CHAPTER 2

Materials and Methods

2.1 Chemicals and materials

The name of chemicals, reagents and instruments are shown in Appendix A and B, respectively. The detail of reagents and/or buffers used in this study is shown in Appendix C.

2.2 Preparation of rice extracts

Three species of purple rice, Doisaket (DSK), Nan (NAN), Phayao (PYO) and one species of unpolished white rice Korkhor 6 (RD6) were used in this study. Dichloromethane extract (DSKD, NAND, PYOD, and RD6D) and methanol extract (DSKM, NANM, PYOM, RD6M) of purple rice and unpolished rice were provided by Dr. Kanokwan Sringarm, Faculty of Agriculture, Chiang Mai University. All experiments were used dimethysulfoxide (DMSO) as vehicle control. A schematic diagram of rice extraction is shown in figure 2.1.

The fingerprint of the rice extracts were performed by high performance liquid chromatography (HPLC) in Faculty of Agriculture, Chiang Mai University. Hydrophobic component such as γ-oryzanol, tocopherol could be extracted most effectively using dichloromethane. Hydrophilic component such as anthocyanin could be found in methanol extraction.

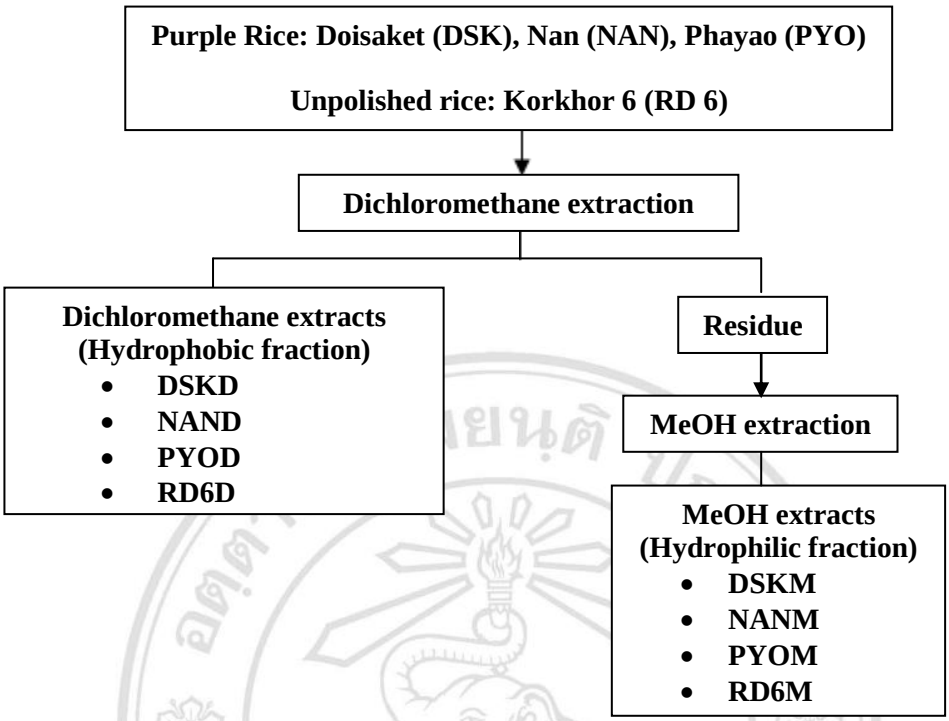

Figure 2.1 A schematic diagram of rice extraction

2.3 Experimental design

In this study (Figure 2.2), murine preadipocyte 3T3-L1 cell line was purchased from Cell Lines Service (CLS, Germany) and used to investigate anti-obesity and anti-insulin resistant effect of the rice extracts. Dichloromethane and methanol extracts of three species of purple rice: DSK, NAN and PYO were tested and compared with the RD6 unpolished rice. Cytotoxicity assay was performed to define non-toxic concentration of rice extracts on both 3T3-L1 preadipocyte and mature adipocyte. The effect of rice extracts on adipogenesis was analyzed by lipid accumulation assay. The expression of genes involved in the adipogenesis including, PPARγ and C/EBPα were examined. To induce the insulin resistance, 3T3-L1 mature adipocytes were pretreated with pro-inflammatory cytokine TNFα. The effect of the rice extracts on lipolysis and glucose uptake were investigated in these cells.

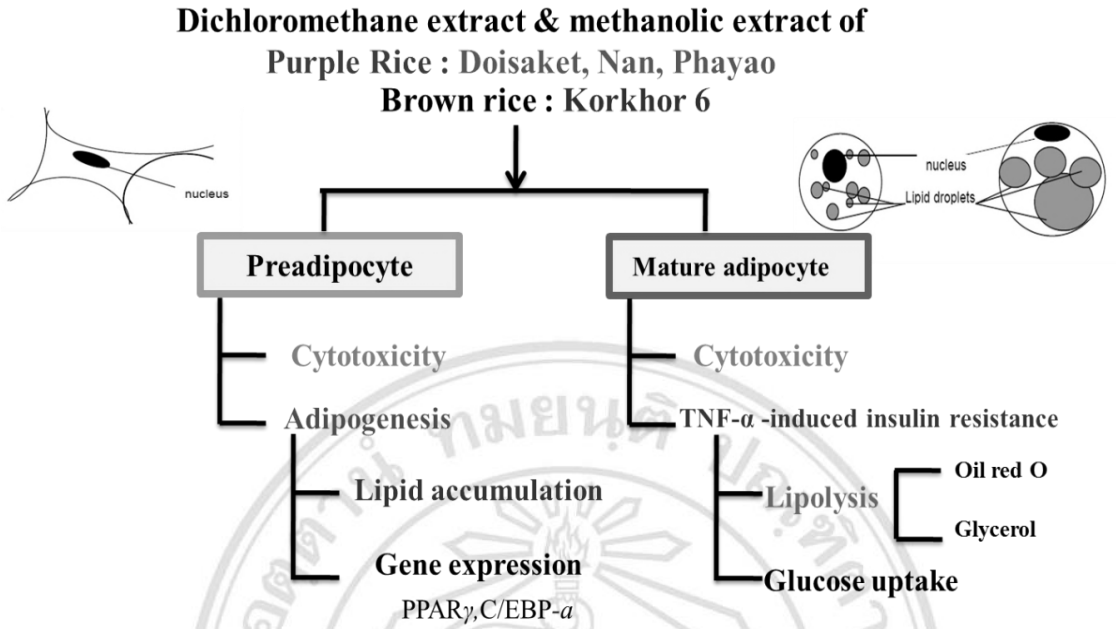

Figure 2.2 Experimental design to determine the anti-adipogenesis and anti-diabetic effect of rice extracts

2.4 Cell culture and differentiation

As shown in figure 2.3, 3T3-L1 preadipocytes were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% calf serum until 80% confluent. The cell differentiation was stimulated in DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone and 1.7 mM insulin. On day 3, the medium was replaced with DMEM supplemented with 10% FBS and 1.7 mM insulin, and further incubated for three days. The maintenance medium was changed every 2 days until the cells ready for experimentation.

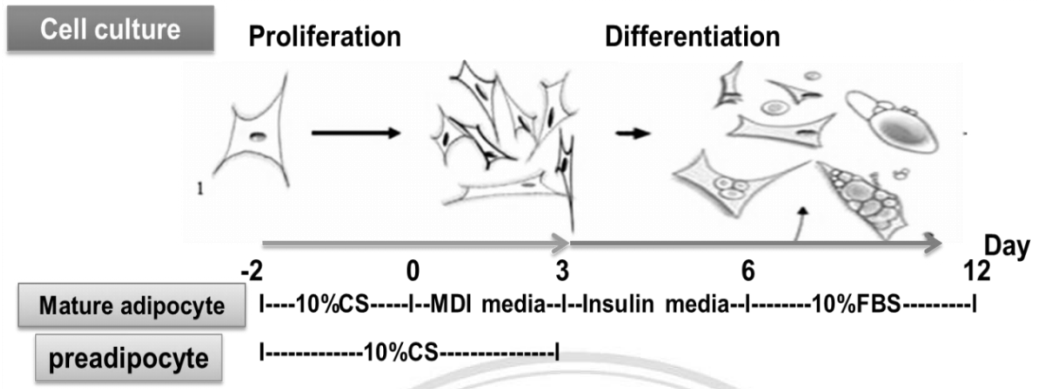

Figure 2.3 A schematic diagram of cell culture and differentiation of 3T3-L1adipocyte

2.5 Effect of rice extracts on 3T3-L1 adipocytecell viability

A treatment diagram for 3T3-L1 adipocyte cell viability assay was shown in figure 2.4. Tests were performed in 96-well plates. Preadipocytes were seeded (1×10^4 cells/well) and performed the assay 2 days later. Cells were treated with varying doses (5-200 μg/mL) of rice extracts for 72 h in 10% calf serum medium (day 0-3). For mature adipocytes, cells were seeded (5×10^3cells/well) and differentiated as described above. Cells were treated with varying doses (5-200 μg/mL) of rice extracts for 72 h in 10% FBS (day 9-12).

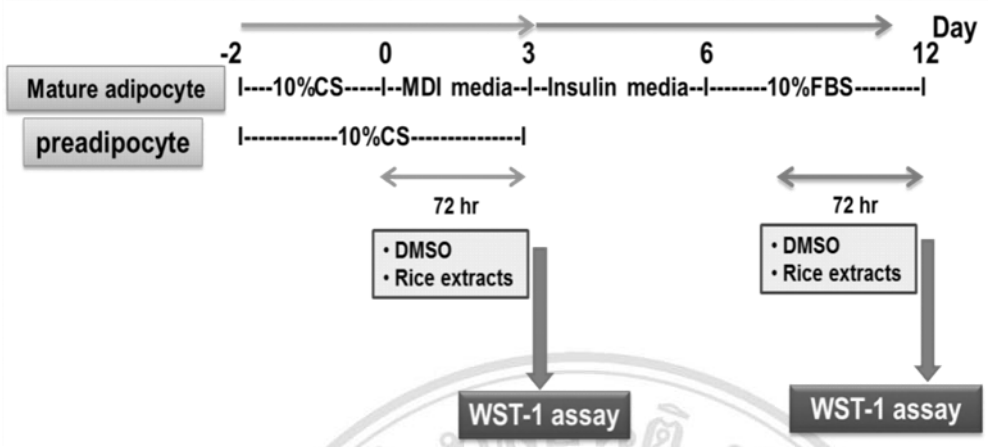

Figure 2.4 A treatment diagram for 3T3-L1 adipocyte cell viability assay

2.5.1 Cytotoxicity assay

Cell viability was quantified using WST-1 reagent according to the manufacturer's instructions (Dojindo molecular technologies, Japan). The assay principle (figure 2.5) is based on the conversion of the tetrazolium salt WST-1 into a colored dye by mitochondrial dehydrogenase enzymes. WST-1 reagents were added followed by incubation at 37 °C for 2 h. The absorbance (Optical density; OD) at 450 nm was measured using a microplate reader. Percentage of cellular viability was calculated using the following equation.

$$\% \text{ Cellular viability} = \frac{(\text{OD of treatment group} - \text{OD of blank}) \times 100}{(\text{OD of control group} - \text{OD of blank})}$$

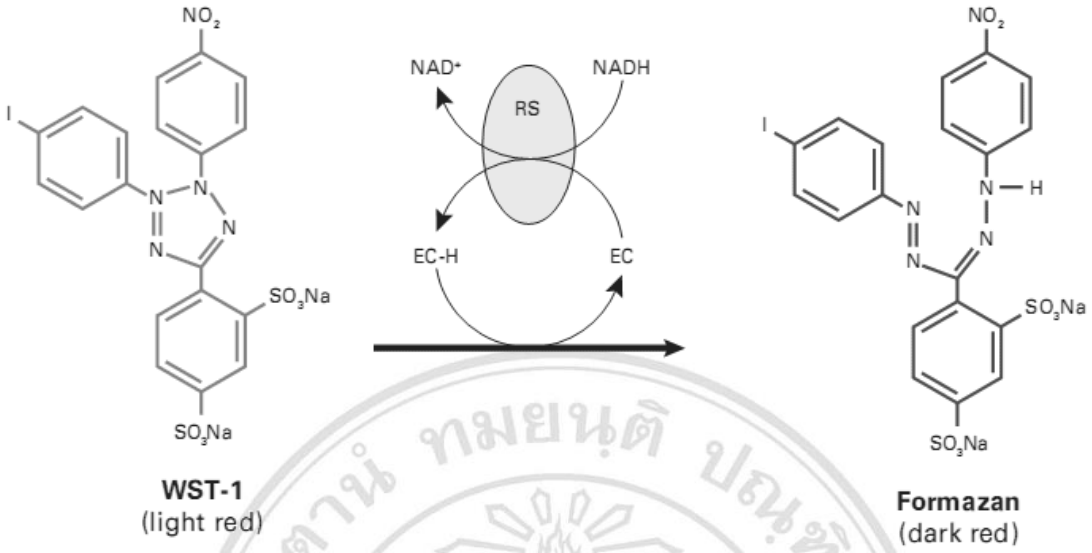

Figure 2.5 Principle of WST-1 assays (78)

2.6 Effect of rice extracts on adipogenesis of 3T3-L1 preadipocyte

2.6.1 Lipogenesis assay

According to treatment diagram (figure 2.6), tests were performed in 96-well plates. Preadipocytes were seeded (1×10^4 cells/well) and performed the assay 2 days later. Cells were treated with varying doses (5-200 μg/mL) of rice extracts for 72 h (day 0-3). All of experiment cell were cultured in 10% calf serum medium. At day 10, lipid accumulation was detected by staining with oil red O dye.

Figure 2.6 A treatment diagram for 3T3-L1 preadipocyte lipogenesis assay

2.6.2 Differentiation assay

For mature adipocytes, cells were seeded (5×10^3cells/well) and differentiated as described above. Cells were treated with varying doses (5-200 μg/mL) of rice extracts for 72 h during incubation in MDI media (day 0-3). The end of differentiation (day 10), cell were stained with Oil Red O for lipid accumulation. A treatment diagram for 3T3-L1 differentiation assay was shown in figure 2.7.

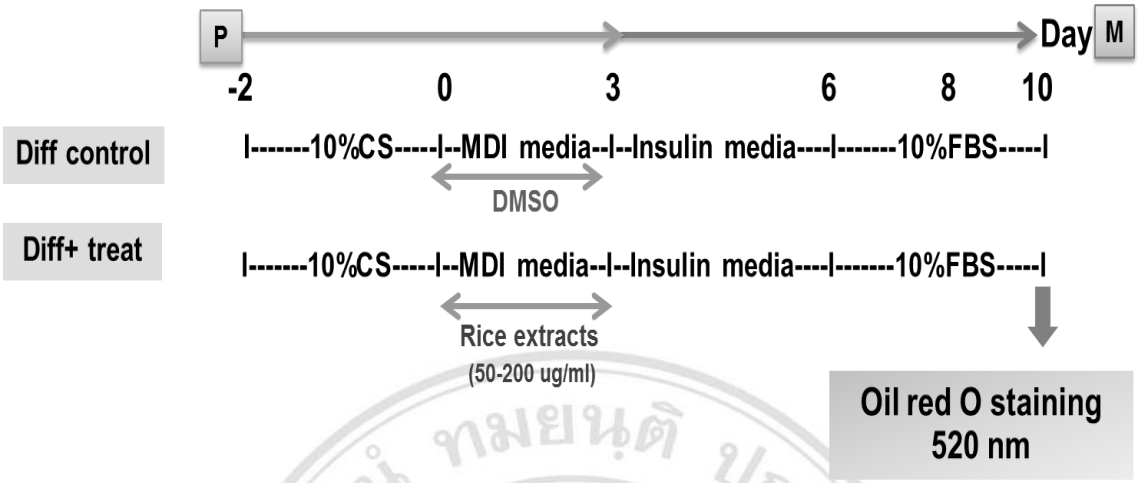

Figure 2.7 A treatment diagram for3T3-L1 differentiation assay

2.6.3 Oil red O staining

At the end of differentiation (day 10), cells were washed twice with phosphate-buffered saline (PBS) and fixed in a 4% buffered paraformaldehyde for 10 min. Cellular lipid was stained with 0.5% Oil Red O solution for 15 min at 60 ^{o}C. Excess Oil Red O dye was washed with water. Cells were left air-dried at room temperature. The photographs of stained cells were recorded. Next, the stained adipocytes were dissolved with 100% isopropanol, and then the absorbance was measured at 510 nm.

2.7 Effect of rice extracts on the expression of master regulators of adipocyte differentiation

Tests were performed in 96-well plates. Preadipocytes were seeded (1×10^4 cells/well) and assay performed 2 days later. According to treatment diagram (figure 2.8), cells were treated with varying doses (5-200 μg/mL) of rice extracts for 72 h during incubation in MDI media (day 0-3). Master regulators for 3T3-L1 adipocyte differentiation, C/EBPα and PPARγ were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

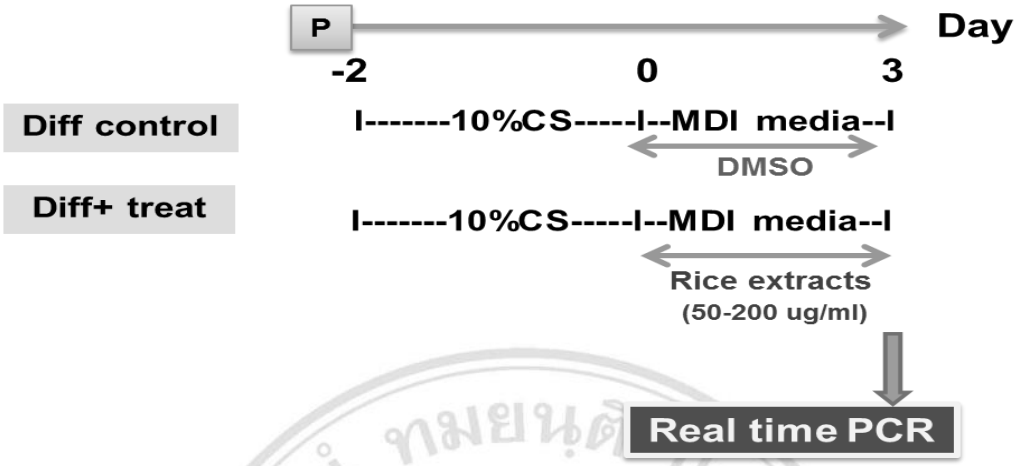

Figure 2.8 A treatment diagram for the expression of master regulators of adipocyte differentiation

2.7.1 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR is the gold standard technique for mRNA quantification. First, mRNA must be transcribed into complementary DNA (cDNA) using revese trancriptase enzyme. The cDNA is then used as a template for amplification in qPCR. The qPCR reaction is standard PCR which target cDNA can be amplified using specific oligonucleotide primers to the target. At the end of each PCR cycle, the amount of amplified DNA can be detected using fluorescence dye.

2.7.1.1 RNA Extraction

Total mRNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction. In brief, the cell monolayer was directly lysed in a culture dish by adding 1 mL of Trizol reagent to 35 mm diameter dish, and passed several times through a pipette. The cleared homogenate solution was transferred to a fresh tube. The homogenized cells were incubated for 5 min at RT to allow the complete dissociation of nucleoprotein complexes. Next, 0.2 mL of chloroform was added per 1 mL

of Trizol reagent used in each tube. The tubes were then vigorously shaken for 15 sec and incubated at RT for 3 min. After centrifugation at 12,000g for 15 min at 4 ˚C, the mixture was separated into a lower red, phenol-chloroform phase, an inter-phase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropanol, incubated at -20 ˚C for 10 min and centrifuged at 12,000g for 10 min at 4 ˚C. After removing the supernatant, the RNA pellet was washed once with 75% ethanol, mixed by a brief vortex and centrifuged at 7,500xg for 5 min at 4 ˚C. At the end of the procedure, the RNA pellet was air dried for 5-10 min and then dissolved in diethylpyrocarbonate (DEPC)-treated water. The quantity and quality of total RNA were assessed by the ratio of OD260/OD280. The concentration of RNA was determined by NanoDrop spectrophotometer (Thermo scientific, USA).

2.7.1.2 cDNA Synthesis by Reverse Transcription

In reverse transcription reaction, 1.0 µg of total RNA was reverse-transcribed into cDNA by oligo-$(dT)_{18}$ primer and AMV reverse transcriptase using RevertAid^{TM} First Stand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instructions. Briefly, the 20 µL reaction mixture contained 1 µg of extracted RNA, 0.5 µg of oligo-$(dT)_{18}$, 1 mM dNTPs, 20 unit of ribonuclease inhibitor, 200 units of reverse transcriptase, and adjusted volume to 20 µL with DEPC-treated water. The reaction mixture was incubated at 70 ˚C for 5 min, 4 ˚C for 1 min, and then 42 ˚C for 60 min.

2.7.1.3 Quantitative Polymerase Chain Reaction (qPCR)

For determination of target genes expression, The 25 µL qPCR reaction mixture contained 5 µL of cDNA (dilute 1:5), 1X THUNDERBIRD™ SYBR® qPCR Mix (Toyobo, Japan), 10 nM ROX solution and 0.3 µM of primers. The sequence primers of target genes are shown in Table 2.1. Each reaction mixture was then placed on an ABI 7500 Real-time PCR system (Applied Biosystems, USA). The temperature profile was as follows: initial denaturation at 95 ˚C for 10 min, followed by 45 cycles of the amplification process, which are denaturation at 95 ˚C for 15 sec, annealing and extension at 60 ˚C for 60 sec. Relative expression levels for targets genes were normalized to the expression of β-actin by the $2^{-\Delta CT}$ method (79).

Table 2.1 Primer Sequences for qPCR

Primer name	Sequence
C/EBPα	Forward: 5′-TTACAACAGGCCAGGTTTCC-3′
	Reverse: 5′-CTCTGGGATGGATCGATTGT-3′
PPARγ	Forward: 5′-CTGGCCTCCCTGATGAATAA-3′
	Reverse: 5′-GGCGGTCTCCACTGAGAATA-3′
β–actin	Forward: 5′-GACAACGGCTCCGGCATGTGCAAAG-3′
	Reverse: 5′-TTCACGGTTGGCCTTAGGGTTCAG-3′

2.8 Effect of rice extracts on TNF-α-induced insulin resistance in adipocyte

2.8.1 TNF-α-induced insulin resistance in adipocyte

To induce insulin resistance (figure 2.9), the mature adipocytes were incubated with 50 ng/mL of TNF-α on day 10. Next, rice extracts concentrations 50-200 µg/mL were added into TNF-α-treated cells on day 11, and incubated for

24 h. Inflammation- induced insulin resistance were determined by glucose uptake assay and lipolysis assay.

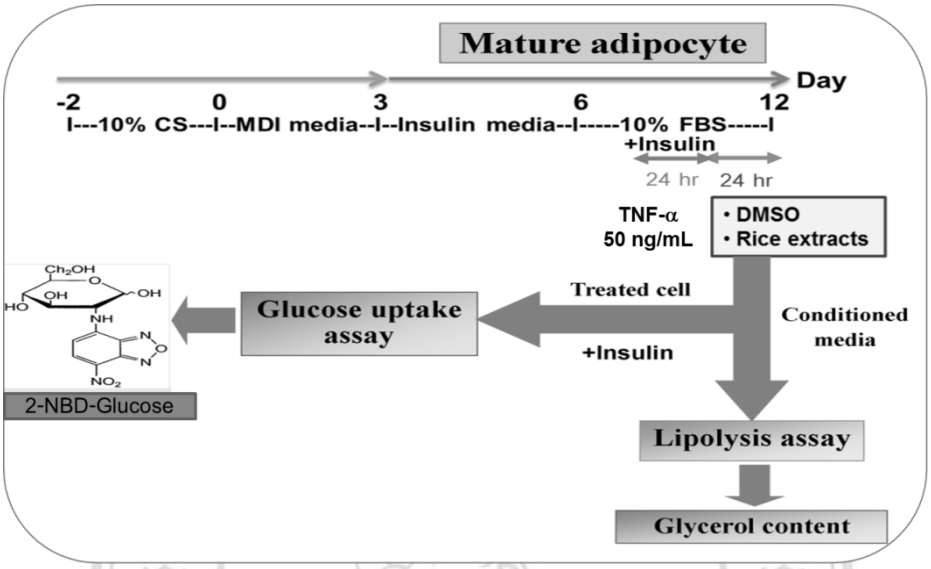

Figure 2.9 A treatment diagram of TNF-α-induced insulin resistance in adipocyte

2.8.2 Glucose uptake assay

A fluorescent glucose analog as shown in figure 15, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-glucose (2-NBDG; Invitrogen, USA), was used to measure glucose uptake in mature adipocyte. At the end of treatment, cells were washed with PBS and cells were incubated with incomplete DMEM for 3 h at 37 ^{O}C. Next, cells were replaced by a 1 mg/ml BSA containing 100 mM 2-NBD-glucose and 100 nM insulin. The cells were incubated for 1 h at 37 ^{O}C. After washing with PBS, fluorescence was measured in a fluorescence microplate reader (λex =466 nm and λem = 540 nm).

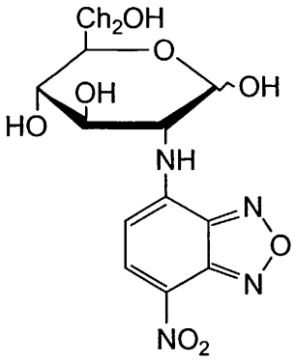

Figure 2.10 2-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-glucose (2-NBDG) structure

2.8.3 Lipolysis assay

Cellular lipolytic activity was determined by measuring the amount of free glycerol released into the culture medium during the 24 h culture, using Free Glycerol assay Reagent (Sigma Aldrich, USA). Briefly, at the end of treatment, cell culture supernatants was collected (50 μl) from each well and placed in a new 96-well. A 150 μl of free glycerol assay reagent was added into each well of the standards and the samples. The reactions were incubated for 15 min at a room temperature. The absorbance of color product was read at 540 nm. The glycerol concentration of the samples was determined using glycerol as a standard.

Glycerol Assay Enzymatic Reaction:

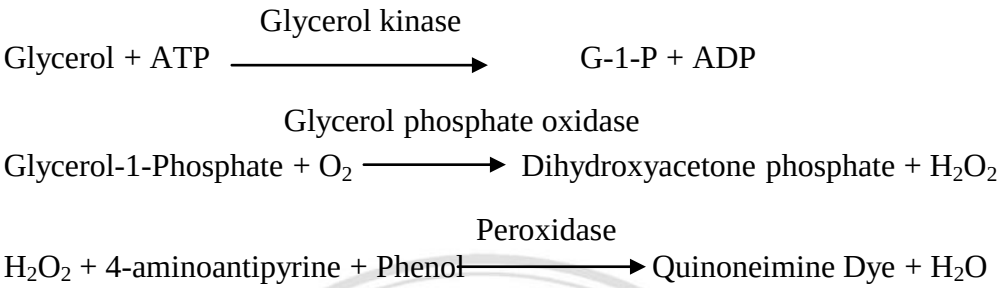

Figure 2.11 Glycerol Assay Enzymatic Reaction

2.9 Statistical Analysis

All values were given as mean ± standard derivation (mean ± SD) from triplicate samples of three independent experiments. Overall differences among the treatment groups were determined using one-way analysis of variance (ANOVA), and differences between individual using the Student-t-test by SPSS 16.0 software package. P value<0.05 is considered as statistical significant.