td>
<td>ACATCTCTTTCTTCTCTTTC</td>
</tr>
<tr>
<td>FAS</td>
<td>FAS F</td>
<td>AAAAGGAAAAGTCTAGAGTCG</td>
</tr>
<tr>
<td></td>
<td>FAS R</td>
<td>GACACATTCTGTCTACACTACAG</td>
</tr>
<tr>
<td>ACSL</td>
<td>ACSL F</td>
<td>ACATTATAAGCAGTTGCTCC</td>
</tr>
<tr>
<td></td>
<td>ACSL R</td>
<td>GCATTACACACTCTCAACAG</td>
</tr>
<tr>
<td>MCAT</td>
<td>MCAT F</td>
<td>AAAACTCTAGGCTCAATCAAC</td>
</tr>
<tr>
<td></td>
<td>MCAT R</td>
<td>GGATGTTGTATTTATGGCCC</td>
</tr>
<tr>
<td>TNFα</td>
<td>TNFα F</td>
<td>CTCACACTCAGATCATCTTC</td>
</tr>
<tr>
<td></td>
<td>TNFα R</td>
<td>GAGAACCT GGAGATGATAAAG</td>
</tr>
</tbody>
</table>

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PPARγ: Peroxisome proliferator-activated receptor γ; SREBP1: Sterol regulatory element binding protein 1; NFκβ: Nuclear factor kappa β; FAS: Fatty acid synthase; ACSL: Long chain acyl-CoA synthetase; MCAT: Malonyl-CoA-acyl carrier protein transacylase; TNFα: Tumor necrosis factor α; F: Forward primer sequence; R: Reverse primer sequence.

4.14 Liver Histology:

Portion of the liver tissues (left medial lobe) from rats of all groups was used for the histopathological analysis. The tissues were excised immediately, washed in saline, weighed and transferred in 10% neutral-buffered formalin solution for fixation and later on processed for histopathological studies. The sections were cut on rotating microtome (Microm, HM 315), processed, and stained with hematoxylin and eosin (H&E staining) for microscopic examination. The stained tissues were observed under...
binocular microscope (Make: Olympus IX71 and camera DP71) at 20X magnifying power and photographed using Image Pro Plus (v5.1.2.59).

4.14.1 Fixation:

Fixation is the process of preserving, hardening and preventing changes in the tissues. The tissues were excised out immediately after sacrificing the animals and cut into pieces of such thickness that the fixative readily penetrated throughout the tissue to be fixed. Tissue was transferred to 10% formalin solution and allowed to remain in it till they were processed further.

4.14.2 Tissue Processing:

Tissue processing involves dehydration, clearing and infiltration of the tissue with paraffin (Mayer, 1893). The usual dehydrating agent is ethyl alcohol; acetone and isopropyl alcohol can also be used. Following dehydration, the tissue was transferred to a paraffin solvent, which is miscible with the dehydrating agent as well. These are known as clearing agents such as chloroform and xylene. Tissues were thoroughly washed by placing them under running tap water and then conveyed through a series of following solvents as per schedule for dehydration, clearing and paraffin infiltration (Table 6).

<table>
<thead>
<tr>
<th>Solvent Grades</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol 70%</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Alcohol 80%</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Alcohol 90%</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Alcohol 95%</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Absolute Isopropyl alcohol</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Acetone</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Chloroform</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Melted paraffin wax (60°C)</td>
<td>20 minutes</td>
</tr>
</tbody>
</table>

The tissues were then embedded in paraffin wax to prepare tissue blocks, which were oriented so that sections could be cut in desired plane of the tissue. Tissues were then fixed in cassette after trimming the tissue block to suitable size.
4.14.3 Section Cutting:

A smear of 5% Mayer’s egg albumin was prepared and smeared onto the slide and dried. The tissue sections of 4 µM thickness were cut with the help of spencer type rotating microtome (Make: Microm, HM 315). The tissue sections were put on slide and then sections were floated in water on slide at 55-60°C, water was drained off and slides were dried on hot plate at 50°C for 30 minutes. The sections were thus ready for staining.

4.14.4 Staining Procedure:

Reagents:

1) Mayer’s hematoxylin stain
2) Eosin stain, 2% w/v in alcohol

After fixing the sections on slides, they were stained by serially passing them through following reagents (Table 7).

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylol</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Acetone</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Alcohol 95%</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Haematoxylin stain</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Running water</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Eosin stain</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Alcohol 95% (3 changes)</td>
<td>3 minutes each</td>
</tr>
<tr>
<td>Acetone (2 changes)</td>
<td>3 minutes each</td>
</tr>
<tr>
<td>Xylol (2 changes)</td>
<td>3 minutes each</td>
</tr>
</tbody>
</table>

After passing through all the above reagents and stains, the sections were mounted on D.P.X. (Diphenyl Phthalate Xylene) and cover slip was placed. Care was taken to avoid air bubbles while mounting the tissue.
4.14.5 Observation:

The histopathological characteristics were observed for all the slides under binocular microscope (Make: Olympus IX71) at 20X magnifying power and photographs were taken by using Image Pro Plus (v 5.1.2.59) software.

4.15 Statistical Analysis:

Results were presented as mean ± standard error (SE). All the statistical analyses were performed using SPSS PC+ package (Version 20, Chicago IL). The data were checked for normal distribution by testing for skewness. Skewed variables were transformed to normality using log to the base 10 transformation. Statistical differences between means in different groups were determined using one way analysis of variance (ANOVA) followed by Boneferroni multiple comparison test. Mean values of various parameters from each group at day 15 were compared with those at day 30 using Student’s t-test. P≤0.05 was considered statistically significant.