Chapter-4

MATERIALS AND METHODS

4.1 Ethical Approval

The animal ethical committee of the Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India authorized the experimental protocol (Reference No: Dean/2017/CAEC/715). It was performed in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 86-23, revised 1996).

4.2 Drugs, chemicals, antibodies, and ELISA kits

The list of drugs and chemicals and antibodies and ELISA kits are provided in **Table 4.1.1** and **Table 4.1.2**, respectively.

Table 4.1.1: Drugs and Chemicals

S. No.	Drugs and Chemicals	Source
1.	Sitagliptin	Shanghai Yongi Biotechnology Co., Ltd Shanghai, China
2.	Metformin	Cipla Laboratories, Maharashtra, India
3.	Casein	Zuventus Healthcare Ltd
4.	Cornstarch	Naturevibe Botanicals
5.	Sucrose	Sigma-Aldrich, USA
6.	Soya bean oil	Nature Fresh, India
7.	Saturated fat	Amul, India
8.	Cellulose	Sigma-Aldrich, USA
9.	Mineral mixture	Cipla Limited, India

10.	Vitamin mixture	Cipla Limited, India
11.	Choline	Sigma-Aldrich, USA
12.	L-cystine	Sisco-Research Laboratories (SRL) Pvt. Ltd., India
13.	Fructose	SRL Pvt. Ltd., India
14.	Glucose	SRL Pvt. Ltd., India
15.	Chloroform	SD Fine Chemicals, Mumbai, India
16.	Methanol	SD Fine Chemicals, Mumbai, India
17.	Sodium chloride	Hi Media, Mumbai, India
18.	Bradford reagent	Sigma-Aldrich, USA
19.	Tris-hydrochloric acid	SD Fine Chem Ltd, Mumbai, India
20.	Pyrogallol	Sigma-Aldrich, USA
21.	Glacial-meta phosphoric acid	Hi Media, Mumbai, India
22.	Disodium EDTA	SD Fine Chem Ltd, Mumbai, India
23.	5,5'-dithio-bis-2-nitrobenzoic acid (Ellman's reagent)	Sigma-Aldrich, USA
24.	Trichloroacetic acid	Sigma-Aldrich, USA
25.	Thiobarbituric acid	Sigma-Aldrich, USA
26.	Formaldehyde	Merck Ltd., Mumbai, India
27.	Paraffin wax	Sigma-Aldrich, USA
28.	Xylene	SD Fine Chemicals, Mumbai, India
29.	Hematoxylin and Eosin (H & E) dye	Hi Media, Mumbai, India
30.	Disodium hydrogen phosphate	SD Fine Chemicals, Mumbai, India
31.	Potassium dihydrogen phosphate	SD Fine Chemicals, Mumbai, India
32.	Potassium chloride	Ranbaxy laboratories Ltd. Punjab, India
33.	RIPA buffer	Thermo Fisher Scientific, UK
34.	EDTA	SD Fine Chem Ltd, Mumbai, India
35.	EGTA	SD Fine Chem Ltd, Mumbai, India
36.	PMSF	SD Fine Chem Ltd, Mumbai, India

37.	2- mercaptoethanol	SD Fine Chem Ltd, Mumbai, India
38.	Bromophenol blue dye	Bio-Rad, USA
39.	Sodium dodecyl sulphate (SDS)	Hi Media, Mumbai, India
40.	Polyacrylamide gel	Hi Media, Mumbai, India
41.	Ammonium persulphate	Hi Media, Mumbai, India
42.	Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, USA
43.	Glycerol	Hi Media, Mumbai, India
44.	Tris-base	Sigma-Aldrich, USA
45.	Acrylamide gel	Hi Media, Mumbai, India
46.	Glycine	Hi Media, Mumbai, India
47.	Skimmed milk	Amul, India
48.	Hydrogen peroxide	Sigma-Aldrich, USA
49.	Bromophenol blue	Hi Media, Mumbai, India
50.	Protein ladder	Cell Signaling Technology (CST), USA
51.	Agarose	Sigma-Aldrich, USA
52.	Nitrocellulose membrane	Millipore, USA
53.	Tris-buffered saline	SD Fine Chem Ltd, Mumbai, India
54.	Bovine serum albumin	Sigma-Aldrich, USA
55.	Qiazol lysis reagent	Qiazen, Germany
56.	3, 3'-diaminobenzidine, tris-base, hydrogen peroxide (DAB)	Sigma-Aldrich, USA
57.	Revertaid first strand cDNA synthesis kit	Qiazen, Germany
58.	Quantifast SYBR green master mix	Qiazen, Germany
59.	Nuclease-free water	Qiazen, Germany
60.	DEPC-treated water	Qiazen, Germany
61.	5X Prime Script RT master mix	Qiazen, Germany

Table 4.1.2: Antibodies and ELISA kits

S. No.	Antibodies and ELISA Kits	Source
1.	Anti-p-AMPKα (Thr172) (p-AMPKα) antibody	CST, USA
2.	Anti-p-ACC (Ser79) (p-ACC) antibody	CST, USA
3.	Anti-β-actin antibody	CST, USA
4.	Anti-horseradish peroxidase (HRP) antibody	CST, USA
5.	Insulin ELISA kit	Thermo Fischer Scientific, USA
6.	Adiponectin ELISA kit	Abcam, UK
7.	Leptin ELISA kit	Abcam, UK
8.	IL-6 ELISA kit	Abcam, UK
9.	TNF-α ELISA kit	Abcam, UK
10.	GLP-1 ELISA kit	Raybiotech, USA
11.	MCP-1 ELISA kit	Raybiotech, USA
12.	ALT colorimetric kit	Abcam, UK
13.	AST colorimetric kit	Abcam, UK
14.	mRNA Primers	Integrated DNA Technologies, USA
15.	Uric acid colorimetric kit	Abcam, UK

4.3 Equipment and Software

The equipment and software used in the study are mentioned in **Table 4.2.1** and **Table 4.2.2**, respectively.

Table 4.2.1: Equipments

S. No.	Instruments	Source
1.	Digital electronic balance	Eutech Instruments, India
2.	Digital magnetic stirrer	IKA®, USA
3.	Digital pH meter	Eutech Instruments, India

4.	Cooling centrifuge	C-24BL, REMI Centrifuge, India
5.	Table top centrifuge	Eutech Instruments, India
6.	Digital microscope	Dewinter optical. Inc., India
7.	Electric Oven	Cintex, Mumbai, India
8.	Disposable syringes	Hindustan Syringes & Medical Devices Ltd., Faridabad, India
9.	Optical plate reader	Bio-Rad Laboratories, California, USA
10.	Water Bath Sonicator	PCI Analytics Pvt. Ltd, Mumbai, Maharashtra, India
11.	UV-Visible Spectrophotometer	Shimadzu, Japan
12.	Vortex mixer	REMI Instruments, Mumbai, India
13.	Optical plate reader	Bio-Rad Laboratories, California, USA
14.	Micropipettes	Eppendorf, Germany
15.	Glucometer	One Touch Select, UK
16.	Automated Blood analyzer	ERBA Diagnostics, Germany
17.	Deep Freezer	Thermo Fischer Scientific, USA
18.	Polytron homogenizer	Kinematica, Switzerland
19.	Refrigerator	Godrej, India
20.	Microtome	Leica, Bensheim, Germany
21.	Distilled water assembly	Saritorius, USA
22.	Rotatory shaker	Eutech Instruments, India
23.	Western blot assembly	Bio-Rad, USA
24.	Electrophoretic Transfer assembly	Bio-Rad, USA
25.	Gel-Documentation system	Bio-Rad, USA
26.	Nanodrop 1000 spectrophotometer	Thermo Fischer Scientific, USA
27.	Rotor gene Q (2plex HRM) RTPCR machine	Qiazen, Germany

Table 4.2.2: Software

S. No.	Software	Source
1.	Dewinter Biowizard	Dewinter, India
2.	GraphPad prism 7	GraphPad software, Inc., USA
3.	Image J software	Downloaded from NIH website
4.	Gene runner primer design software (version 6.5.52X64 Beta)	Gene runner.net

4.4 Animal husbandry

The animal house of BHU, Varanasi, India, supplied the forty-two male Swiss albino mice, six weeks old with body weight 25 ± 2.0 g. The mice were kept in polypropylene cages in the animal house quarantine facility of Department of Pharmaceutical Engineering and Technology, IIT (BHU) and before the onset of the experiment, they were acclimatized for ten days with standard climatic conditions (humidity: 55% - 60%, room temperature (RT): 22 ± 2˚C, 12/12h light and dark phases) with *ad libitum* food and water entitlements.

4.5 Experimental designs

4.5.1 Experimental design for the selection of obese and insulin-resistant animals

The experimental design for the selection of obese and insulin-resistant animals is represented in **Fig. 4.1**.

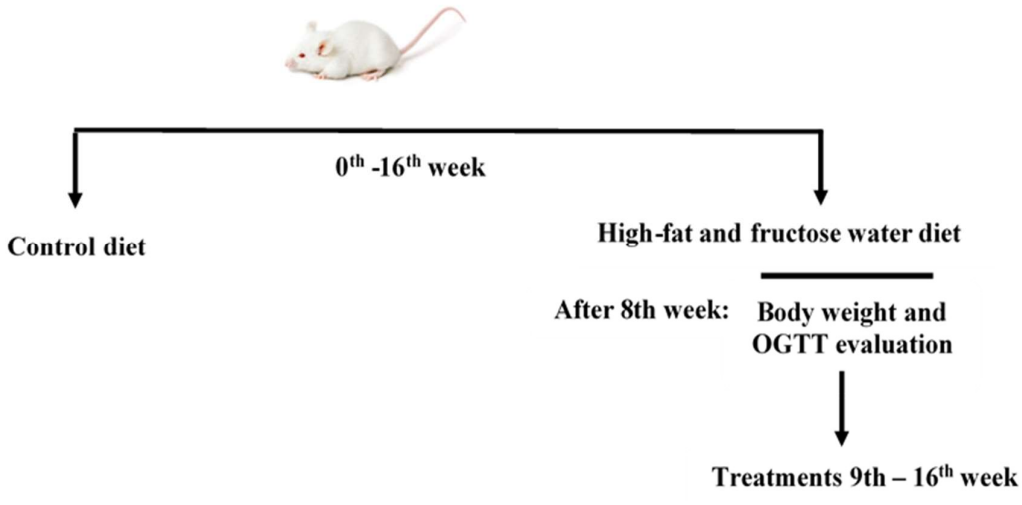

Figure 4.1: Experimental design for obese and insulin-resistant animal selection

4.5.2 Experimental design for Objective 1

The experimental design to study the effect of sitagliptin on metabolic syndrome, fatty liver, and hepatic oxidative stress in a high-fat diet-induced animal model of obesity is represented in **Fig. 4.2**.

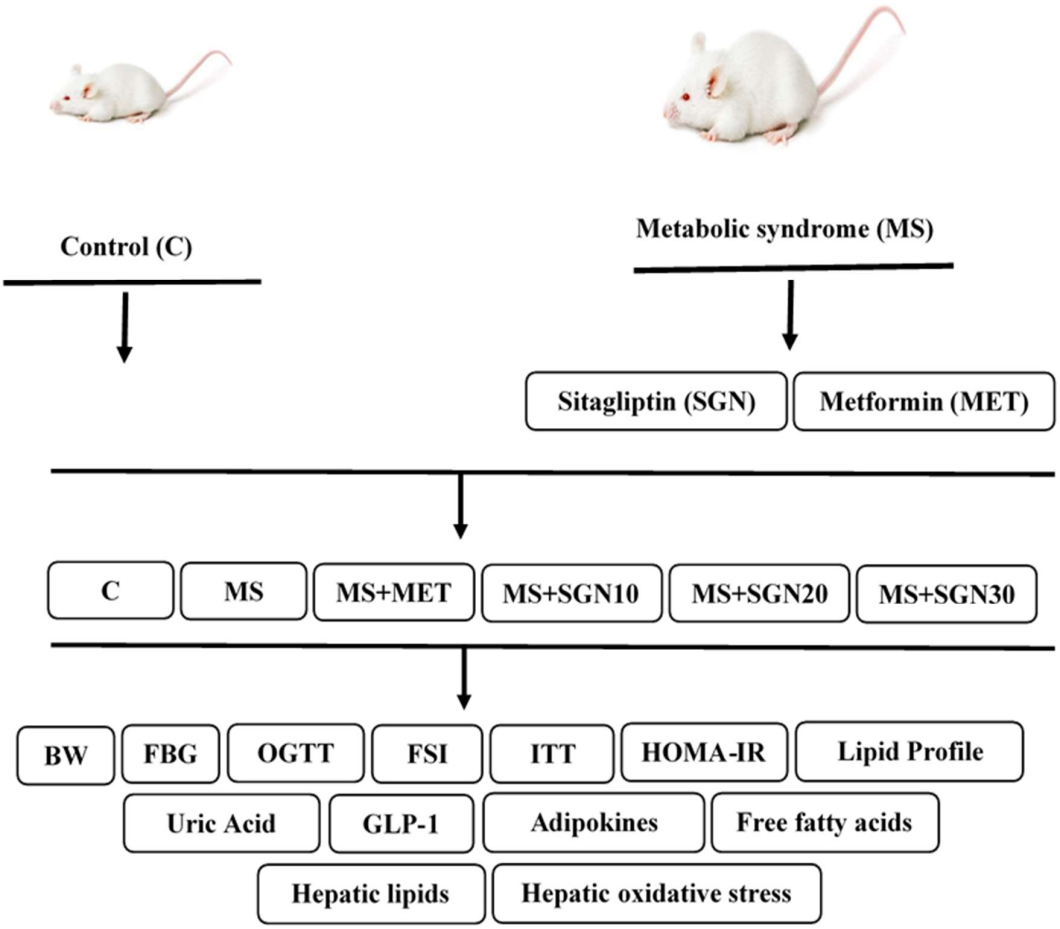

Figure 4.2: Experimental design for Objective 1

4.5.3 Experimental design for Objective 2

The experimental design to analyze the potency of sitagliptin on white adipose tissue inflammation, adiponectin expression and hepatic fatty acid metabolism in experimentally induced obese mice with a focus on AMPK signaling is represented in **Fig. 4.3**.

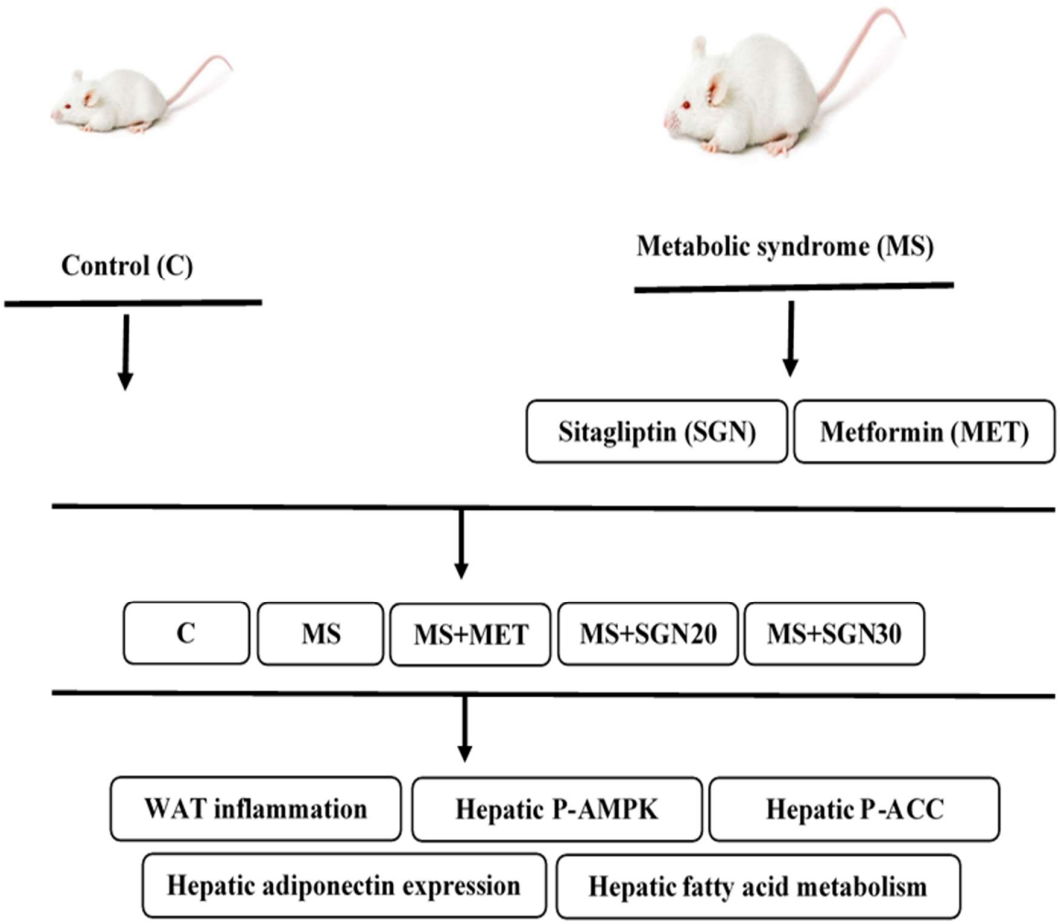

Figure 4.3: Experimental design for Objective 2

4.5.4 Experimental design for Objective 3

The experimental design to investigate the effect of sitagliptin on oxidative stress and mitochondrial biogenesis markers in white and brown adipose tissues in metabolically compromised mice is represented in **Fig. 4.4**.

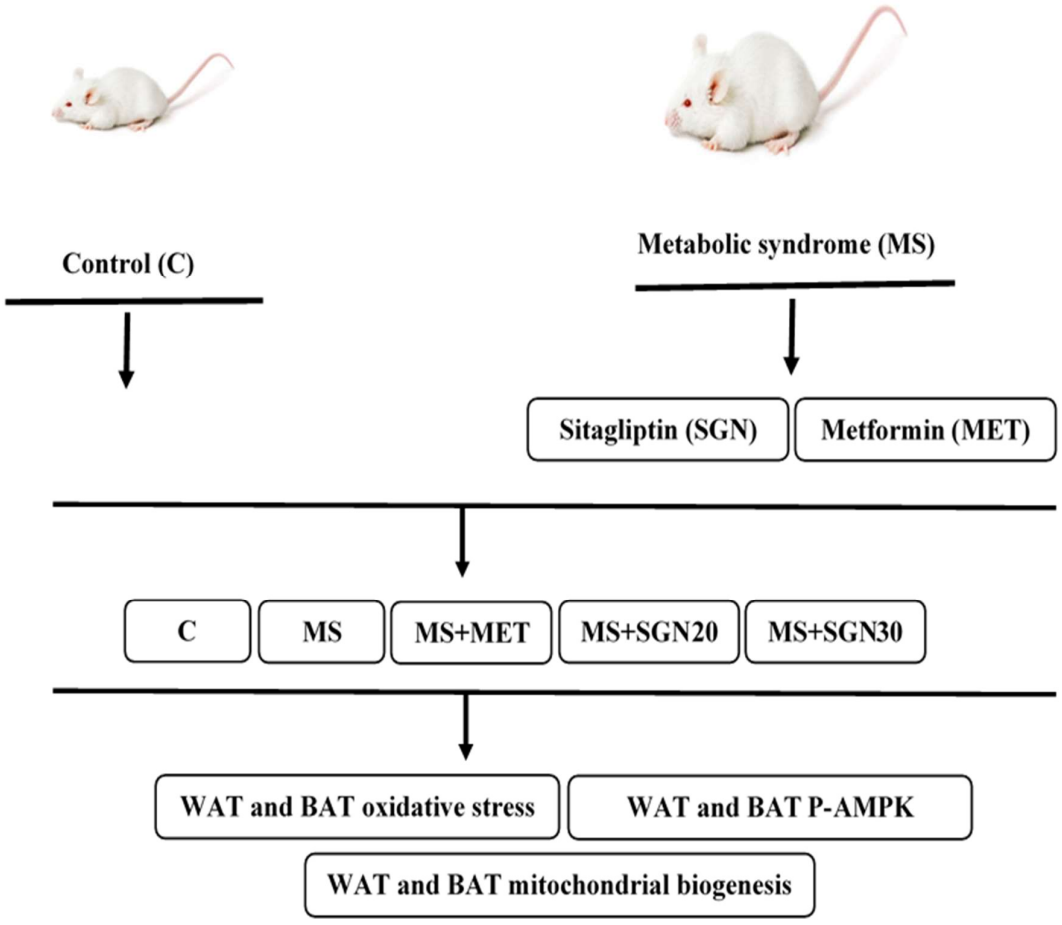

Figure 4.4: Experimental design for Objective 3

4.6 Methods

4.6.1 Preparation of Control diet and High-fat diet

Control diet and high-fat diet were freshly prepared in-house each week and were stored in the refrigerator until use. They are listed in **Table 4.3.1**. Control diet had 10% fat, 23% protein, and 67% carbohydrate with an energy content of only 3.4kcal/g. The high-fat diet had 60% fat, 20% protein, and 20% carbohydrate with an energy content of 5kcal/g. The animals receiving high-fat diet were provided 20% fructose solution in the drinking water, whereas the animals in the control group received regular drinking water [Reuter 2007; Saravanan et al. 2014; Morakinyo et al. 2015]

Table 4.3.1: Composition of experimental diets

S.No.	Ingredients	Control diet		High-Fat diet	
		Mass (g)	Energy (Kcal)	Mass (g)	Energy (Kcal)
1.	Casein	270	958.5	330	1171.5
2.	Cornstarch	500	1830.5	150	549.2
3.	Sucrose	100	400	90	360
4.	Soya bean oil	30	270	30	270
5.	Saturated fat	---	---	300	2700
6.	Cellulose	50		50	
7.	Mineral mixture	35		35	
8.	Vitamin mixture	10		10	
9.	Choline	2		2	
10.	L-cystine	3		3	
	Total	1000	3,459	1000	5072.9

4.6.2 Induction of obesity and metabolic syndrome in mice

After ten days of adaption period, mice were impartially segregated in two groups, one group received control diet and normal drinking water (without fructose) (n=6) and other group received 60% high-fat diet and 20% fructose drinking water (HFFW) (n=36) *ad libitum* for eight weeks. After eight weeks (56 days), body weight and oral glucose tolerance test (OGTT) (section 4.6.6.1) were observed (Fig. 4.1). Only those animals with a body weight between 11-15% of original and postprandial blood glucose levels between 12mmol/l to 15mmol/l were considered for the study. The respective diets were continued till the sixteenth week.

4.6.3 Experimental groups

Animals having significantly increased body weight, hyperglycemia, and decreased insulin sensitivity were segregated into five groups (n=6 each) and were randomly allocated into the following groups:

Table 4.3.2: Experimental groups

Groups		Diet	Treatment
Group I	C	Control diet and normal drinking water	Distilled water
Group II	MS	HFFW	Distilled water
Group III	MET	HFFW	Metformin 100mg/kg
Group IV	MS+SGN10	HFFW	Sitagliptin 10mg/kg
Group V	MS+SGN20	HFFW	Sitagliptin 20mg/kg
Group VI	MS+SGN30	HFFW	Sitagliptin 30mg/kg

4.6.4 Serum and tissue preparation

When the experimental protocol was finished, mice were euthanized. The blood was acquired by cardiac puncture and serum was obtained by centrifuging at 4000 rpm, 4˚C for 20 min (C-24BL, REMI Centrifuge, India). The organs of interest, i.e., liver, visceral adipose tissues: omental white adipose tissue, epididymal white adipose tissue (eWAT), retroperitoneal white adipose tissue, and intercapsular brown adipose tissues (iBAT) were removed and weighed. They were further washed with cold saline and preserved at -80˚C till subsequent studies.

4.6.5 Estimation of body weight, organ weight, and cumulative food intake

Body weight was monitored weekly, and food intake daily during the experimental period. The liver weight index and visceral fat index were estimated from the weight of the respective organs. For estimation of food intake, the animals were fed respective diets sufficient for three days, and thereafter, on the fourth day, the leftover diet was measured and subtracted from the fed diet. The cumulative food intake was measured over the entire period by cumulating the food consumed by the animals [Morakinyo et al. 2015; Zhong et al. 2017].

4.6.6 Biochemical estimations

4.6.6.1 Estimation of Fasting blood glucose (FBG) and OGTT

FBG was measured every week at 9 a.m. after overnight fasting, throughout the experimental period. It was estimated by collecting blood samples from tail vein by the tail-prick method through simple glucometer (One-touch Select) where one drop of blood was placed on the glucose strips, which gave accurate glucose levels. At the end of eight weeks and sixteen weeks, OGTT was performed after overnight starvation. Initially,

blood glucose level was estimated at 0 hr (baseline level) followed by oral administration of glucose load to the animals at 2 g/kg body weight and thereafter blood glucose levels were measured at 30, 60, 90, and 120 min [Morakinyo et al. 2015; Kosuru et al. 2017].

4.6.6.2 Estimation of Insulin tolerance test (ITT), fasting serum insulin (FSI) and Homeostasis model of insulin resistance (HOMA-IR)

Insulin tolerance test (ITT) estimation was performed at the end of the experimental protocol, where the animals were fasted overnight, and the next morning, baseline blood glucose levels were first estimated at 0 min. Thereafter, insulin solution (1 IU/kg body weight) was given to the animals through the intraperitoneal route. The blood glucose levels were then measured at 30, 60, 90, and 120 min. The FSI levels were measured by mouse insulin ELISA kit (Thermo Fischer Scientific, USA), with the optical plate reader (Bio-Rad Laboratories, California, USA) and performed according to the manufacturer's instructions. The estimation of insulin resistance was done as per the homeostasis model of insulin resistance (HOMA-IR) [Matthews et al. 1985].

$$HOMA - IR = \frac{\left\{\text{FBG}\ \left(\frac{\text{mmol}}{\text{L}}\right) * \text{FSI}\ \left(\frac{\text{mIU}}{\text{L}}\right)\right\}}{22.5}$$

4.6.6.3 Estimation of serum lipid profile, ALT, and AST levels

The diagnostic kits from Siemens, India were used for the estimation of triglyceride (TG), total cholesterol (TC), and high-density lipoprotein (HDL-C) in serum by the autoanalyzer (ERBA diagnostics, Germany). Low-density lipoprotein cholesterol (LDL-C) was derived from the formula: TC-[(TG/5) + HDL] and very low-density lipoprotein cholesterol (VLDL-C) was derived according to the formula: TG/5

[Friedewald et al. 1972; Kosuru et al. 2017]. ALT and AST estimation were done from the commercial colorimetric assay kits: sensitivity-10 mU/well, (Abcam, UK).

4.6.6.4 Estimation of serum leptin and adiponectin

Leptin level was measured in serum by the ELISA kit (Abcam, UK) with the optical plate reader at 450nm (sensitivity: <4pg/ml) as directed by the manufacturer's instructions. Adiponectin level was measured in serum with sandwich assay type mouse adiponectin ELISA kit (Abcam, UK) following the manufacturer's directions. The absorbance of samples were taken through the optical plate reader [Bio-Rad Laboratories, California (USA)] (sensitivity: 0.62ng/ml) at 450nm.

4.6.6.5 Estimation of serum free fatty acids, uric acid, and GLP-1 level

Serum free fatty acid and uric acid (sensitivity: >2 μM) levels were measured with the colorimetric assay kit (Abcam, UK), following the guidelines of the manufacturer. GLP-1 level was measured from the ELISA kit (Raybiotech, USA) by the optical plate reader (Bio-Rad Laboratories, California, USA) according to the manufacturer's protocol (sensitivity: >1.17 pg/ml).

4.6.7 Estimation of hepatic lipid and hepatic oxidative stress

The hepatic lipid content was measured according to Folch's method [Folch et al. 1957]. The liver tissues collected from the mice were first homogenized in the extraction solution of chloroform: methanol (in the ratio of 2:1 v/v) followed by centrifugation and filtration. Thereafter, a biphasic system was made by adding distilled water to the filtrate and performing centrifugation at 3000 rpm, 4˚C, 10 min. The lipid content estimation was done by gravimetric analysis. For the estimation of oxidative stress, liver tissues were homogenized in 0.9% NaCl, followed by centrifugation at 10,000 rpm, 4˚C, 10 min. The

clear supernatants were obtained after filtration. The total protein content was measured through the Bradford assay. Lipid membrane damage was evaluated by measuring malondialdehyde (MDA) levels [Ohkawa et al. 1979]. The level of antioxidants was determined by measuring superoxide dismutase (SOD) and glutathione (GSH) in the tissues [Saha et al. 2010; Kushwaha et al. 2015].

4.6.8 H & E staining

The most widely used technique for evaluating the histopathological changes is H & E staining of tissue sections [Feldman et al. 2014]. The following methodology was adopted in visualizing the histopathological changes in the hepatic tissues, eWAT, and iBAT.

Procedure

i. **Fixation:** The tissues of interest were initially fixed in freshly prepared 10 % formalin solution, as mentioned in **Table 4.3.3.** They were then impregnated with paraffin wax.

ii. **Microtomy:** 4-5µm thick sections of paraffin immersed tissues were cut with a microtome and adhered to the slides.

iii. **Dewaxing and hydration:** For deparaffinizing, the tissue sections were given the following treatments. They were kept in xylene for 5-7 min. They were then hydrated by passing through decreasing concentration of alcohol and water as following:

- 100 % alcohol for 5 min – twice
- 95 % alcohol for 5 min
- 70 % ethanol for 5 min
- DW – 5 min

iv. **Staining:** The sections were then stained with hematoxylin (depending on the intensity).

v. Following treatments were given to the sections in case of over-staining:

- Rinse with DW – 5 min
- 10 % acetic acid in 95 % ethanol – 1 min
- Rinse with DW – 5 min
- Wash with tap water – 3 times

vi. **Counterstain:** Treated with eosin for 40-45 sec, followed by rinsing with DW for 5 min.

vii. **Dehydration:**

- 70 % alcohol for 30 sec
- 95 % alcohol for 30 sec - twice
- 100 % ethanol for 1 min - twice
- Xylene for 5 min - thrice

viii. The sections were covered with a cover slip and left for overnight drying at RT. It was then viewed under a microscope (Dewinter Olympus).

Table 4.3.3: 10 % Formalin solution

S. No.	Components	Volume/weight
1.	Formalin (37%)	100 mL
2.	Monobasic sodium phosphate	4 g
3.	Sodium phosphate dibasic	6.5 g
4.	Distilled water (DW)	Upto 900 mL

4.6.9 Estimation of IL-6, TNF-α, and MCP-1 levels in eWAT

eWAT was homogenized in ice-cold phosphate buffer saline (PBS), and the supernatant was obtained by centrifuging the homogenate at 10,000 rpm, 4°C, 10 min [Li

et al. 2018]. PBS was prepared, as mentioned in **Table 4.3.4**. IL-6, TNF-α, and MCP-1 levels were estimated by the optical plate reader (Bio-Rad Laboratories, California, USA) with the respective ELISA kits following the guidelines of the manufacturer.

Table 4.3.4: PBS composition

S. No.	Components	Weight (g)
1.	Sodium chloride	8.0
2.	Potassium Chloride	0.2
3.	Sodium hydrogen Phosphate	1.44
4.	Potassium dihydrogen phosphate	0.24
5.	DW	Upto 1000 ml

4.6.10 Estimation of oxidative stress in eWAT and iBAT

The eWAT and iBAT homogenates were prepared in 0.9% NaCl with centrifugation at 10,000 rpm, 4˚C for 10 min. It was then filtered, where clear supernatants were used for the enzyme analysis. MDA, SOD, and GSH levels were estimated following the earlier methods as discussed in section 4.6.7. The total protein content in the tissue was evaluated through the Bradford assay.

4.7 Western blotting

I. Preparation of lysate from tissues

i. **Homogenization of tissue samples:** 300±20 mg of hepatic tissue, eWAT, and iBAT were dissected with ethanol wiped tools on ice. They were then weighed and placed separately in eppendorf tubes.

ii. An appropriate volume of ice-cold lysis buffer containing RIPA buffer, EDTA, EGTA, PMSF, and the cocktail was added to the eppendorf tubes followed by

homogenization with the manual homogenizer. The volume of lysis buffer was determined according to the weight of the tissue samples. The protein extract should not be too dilute to avoid loss of protein or large quantities of samples to be loaded on gels.

iii. The homogenized tissue samples were incubated at 4°C for 2 hr. It was then sonicated for 15-20 s pulse for 4 min, with 1 min gap after each pulse. Thereafter, the samples were centrifuged at 15000 rpm, 4˚C, 20 min followed by supernatant collection.

II. Determination of protein content

i. The protein content was estimated by the Bradford assay. Blank, test, and standard samples were prepared accordingly (**Table 4.4.1**). The standard curve was plotted by taking absorbance at 595 nm.

ii. The protein content was obtained with the equation y = mx + c (y-absorbance; m-slope; x-protein conc. and c-constant).

III. Sample preparation

i. 40 µg of each sample was taken in a fresh eppendorf tube, and volume make up was done upto 40 µL with RIPA buffer. 8 µL of laemmli buffer (sample loading dye) was added with a final volume of 48 µL.

ii. The composition of the laemmli buffer is mentioned in **Table 4.4.2**.

iii. The samples were subjected to denaturation by heating in a water bath at 95° C for 5 min.

IV. Loading of samples and Running of gel

i. The 8 % resolving gel was first prepared according to the composition listed in **Table 4.4.4**. It was poured in between the plates and allowed to stand for some time. To ensure no air bubble was present, water was poured above the gel. The gel was left for solidification.

ii. The stacking gel was then prepared according to the composition listed in **Table 4.4.5.** It was also similarly poured between the plates as resolving gel, with combs placed immediately and left for solidification. The comb was removed after solidification.

iii. The prepared protein samples were loaded in the respective wells. The protein ladder was loaded in the first column.

iv. The running buffer was prepared with the composition mentioned in **Table 4.4.6**, and poured in the tank.

v. Initially, it was run at 50 V for 35 min, till the protein sample was running in the stacking gel, and thereafter when the protein sample moved to the resolving gel, it was adjusted to 100 V for 70 min.

V. Blotting and Blocking of membrane

i. Every component of transfer assembly was soaked in transfer buffer and put in the following order along with the gel: pad-whatman filter paper-gel-nitrocellulose membrane (NC)-whatman filter paper-pad.

ii. The transfer buffer was prepared with the components listed in **Table 4.4.7** and poured in the tank. The voltage was adjusted to 350 mA for 70 min.

iii. After 70 min, the NC membrane was removed and washed with Tris-buffer saline (TBS) (**Table 4.4.8**).

iv. The NC membrane was blocked in the blocking buffer (**Table 4.4.9**) for 2 hr on a rotary shaker, and further washing was done in tris-buffered saline (TBST) solution thrice for 10-15 min each.

VI. Antibody incubation

i. The primary antibody solution was prepared in 20 ml of 5 % BSA (at appropriate dilution as recommended in the product datasheet). The NC membrane was incubated with specific primary antibodies by keeping it with gentle agitation overnight at 4°C.

ii. Subsequently, the primary antibody solution was discarded, and the membrane was washed thrice with TBST for 10 min each on the rotatory shaker.

iii. The secondary antibody (anti-rabbit HRP) solution was prepared in TBST (1:5000). It was poured over the NC membrane and shaken for 1 hr on a rotatory shaker at RT. Further washing of the NC membrane was done with TBST thrice for 10 min each.

VII. Membrane development and Signal detection

i. DAB solution was prepared, as mentioned in **Table 4.4.10.** It was kept in contact with the NC membrane for 2-3 min in the dark. The solution was then removed, and DW was poured over the NC membrane.

ii. The membrane was subjected to imaging by the Gel-Documentation imaging system. The bands obtained were analyzed by densitometry by Image J software (NIH, USA).

Table 4.4.1: Standard dilution of BSA

S. No.	Protein concentration (nmol/well)	BSA (µL)	Assay buffer (µL)
1.	0	0	60
2.	2	6	54
3.	4	12	48
4.	6	18	42
5.	8	24	36
6.	10	30	30

Table 4.4.2: Composition of Laemmli buffer

S. No.	Components	Weight/Volume
1.	SDS	1.2 g
2.	2- mercaptoethanol	1.8 ml
3.	Glycerol	4.7 ml
4.	Bromophenol blue	0.72 mg
5.	Tris- HCl (pH-6.8)	0.375 M

Table 4.4.3: Composition of Tris-Base (pH-7.6)

S. No.	Components	Weight/Volume
1.	Tris base	0.6057 g
2.	DW	100 ml

Table 4.4.4: Composition of SDS PAGE Resolving gel (pH-8.8)

S. No.	Components	Volume (6X)
1.	DW	2.28 ml
2.	Polyacrylamide	2.04 ml

3.	1.5 M Tris base	1.56 ml
4.	APS (10X)	60 µL
5.	SDS (10X)	50 µL
6.	TEMED	5 µL

Table 4.4.5: Composition of Stacking gel (pH-6.8)

S. No.	Components	Volume (6X)
1.	DW	2.1 ml
2.	Acrylamide (4 %)	0.5 ml
3.	1 M Tris base	0.38 ml
4.	APS	30 µL
5.	SDS	30 µL
6.	TEMED	3 µL

Table 4.4.6: Composition of Running buffer (pH-8.3)

S. No.	Components	Weight/Volume
1.	25 mM Tris base	3.025 g
2.	190 mM Glycine	14.25 g
3.	0.1 % SDS	5 g
4.	DW	Upto 1000 ml

Table 4.4.7: Composition of Transfer buffer (pH-8.3)

S. No.	Components	Weight/Volume
1.	25 mM Tris base	3.025 g
2.	190 mM Glycine	14.25 g
3.	Methanol	200 ml
4.	DW	Upto 1000 ml

Table 4.4.8: Composition of Tris buffered saline (TBS)

S. No.	Components	Weight/Volume
1.	Tris base	6.05 g
2.	NaCl	8.76 g
3.	DW	1000 ml
4.	HCl (for adjusting pH upto 7.5)	

Table 4.4.9: Blocking buffer

S. No.	Components	Weight/Volume
1.	Skimmed milk	1 g
2.	TBST	20 ml

Table 4.4.10: Composition of DAB solution (pH-7.5)

S. No.	Components	Weight/Volume
1.	DAB	7-8 mg
2.	Tris base	13 ml (pH-7.6)
3.	Hydrogen peroxide	13 µL
4.	HCl (for adjusting pH)	Quantity sufficient

4.8 Reverse transcriptase-Polymerase Chain reaction (RT-PCR)

Two-step RT-PCR was performed. The primers used in the study were designed using the gene runner primer design software (version 6.5.52) and obtained from Integrated DNA Technologies (IDT), USA. They are listed in **Table 4.5.1**. The conditions followed for designing the primers were as following:

i. The primer length between 18-20 nucleotide

ii. GC content of the primers ≤ 60%

iii. Location of forward and reverse primers < 200 base pair

iv. Melting temperature (Tm) around 60°C

All tubes and micro-pipette tips used in the protocol were treated with DEPC water before the start of the experiment to avoid RNase contamination.

Table 4.5.1: List of Primers with forward and reverse sequence

Target Gene	Gene Bank Accession no.	Amplicon	Primer sequence	
ADIPOQ	U49915.1	151	Forward	CAGGAAAAGAATGTGGACCAG
			Reverse	AGAGAAGAAAGCCAGTAAATG
CPT-1A	AF017174.2	169	Forward	GACAGGCATTTTCTTCTTCC
			Reverse	AGGCAGTGACGTTTGGAAGC
FASN	BC046513.1	89	Forward	GCTCTCTTTCTTCTTCGA
			Reverse	GGTAGGCATTCTGTAGTG
PPARα	BC016892.1	179	Forward	AAGACTACCTGCTACCGA
			Reverse	ACAGACCGCTCAGACTTC
PPARGC1-A	BC066868.1	98	Forward	GCAGCCACTCCACCAAGAAA
			Reverse	AGTCTTCCTTTCCTCGTGTC
NRF-1	AF098077.1	97	Forward	ACCCAGGCATTACGGACCAT
			Reverse	GCCTGAGTTTGTGTTTGCTG
TFAM	BC001987.1	100	Forward	CTGAAGTTGGACGAAGTGAT
			Reverse	GCCTAATCCCAATGACAAC
UCP-1	BC012701.1	151	Forward	GATCTCAGCCGGCTTAATG
			Reverse	CATTAAGCCGGCTGAGATC
ACTB	BC138614.1	135	Forward	TTACTGCTCTGGCTCCTAGCAC
			Reverse	ACTCCTGCTTGCTGATCCAC

I. RNA isolation

i. **Homogenization of tissue samples:** 100 mg of liver, eWAT, and iBAT were weighed and homogenized separately and completely in Qiazol lysis reagent at RT with manual homogenizer following the manufacturer's instructions.

ii. The homogenates were left at RT for 5 min and then centrifuged at 12,000 X g rpm, 4°C, 10 min. The pellets were obtained, which consisted of polysaccharides, extracellular matrix, and high molecular weight DNA, while the supernatant contained RNA.

iii. The centrifuge tubes containing the homogenates were kept undisturbed for 5 min at RT.

iv. The supernatant containing RNA was separated out into a new centrifuge tube.

v. **Phase separation:** 0.2 ml of chloroform per 1 ml of Qiazol lysis reagent was added to the separated RNA and vortexed for 15 sec.

vi. The mixture was kept for 5 min and again centrifuged at 12,000, 4°C, 5 min. Three layers were separated, one upper colorless aqueous phase, white interphase and, the third lower red organic phase. RNA was present in the upper aqueous phase.

vii. The upper aqueous layer was then pipetted out to a new centrifuge tube carefully by angling at 45°C.

viii. **RNA isolation:** 0.5 ml of 100% isopropanol per 1 ml Qiazol lysis reagent was added to the separated RNA and mixed thoroughly by vortexing for 1 min. The tubes were again left undisturbed for 10 min.

ix. They were then centrifuged at 12, 000 x g, 4°C, 10 min. White RNA pellets were obtained at the bottom of the tube. They were aspirated, and supernatants were discarded.

x. **RNA wash:** 1 ml of 75% ethanol per 1 ml of Qiazol lysis reagent was added to the obtained RNA pellets. They were then centrifuged at 7,500 x g, 4°C, 5 min.

xi. The supernatants were removed completely, and the precipitate containing RNA pellets were briefly air-dried.

xii. **RNA resuspension:** RNA was dissolved in 20-50 µl of RNAse-free water by passing the solution up and down several times using a pipette.

xiii. It was incubated in the heat block set at 60°C for 10 min.

xiv. **RNA quantification:** The yield of RNA was determined by measuring the absorbance at 260 nm using the Nanodrop 1000 spectrophotometer (**$A_{260/280}$**).

II. Genomic DNA removal

i. The template RNA was added along with DNase I and RNase-free to RNAse-free centrifuge tube, as mentioned in **Table 4.5.2.**

ii. It was incubated at 37°C for 30 min.

iii. 1 µL of 50 mM EDTA was added to the above tube and incubated at 65°C for 10 min.

iv. The prepared RNA served as a template RNA for the enzyme reverse transcriptase.

v. Again, the yield of RNA was determined by measuring the absorbance at 260 nm using the Nanodrop 1000 spectrophotometer (**$A_{260/280}$**).

III. First strand cDNA synthesis

i. The components of cDNA synthesis were added step by step to the nuclease-free tube on ice in the order mentioned in **Table 4.5.3.** They were mixed gently and briefly centrifuged at 5, 000 rpm, 4°C, 5 min.

ii. The reaction mixture was incubated initially for 5 min at 25°C followed by 60 min at 42°C.

iii. The reaction was terminated by heating at 70°C for 5 min.

IV. Polymerase Chain reaction of First strand cDNA

i. **Primer preparation:** The primers were obtained in dry form. The tubes were spinned gently before opening. The oligonucleotides were dissolved in nuclease-free water (NFW), and a stock solution of 100 µM was prepared. From the stock solution, 10 µM working solution was prepared by dissolving NFW in the ratio of 1:10 to set the primer reactions.

ii. 10µl of PCR reaction mixture was prepared according to the components mentioned in **Table 4.5.4** in 0.5 ml PCR reaction tubes.

iii. The reaction for each sample was run in triplicate with the calibrator gene and no template control (NTC).

iv. After sample preparation, the PCR tube was centrifuged for at 1000 rpm, 1 min, RT in the table-top centrifuge machine for proper mixing of the sample.

v. The reaction was run in three steps: the first step involved cDNA denaturation for 10 sec at 95°C, the second step involved primer annealing, and third step involved primer extension for 40 sec at 60°C in Rotor gene Q (2plex HRM) RT-PCR. Signal acquiring was set for the green channel.

vi. Each reaction was run for 40-45 cycles or till the plateau phase was observed for each sample.

vii. After the protocol was completed, relative expression levels for desired genes were computed with respect to the normalizer β-actin. ΔCt values were calculated according to the formula: $\Delta Ct = [Ct_{target} - Ct_{reference}]$.

viii. Finally, $2^{-\Delta\Delta Ct}$ values with the calibrator gene gave the fold change.

Table 4.5.2: Components for genomic DNA removal from obtained RNA

S. No.	Components	Volume
1.	RNA	1 µg
2.	10X Reaction buffer with $MgCl_2$	1 µL
3.	DNase I, RNase-free	1 µL
5.	NFW	Upto 10 µL
Total		10 µL

Table 4.5.3: Components for cDNA synthesis

S. No.	Components	Volume/weight
1.	Template RNA	0.1 ng -5 µg
2.	5X Reaction buffer	4 µL
3.	RiboLock RNase Inhibitor (20 U/ µL)	1 µL
4.	10 mM dNTP mix	2 µL
5.	RevertAid RT (200 U/ µL)	1 µL
6.	NFW	Upto 12 µL
Total		20 µL

Table 4.5.4: PCR reaction mixture

S. No.	Components	Volume for tissue sample	Volume for NTC
1.	Quantifast SYBR green master mix	5 µL	5 µL

2.	cDNA	1 µL	1 µL
3.	Forward primers	1 µL	-
4.	Reverse primers	1 µL	-
5.	NFW	2 µL	4 µL
Total		10 µL	10 µL

4.9 Statistical analysis of Data

GraphPad Prism-5 software, USA, was used for statistically analyzing the results. Obtained values are demonstrated as Mean ± Standard Error of Mean (SEM). One-way ANOVA gave differences among group means. For finding statistical significance in mean among different experimental groups, Tukey's multiple comparison post hoc test was applied unless otherwise stated. Two-way ANOVA with Bonferroni post hoc test was applied to ascertain significant changes in body weight of animals.