CHAPTER 2: METHODOLOGY

2.1. CELL CULTURE CONDITIONS

Human adipose tissue derived mesenchymal stem cells

Human adipose tissue derived mesenchymal stem cells (ADSCs) were procured from ANSA and were cultured in knock out-DMEM with fetal bovine serum (10%) and Glutamine (2mM). Cells were incubated in a humidified chamber maintained at 37°C with 5% CO2. Once the cells attained 90% confluency, they were trypsinized using trypsin/EDTA solution (0.5%), incubated at 37°C for ~2 mins. Effect of trypsin was neutralized using the culture media and the detached cell suspension was centrifuged at 1500 rpm for 5 mins. The supernatant was discarded and the pellet was resuspended in the fresh culture medium. Cells were counted using a hemocytometer with trypan blue (0.4%). Number of viable cells were calculated and 5 x 10^5 cells resuspended in 0.1ml of the fresh culture media was used for injecting each animal subcutaneously/ intramuscularly.

3T3L1 Cell Culture

3T3L1 preadipocyte cells were initially grown in culture medium containing DMEM high glucose (25mM), fetal bovine serum (10%), L- Glutamine (2mM) and Pen Strep (1% -10,000 units /mL Penicillin and 10,000 μg /mL Streptomycin). Once the cells attained 70% confluency, they were trypsinized as mentioned in ADSCs culture. Cells were seeded at a specific density for each assay.

3T3L1 Cell Differentiation

When the cells were 100% confluent, they were differentiated with differentiation medium containing DMEM high glucose (25mM), fetal bovine serum (10%), L- Glutamine (2mM), Pen Strep (1% -10,000 units /mL Penicillin and 10,000 μg /mL Streptomycin), dexamethasone (1μM), IBMX (3-isobutyl-1-methylxanthine, 500μM) and Insulin (100nM).
After 48h of incubation, cells were replenished with maintenance medium which contains DMEM high glucose (25mM), fetal bovine serum (10%), L-glutamine (2mM) and Insulin (100nM). Medium change was done after 48h. Cells were cultured in culture medium for 2-5 days based on the assay requirement with media change every 48h. All the cells were incubated at 37°C with 5% CO₂.

**C2C12 Cell Culture**

C2C12 myoblast cells were grown in culture medium containing DMEM high glucose (25mM), fetal bovine serum (10%), L-Glutamine (2mM) and Pen Strep (1% -10,000 units/mL Penicillin and 10,000 μg/mL Streptomycin). Once the cells attain confluency, they were trypsinized as mentioned above and seeded at specific cell density for the assay.

**C2C12 Cell Differentiation**

C2C12 myoblast cells were differentiated into myotubes by culturing the cells in DMEM high glucose (25mM), fetal bovine serum (2%), L-Glutamine (2mM) and Pen Strep (1% -10,000 units/mL Penicillin and 10,000 μg/mL Streptomycin) till Day 7 with medium change every alternate day.

**Freezing the cells**

The cultured cells were trypsinized as mentioned earlier. Cell pellet was gently resuspended in the freezing mixture containing heat inactivated fetal bovine serum (90%) and Dimethylsulphoxide (10%). The suspension was carefully transferred into a freezing vials, previously labelled. The vials were then placed in Mr. Frosty™ freezing container and stored at -80°C for 16-24h, allowing the cooling of cells at a slower rate. The vials were then stored in a liquid nitrogen tank (-196°C) until further use.

**Reviving the cells**

Respective medium for the cells to be revived stored at 4°C was kept at 37°C water bath to thaw. Vials containing cells were taken from the liquid nitrogen tank and dipped quickly in a
37°C water bath for few seconds. Pre-warmed media is used to resuspend the frozen lump of the cells in the vial. After trituration, once the cells are in suspension; they are collected in a falcon tube containing 1 ml of medium. Cell suspension is the spun at 1000 rpm for 5 mins. Supernatant is gently discarded and the pellet resuspended in 1 ml of fresh pre-warmed medium. For a 25 cm² flask, the volume of the suspension is made upto 5 ml and the cell suspension is transferred to the flask and the suspension is spread around. The flask containing the cell suspension is the moved to a humidified incubator set at 37°C, 5%CO₂.

2.2. CHARACTERIZATION OF ADSCS

ADSCs Flow Cytometry Analysis
ADSCs were cultured as mentioned. Cells were trypsinized and spun down. Cells were then washed with cold PBS. The pellet was then dislodged and further incubated with the primary antibody *viz.* CD105, CD90, CD34 and HLA-DR for 1hr on ice and then fixed with 4% paraformaldehyde. Cells were incubated on ice. The cell suspension was spun again and washed with cold PBS. Percentage of positive and negative cells were the analysed by flow cytometry using FACS CALIBUR (BD Biosciences, San Jose, CA, USA) and data was analysed using Cell Quest Pro software (BD Biosciences, San Jose, CA, USA). 10,000 cells were acquired for each run.

Tri-lineage Differentiation of ADSCs
Cells were seeded in 6 well tissue culture plate at a density of 2 x 10⁴ cells per dish for differentiation into adipocytes, chondrocytes and osteocyte to confirm the trilineage differentiation potential of the cells. Cells differentiated with adipogenic, osteogenic and chondrogenic media induced cells to form adipocytes, osteocytes and chondrocytes respectively. Cells differentiated with adipogenic media showed matured adipocytes with oil droplets stained positive for Oil Red O. Osteocytes showed deposition of calcium matrix and
were positive for Alizarin red staining. Chondrocytes formed matrix which was positive with Alcian blue staining.

**ADSCs Conditioned Medium (ADSCs CM) preparation**

ADSCs were grown as mentioned above. After 48h of incubation, the conditioned media was collected from the same passage (Passage 3) centrifuged at 2000 rpm for 10 minutes, filtered using 0.2µm filter and stored at -80ºC until further use. 50% of the CM was prepared by diluting with the culture media. 50% of the CM was used for the treatment following Hao et al., 2014. They showed that CM derived from adipose tissue exhibits reduced glutamate-induced neuronal injury in a concentration-dependent manner, the maximum protective effect being at 50 % CM. They further state that lower than 50 % CM or at 70 % CM, protective effects were less evident. 100 % CM do not mediate any protection.

**2.3. DETAILS OF ASSAYS PERFORMED**

**2-N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) uptake assay**

*Acute treatment with TNFα: 3T3L1 preadipocyte cells and C2C12 myoblast cells were seeded at a density of 2x 10^3 cells per well in Corning® 96 Well Flat Clear Bottom Black Polystyrene TC-Treated microplatesand differentiated as previously described. For 2-NBDG uptake assay, fully differentiated 3T3L1 and C2C12 cells were treated with sodium palmitate (500µM) for 24h with and without CM. Metformin at a concentration of 1.5 mM was used as a positive control. Cells were serum and glucose starved for 2h in krebs ringer phosphate buffer (KRPH) with 0.5% BSA. Cells were then stimulated with 10ng/ml of Tumor Necrosis Factor α (TNFα) for 1h and then washed with PBS. Furthermore, cells were treated with insulin (100nM) for 15 minutes. Then, washed with PBS once and incubated with 2-NBDG dye was added at a concentration of 50µM for 20 minutes. All the incubations were carried out at 37ºC, 5% CO₂. After PBS wash, fluorescence intensity was measured at 460/540nm.*
wavelength using EnSight Multimode Plate Reader (HH34000000) and analysed using the Kaleido™ data acquisition & analysis software.

_Hypertrophied 3T3L1 cells:_ To mimic the chronic condition of insulin resistance _in vitro_, hypertrophic 3T3L1 cells were generated. 3T3L1 cells were treated with 200µM sodium palmitate from the Day 0 of differentiation along with all the components in the media mentioned above until Day 7. On Day 8 of the differentiation, cells were treated with 500µM palmitate and 1ng/ml TNFα for 24h. Cells were then assayed for 2-NBDG uptake as mentioned earlier.

_Note: Sodium Palmitate (5M stock concentration) was prepared in 50% ethanol. Further, conjugated with 10% Bovine serum albumin prepared in distilled water (5mM). Finally, used as required from the conjugated stock._

**Adipogenesis in 3T3L1**

For adipogenesis, 3T3L1 preadipocyte cells were seeded at a density of 2x 10³ cells per well in a 96 well tissue culture treated microplate. After the cells were 90-100% confluent, differentiation was carried out as mentioned earlier with and without CM until Day 7 at every stage of differentiation media change. Gemfibrozil (200µM) was used as a positive control as an inhibitor of adipogenesis. Cells were then washed with PBS and 100 µl of isopropanol was added to each well and incubated at room temperature with mild shaking for 20 mins facilitating the release of lipids from the cells to the isopropanol in the supernatant. 20 µl of the isopropanol and 200 µl of the Triglycerides FS 5' from DiaSyS, Diagnostic Systems was added into a fresh 96 well assay plate. Incubated for 15 mins at room temperature. Optical density was measured at 500nm wavelength.
Intramuscular triglyceride accumulation in C2C12 cells

Fully differentiated C2C12 cells were treated with 750µM palmitate for 48h with and without the CM. Cells were washed with PBS and triglyceride was estimated using the same procedure as mentioned for 3T3L1 cells adipogenesis.

Quantitative real-time reverse transcription-polymerase chain reaction

Fully differentiated 3T3L1 and C2C12 cells were treated with and without CM with 500µM palmitate and 1ng/ml TNFα for 48h. Cells were harvested and total RNA was extracted using TRIzol™ Reagent. 2µg of RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (4368814). Primers for the corresponding genes were procured from Sigma Aldrich. qPCR was conducted using KAPA SYBR Green/ROX qPCR master mix on a StepOnePlus™ Real-Time PCR System (4376600), using a SYBR green detection method. Comparative Ct method of \(2^{-\Delta\Delta Ct}\); where \(\Delta\Delta Ct = \Delta Ct\) (target control) minus \(\Delta Ct\) (housekeeping gene control (18s)). Final results were obtained using the StepOne™ Software and are then represented as fold change values relative to the housekeeping gene.

C2C12 cell fractionation

A rapid increase in the uptake of glucose is induced by insulin by translocating GLUT4 from cytosol to the plasma membrane (Cushman and Wardzala, 1980). It has been established that type 2 diabetes mellitus subjects have decreased insulin-stimulated glucose uptake in skeletal muscle due to the occurrence of insulin resistance (Alvim et al., 2015). To study the translocation of GLUT4 from the nucleus to the plasma membrane, fully differentiated C2C12 cells treated with and without CM were harvested and fractionated using the cell fractionation kit from Cell Signalling Technology as per the instructions provided by the manufacturer. The plasma membrane fraction and the whole cell lysate were compared by
performing western blot in order to understand the effect of CM treatment on glucose uptake via GLUT4 translocation.

**Western blotting**

Western blot for GLUT4 protein translocation was carried for the membrane fraction and the total GLUT4. Phospho Akt and total Akt western blot was performed. All the antibodies were obtained from Cell Signalling Technology. Anti β tubulin antibody was used as an internal control (BD Biosciences). Protein bands were developed with TMB Substrate Solution.

### 2.4. CARRAGEENAN INDUCED ACUTE MODEL OF INFLAMMATION

*db/db* mice was a generous gift from Connexios Life Sciences; Bangalore, India. Mice were housed in a controlled environment with research diet-and access to water *ad libitum*. Animal experiment protocols and experimental procedures were approved by the Connexios Institutional Animal Ethics Committee which are in agreement with the ARRIVE guidelines (Anil et al., 2014; Kilkenny et al., 2010). To test the anti inflammatory effect of ADSCs and its CM; mice model of acute inflammation was developed by inducing paw oedema using carrageenan. Carrageenan (2%) was prepared in PBS and injected subcutaneously to right paw of the mice using 26 gauge needle syringe for the development of inflammation. Mice were divided in four groups (n=3) for the treatment viz. PBS control, Carrageenan control, ADSCs and CM. 50 microlitres of carrageenan was injected to each animal 30 minutes after the injection of ADSCs and CM at the site of paw oedema subcutaneously.

*Note: While preparing carrageenan solution, it should be mixed homogenously without lump formation.*

**Measurement of paw volume**

Changes in the paw volume were measured using a digital plethysmometer (INCO, Nivique) which is an instrument designed to measure changes in small volumes. Immersion of the
animal paw causes water displacement in the measuring tube and the output signal displays the measured volume of displacement in millilitres. Paw volume was measured at 24h from the time of treatment.

**Enzyme Linked Immunosorbent Assay (ELISA)**

ELISA was performed to quantify the amount of protein present in the CM as well as for the serum collected from mice at different time points at the end of the study. All the ELISA was carried out as per the manufacturer’s instruction for the respective ELISA kit.

**Histopathological Examinations**

All the tissues which were investigated for histology were fixed in 10% formalin and 4μm sections were taken and stained with hematoxylin and eosin (H&E) stain. The images were captured using inverted microscope (Nikon Eclipse TE2000-5).

**Immunohistochemistry**

Tissue sections were put onto the slides. The sections were then deparaffinized and rehydrated. For antigen retrieval, slides with the tissue sections were incubated for 5 minutes in boiling citrate buffer (10mM sodium citrate, 0.05% Tween; pH 6). Then, the slides were placed in warm PBS and allowed to cool for 20 minutes. Tissue sections were then blocked for 30 minutes using 5% goat serum and 1% bovine serum albumin in PBS, washed in PBS thrice at an interval of 5 minutes each. Sections were incubated with GLUT4 Antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C in a humid chamber, washed in PBS thrice and secondary antibody conjugated to horseradish peroxidase was added, incubated for 1h at room temperature, washed as mentioned. Diaminobenzidine (DAB) substrate was added for 30 minutes and counterstained with hematoxylin. Slides were then dehydrated and mounted with DPX mountant. The images were captured at 100X magnification using inverted microscope (Nikon Eclipse TE2000-5, Japan).
2.5. DIET INDUCED OBESITY MODEL DEVELOPMENT

Six weeks old male C57BL/6 mice were procured from Vivo biosciences, Hyderabad, India. Animals were housed in polypropylene cages, maintained at 23 ± 1°C, 60 ± 10% humidity, exposed to 12 h cycles of light and dark and had access to diet and water ad libitum throughout the experimental period. After acclimatization for a week, mice were fed either on chow diet (10% Kcal fat; D12450B) or High fat diet (HFD) (60% kcal from fat; D12492) for 10 weeks. Animal experiment protocols and experimental procedures were approved by the Connexios Institutional Animal Ethics Committee which are in agreement with the ARRIVE guidelines as mentioned in section 2.11.

Treatment of mice with ADSCs in three different forms

After the mice attained the hyperglycemic state which was confirmed by fasting glucose levels; mice were randomized and grouped (n=6) based on the body weight and oral glucose tolerance test (OGTT) at the end of week 10 on high fat diet. Animals were grouped as, lean control fed on chow diet, Diet Induced Obese control (DIO), Metformin treated DIO, ADSCs Suspension (CS) treated DIO, conditioned media (CM) treated DIO and cell lysate (CL) treated DIO. Further, mice were treated with CS (5 x 10^5 cells in culture media/ animal), CM, CL (lysate from 5 x 10^5 cells in culture media/ animal). All the CS, CM and CL treatment was given in 0.1ml volume using 26G needle once in 4 weeks intramuscularly in the thigh muscle whereas metformin was used as a positive control (300 mg per kg body weight, p.o. daily).

ADSCs preconditioned with metformin treatment

Mice were randomized and grouped as lean control, DIO control, metformin, ADSCs and MetADSCs (n=4) based on their body weight and Oral Glucose Tolerance Test (OGTT). Metformin and ADSCs treatment was given as mentioned in 2.16. For preconditioning of
ADSCs with metformin (MetADSCs); 80% confluent cells were treated with metformin (1mM) for 16h, trypsinized and $5 \times 10^5$ cells were injected per animal through i.m. route once.

**2.6. DIFFERENT PARAMETERS MEASURED IN DIO MICE**

**Body weight**

Body weight was measured for all the mice once in a week from week 0 till the end of the study (Week 18). Data is represented in grams.

**Fasting Glucose measurements**

One week after the animals were injected with the respective treatment, they were kept on fasting for 6h (6 am to 12 noon) and the blood was collected from tail vein by pricking the end of the tail with a 26G needle. One drop of blood was used for the measurement of fasting glucose using Accu-check glucometer (Roche Diagnostics) every week until the end of the study.

**Oral glucose Tolerance test (OGTT)**

Animals were fasted as mentioned earlier. Oral glucose load of 2g/kg body weight was given with an oral gavage and OGTT was determined. Glucose was measured at different time points viz. 0, 30, 60, 120 mins.

**Triglyceride Measurements**

At the end of the study, animals were fasted overnight and triglyceride was measured from the tail vein blood using Accutrend Plus system (Cobas).

**Serum Preparation**

Blood was drawn from mice by retro orbital puncture. Blood collected in the microfuge tube was allowed to stand for 30 mins at room temperature. The tubes containing blood were then centrifuged at 10,000rpm for 10 mins. Supernatant was collected in a fresh tube and was stored at -80°C until further use.
**Homeostatic model assessment of Insulin Resistance (HOMA-IR)**

HOMA-IR was calculated to assess the state of Insulin resistance using the predefined formula which is (Fasting insulin [mg/dL] x Fasting glucose [mg/dL] / 405)

**Triglyceride Glucose index (TyG)**

TyG was calculated using the formula (TG [mg/dL] x glucose [mg/dL]/2)

**miRNA Analysis**

miRNA from muscle tissue was isolated using miRNeasy Mini Kit from Qiagen. After quantifying the miRNA using Nanodrop Spectrophotometer, cDNA conversion was carried out using TaqMan® MicroRNA Reverse Transcription Kit from Applied Biosystems. miR-206 gene expression was performed by using TaqMan® Universal Master Mix II from Thermo fisher scientific. Relative gene expression was calculated by normalising to the U6 snRNA levels. STEP one plus PCR machine was used from the Applied Biosystems for the analysis.

**Quantification of protein in the muscle tissue**

For the quantification of protein for tissue samples, at the end of the study mice were sacrificed. 100 mg of the muscle tissue was collected in the protein lysis buffer containing protease and phosphatase inhibitors. Protein quantification was carried out by using Bradford assay and represented as microgram per milligram of protein.

**2.7. STATISTICAL ANALYSIS**

All data presented are mean ± SEM. Treatment groups were compared using one way ANOVA (Bonferroni’s Multiple Comparison Test using GraphPad Prism software). P value ≤ 0.05 were considered as statistically significant.