# CHAPTER 2

# Materials and Methods

## 2.1 Research design, scope, and methods

### 2.1.1 Study protocol

All experiments in this study were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use committee, in compliance with NIH guidelines, and accordance to the ARRIVE guideline for reporting animal research. Male Wistar rats (180-200g.) were purchased from the National Animal Center, Salaya campus, Mahidol University, Bangkok, Thailand. Rats were acclimatized for 1 week then the animals were randomly divided into 2 groups to receive either a normal-diet (ND) or a high fat diet (HFD) for 12 weeks. All rats received reverse osmosis drinking water (R/O) *ad libitum*. Baseline levels of HRV, echocardiography, blood pressure (BP) were determined. Blood was collected for the investigation of metabolic parameters including the plasma levels of insulin, glucose, HDL, LDL, TC, TG and HOMA-index. At week 12, metabolic parameters, serum LPS, HRV, echocardiography, and BP were determined. Then, the rats in each group were randomly divided into 4 subgroups including: ND treated with vehicle (NDV); ND treated with probiotics (NDPO); HFD with vehicles (HFV) and HFD with probiotics (HFPO). These treatments occurred for 12 weeks (i.e. week 13-24). At the post treatment weeks 4, 8 and 12, the HRV, echocardiography and BP were examined. In addition, blood was collected for the investigation of the same metabolic parameters as before. At the end of study, rats underwent overnight fasting for the determination of insulin sensitivity by the oral glucose tolerance test (OGTT). Next, the pressure volume-loop (PV-loop) was determined and all rats were sacrificed by decapitation. The hearts were rapidly removed and used to determine cardiac mitochondrial function, the cardiac apoptosis signaling pathways by western blot analysis including Bax and

Bcl-2 protein expression, and cardiac MDA levels. The study protocol is explained in Figure 2.1.

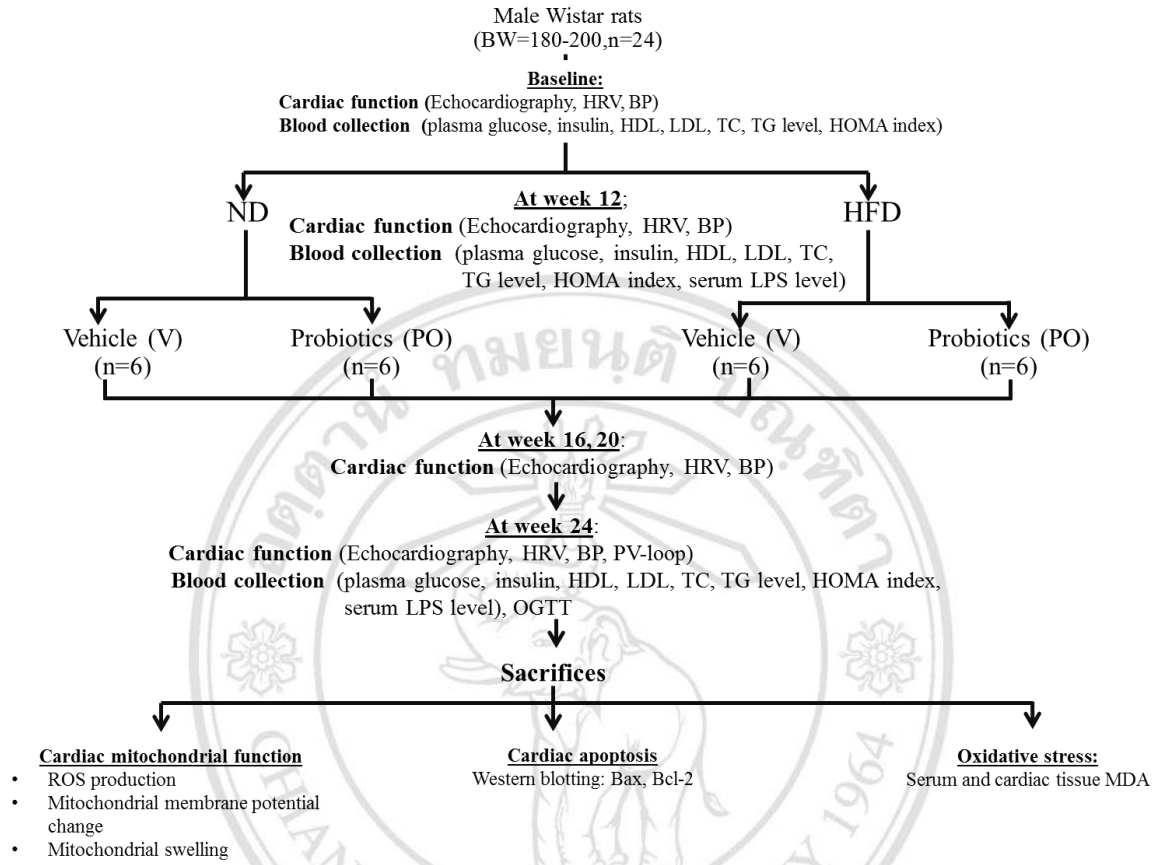


**Figure 2.1** A diagram to demonstrate the study protocol.

### 2.1.2 General Methods

All experiments in this study were approved by the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines.



#### 1) Animal Preparation

Male Wistar rats, weighing 180-200g., were purchased from the National Animal Center, Salaya campus, Mahidol University, Bangkok, Thailand. All of the animals were acclimatized for one week and housed in a 12/12 hour dark/light cycle and temperature control room. During the first week, all of the rats received ND, then rats were randomly divided into two groups to receive either HFD or ND for 12 further weeks. The contents of these two diets are shown in Table 2.1 and 2.2. All of the rats

received R/O drinking water *ad libitum.* The rats' food intake was recorded daily, and body weight was recorded once a week. Fasting blood was collected from the tail tip (1ml) at week 0, week 12 as pre-treatment, and post-treatment at week 4, 8 and 12. Plasma was kept in -80℃ until it needed to be analyzed. At the post-treatment week 12, an OGTT was carried out. At the end of experiment, all of the rats were deeply anesthetized and sacrificed by decapitation.

**Table 2.1 The contents of the normal diet (ND)** (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand) (Apaijai, Pintana, Chattipakorn, & Chattipakorn, 2013; Pratchayasakul et al., 2011)

| Composition | **Normal diet (CP 082)** | | |
|---|---|---|---|
| | g | Kcal | %E |
| Carbohydrate | 495.30 | 981.20 | 51.99 |
| Fat | 83.70 | 753.30 | 19.77 |
| Protein | 269.00 | 1076.00 | 28.24 |
| Vitamins | 65.40 | - | - |
| Fiber | 34.30 | - | - |
| Total | 947.70 | 3810.50 | 100 |
| Kcal/g | 4.02 Kcal/g | | |

**Table 2.2 The contents of the high-fat diet (HFD)** (Apaijai et al., 2013; Pratchayasakul et al., 2011)

| Composition | High fat diet | | |
|---|---|---|---|
| | G | Kcal | %E |
| Carbohydrate | 190.76 | 763.04 | 14.27 |
| Fat | 342.24 | 3080.16 | 59.28 |
| Protein | 353.60 | 1414.40 | 26.45 |
| Cholesterol | 10 | 90 | 1.68 |
| Vitamins | 85.19 | - | - |
| DL-Methionine | 3 | - | - |
| Fiber | 13.21 | | |
| Yeast powder | 1 | - | - |
| Sodium chloride | 1 | - | - |
| Total | 1000 | 5347.60 | 100 |
| Kcal/g | | 5.35cal/g | |

**2) High-fat diet preparation**

The high-fat diet (59.28% E fat) was prepared by mixing the following ingredients; standard rat diet (365 g/kg food), casein (250 g/ kg food), lard (310 g/kg food), cholesterol (10 g/kg food), vitamins (60 g/kg food), DL-Methionine (3 g/kg food), yeast powder (1 g/kg food) and sodium chloride (1 g/kg food). The mixture was molded into a spherical shape and then refrigerated until utilization (Pratchayasakul et al., 2011).



**3) Probiotics preparation**

*Lactobacillus paracasei* ST11 (HP4) was obtained from the Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Thailand. *Lactobacillus paracasei* ST11 (HP4) was mixed in a PBS buffer and the amount of probiotics used was 1x $10^8$ CFU/ ml/ day.

**4) Echocardiography protocol**

Echocardiography is a non-invasive method used for the assessment of LV function by using ultrasonic waves. All rats were anesthetized by 2% Isoflurane with oxygen (2L/min) via inhalation. The rat was placed in a supine position and shaved at the chest area. An echocardiography probe (S12, Hewlett Packard), which was connected to an echocardiography machine (GE vivid i), was placed on the chest and moved along the short axes of the heart for data collection. Signals from the M-mode echocardiogram at the level of papillary muscles was recorded (Inthachai et al., 2015a). Fractional shortening (FS) and Ejection fraction (EF) were calculated using the formula. After investigation, the rat was allowed to fully recover and was returned to the cages.

$$\%FS = \left(\frac{LVIDd - LVIDs}{LVIDd}\right) \times 100$$

$$\%EF = \left(\frac{EDV - ESV}{EDV}\right) \times 100$$

LVIDd = Left ventricular internal diameter during diastole

LVIDs = Left ventricular internal diameter during systole

EDV= End diastolic volume

ESV= End systolic volume

**5) Heart Rate Variability (HRV) protocol**

HRV is a non-invasive method using lead II of electrocardiograms (ECG) for assessment of cardiac autonomic function. Animals were anesthetized with 2% Isoflurane, and three electrodes were inserted at the right arm (RA), left leg (LL), and trunk in the prone position. Rats were laid down within a restrainer to limit their mobility. ECG were recorded from the animal using a signal transducer (PowerLab 4/25T, AD Instrument, Australia) and operated through Chart 5.0 program for 20 minutes. After that, animals were returned to the cages. Data was analyzed using the MATLAB program. The high frequency (HF) component (ranging between 0.15-0.40 MHz) and the low-frequency (LF) component (ranging between 0.04-0.15) are markers of parasympathetic and sympathetic tone respectively. LF/HF ratio was reported as the cardiac sympathetic/parasympathetic balance (Sivaporn Sivasinprasasn et al., 2015).

**6) Blood Pressure (BP) protocol**

BP is a non- invasive method for determining cardiovascular function (Krege, Hodgin, Hagaman, & Smithies, 1995). Rats were placed in a restrainer to limit their mobility. The warming pad was used to control the body temperature. After that a volume pressure recording (VPR) and occlusion cuff (O-cuff) were attached to their tail. Blood pressure was recorded using CODA2 channel non-invasive blood pressure system (Kent Scientific Corporation, Wyoming, USA). Blood pressure values were achieved by taking an average of 20 consecutive measurements at a steady state (Lekawanvijit et al., 2012).

**7) Left ventricular (LV) Pressure-Volume loop (P-V loop) protocol**

Rats were anesthetized using the combination of zoletil (50 mg/kg) and xylazine (3 mg/kg) via intramuscular injection. A thoracotomy was performed to identify the left carotid artery. The P-V catheter was inserted through the left carotid artery, and advanced into the left-ventricular (LV) chamber. LV function including heart rate (HR), end-systolic pressure (ESP), end-diastolic pressure (EDP), dP/dt max, dP/dt min, end-systolic volume (ESV), end-diastolic volume (EDV), and stroke volume (SV) were determined using the admittance P-V system (Transonic Scisense, ON, Canada)(Samniang et al., 2016).

**8) Cardiac mitochondrial function protocol**

**Cardiac mitochondrial isolation**

After the rats were sacrificed, the heart was removed and homogenized at 800 g for 5 min by a homogenizer with an ice-cold buffer containing sucrose (300 mmol/l), TES sodium salt (5 mM), and ethylene glycol bis (2-amino ethylether)-N,N,N,N-tetraacetic acid (EGTA) (0.2 mM) at pH 7.2 and 4 C. After that, the homogenate was centrifuged at 800g for 5 min, and then the supernatant was collected and centrifuged again at 8,800 g for 5 min. The mitochondrial pellet was collected, re-suspended in the ice-cold buffer, and centrifuged again at 8,800g for 5 min. Protein concentration was determined using the bicinchoninic acid (BCA) assay. Cardiac mitochondria ROS

production, cardiac mitochondria membrane potential changes, cardiac mitochondrial swelling and morphology of cardiac mitochondria were determined (Thummasorn, Kumfu, Chattipakorn, & Chattipakorn, 2011a).

**Qualification of protein concentration in Cardiac tissue**

Protein concentration was evaluated using the bicinchoninic acid (BCA) assay (Thummasorn, Kumfu, Chattipakorn, & Chattipakorn, 2011b). Reagent A was prepared with sodium bicinchoninate (0.1 g), $Na_2CO_3.H_2O$ (2.0 g), sodium tartrate (dehydrate) (0.16 g), NaOH (0.4 g) and $NaHCO_3$ (0.95 g) and made up to 100 ml. The pH of this reagent was adjusted to 11.25 with NaOH, if necessary. Then, reagent B was prepared by dissolving $CuSO_4.5H_2O$ (0.4 g) in 10 ml of water. The combination of reagent A (100 ml) and reagent B (2 ml) were used as the standard working reagent (SWR). The color of the reagent was green and stable in room temperature for 1 week. The SWR (1 ml) was mixed with mitochondrial protein (50 µl) and incubated for 30 minutes with a controlled temperature of 60 °C. A spectrophotometer was used to measure absorbance at 562 nm after the sample was cooled down to room temperature. A calibration curve was set up using dilutions of a stock 1 mg/ml solution of bovine serum albumin (BSA) (Thummasorn et al., 2011b).

**Determination of reactive oxygen species (ROS) in cardiac mitochondria**

Mitochondrial ROS levels were measured by 2, 7-dichlorohydrofluorescein diacetate (DCFH-DA) dye staining. DCFH-DA diffused into mitochondrial cells and enzymatically hydoyzed by intracellular esterase to 2, 7-dichlorohydrofluorescein (DCFH). Then, DCFH were oxidized by ROS and changed to 2, 7-dichlorofluorescein (DCF), a fluorescent compound. This is a specific indicator for $H_2O_2$. However, DCFH is oxidized by other ROS including HO·, ROO·, NO·, $ONOO^-$. The mitochondria were incubated with 2µM DCFH-DA for 20 minutes at 25°C. The ROS level was assessed at the wavelength of 485 nm (bandwidth 5 nm) and emission wavelength of 530 nm (bandwidth 10 nm) using a fluorescent microplate reader (BioTek, VT, USA) (Novalija, Kevin, Eells, Henry, & Stowe, 2003; Thummasorn et al., 2011b).

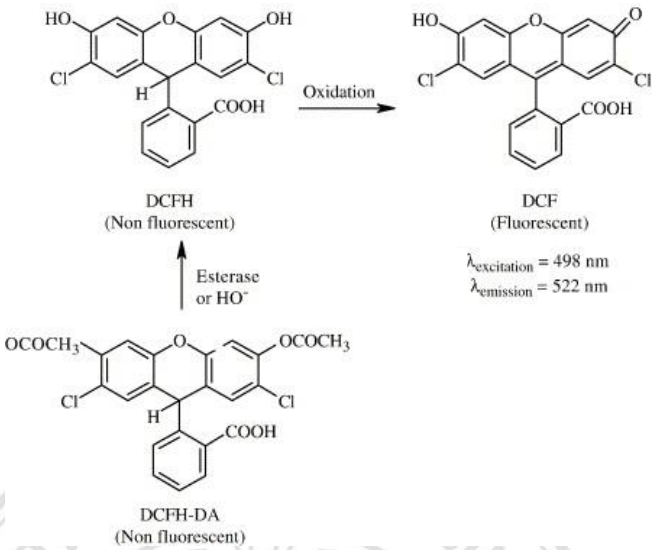


**Figure 2.2** Mechanism of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) de-esterification to 2,7-dichlorodihydrofluorescein (DCFH), and further oxidation to fluorescent 2,7-dichlorofluorescein (DCF) by ROS and RNS (Gomes, Fernandes, & Lima, 2005).

**Determination of mitochondrial membrane potential change (ΔΨm)**

The mitochondrial membrane potentials were measured by staining with the dye 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Isolated cardiac mitochondria were stained with JC-1 (5 μM) at 37 °C for 30 minutes, and then the membrane potentials were determined as fluorescence intensity ratio. JC-1 remained in the mitochondrial matrix as a monomer form (exhibits green fluorescence) and could be changed to its aggregate form (exhibits red fluorescence) after interaction with anions. The green fluorescence was excited at the wavelength of 485 nm and the emission was detected at a wavelength of 590 nm while the red fluorescence was excited at the wavelength of 485 nm and the emission was detected at a wavelength of 530 nm. The changes in mitochondrial membrane potential were calculated as the ratio of red to green fluorescent intensity. Decreasing of the ratio of red/green fluorescence intensity was indicated mitochondrial membrane depolarization (Di Lisa et al., 1995; Thummasorn et al., 2011b).

**Determination of cardiac mitochondrial swelling**

Mitochondrial proteins 0.4 mg/ml were incubated in 1.5 ml of respiration buffer (containing 100 mMKCl, 50 mM sucrose, 10 mM HEPES, 5 mM $KH_2PO_4$, pH 7.4 at 37ºC) with addition of 10 mM pyruvate/malate for 1 minutes. Mitochondrial swelling was evaluated by measuring the change of absorbance by using a spectrophotometer at a wavelength of 540 nm. Decreasing of absorbance indicated cardiac mitochondrial swelling. (Ruiz-Meana, Garcia-Dorado, Miro-Casas, Abellan, & Soler-Soler, 2006; Thummasorn et al., 2011b).

**Cardiac mitochondrial morphology**

Cardiac mitochondrial morphology was identified by transmission electron microscopy (TEM). Cardiac mitochondria were extracted and fixed in 2.5% glutaraldehyde at 4°C. After that, the sample was rinsed in 0.1 M phosphate buffer ($PO_4$) 2 times for 15 min. Post-fixation in 1% cacodylate-buffer osmium tetroxide for 2 hours at room temperature was followed by rinsing in 0.1 M $PO_4$ 2 times for 5 min. For the dehydration process, the sample was dehydrated by graded series of ethanol at 50%, 70%, 85%, 95% and 100%. For the infiltration process, the sample was infiltrated in propylene oxide (PO), Resin: PO 1:2, Resin: PO 1:2, and resin, respectively. Then, the sample was embedded in Embedded 812, overnight. After that, ultrathin sections were cut by a diamond knife and stained in uranyle acetrate and lead citrate. Finally, ultrathin sections of the cardiac mitochondria were identified by Transmission electron microscopy (TEM).

**9) Western blot analysis for Bax and Bcl-2**

LV tissue was homogenized in a lysis buffer (containing 20-mMTris-HCl pH 6.8, 1mM Sodium orthovanadate, 5mM Sodium fluoride and protease inhibitor) in order to extract protein. The homogenate was centrifuged at 13,000 rpm for 10 minutes. Total protein (50-80 mg) was mixed with the loading buffer (consisting of 5% mercaptoethanol, 0.05% bromophenol blue, 75 nM Tris-HCl, 2% SDS and 10% glycerol with pH 6.8) in a 1 mg/ml proportion. The mixture was boiled for 10 minutes and loaded into 10% gradient SDS-polyacrylamide gel. The protein was separated by

electrophoresis. After that, proteins were transferred to nitrocellulose membranes with the presence of glycine/methanol transfer buffer (containing 20 mM Tris, 0.15 M glycine and 20% methanol) in a wet tank transfer system (Bio-Rad). Nitrocellulose membranes were incubated in 5% skimmed milk in TBS-Tween buffer (containing 20 mM Tris-HCl (pH 7.6), 137 nM NaCl, 0.05% Tween-20) for 1 hour at room temperature (Inthachai et al., 2015b). The membrane was detected to anti-Bax, anti-actin (Santa Cruz Biotechnology, Inc., Texas, U.S.A.), and anti- Bcl-2 (Cell Signaling Technology, Danvers, MA, USA) for 12 hours. Horseradish peroxidase conjugated with anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA) was used to detect the primary antibody. Visualize peroxidase reaction products were detected by enhanced chemiluminescence (ECL) detection reagent. Then, Protein was exposed by ChemiDoc$^{TM}$ Touch Imaging System (Bio-rad, California, USA).

**10) Determination of serum lipopolysaccharide (LPS)**

Lipopolysaccharide (LPS) levels in serum were measured by a pierce limulus amebocyte lysate (LAL) chromogenic endotoxin quantitation assay kit (Thermo scientific, USA). Serum samples were diluted 50 to 100- fold and heated at 70° C for 15 minutes. The LAL assay kit includes endotoxin standard, LAL and chromogenic substrate. Endotoxin standard and Samples were added into each well and incubated in 37º C for 5 minutes, LAL reagent was added and incubated again at 37ºC for 10 minutes. After exactly 10 minutes, the chromogenic substrate was added into each well and incubated for 6 minutes at 37ºC. The stop solution was added into samples before measuring the absorbance at 405-410 nm by a spectrophotometer (Tsuji, Steindler, & Harrison, 1980).



**11) HPLC-based assay of malondialdehyde (MDA) concentration**

Malondialdehyde (MDA) concentration in cardiac tissue and serum were measured by a HPLC system (Thephinlap et al., 2011). A 80$\mu$l aliquot of samples were mixed with 176 $\mu$lof 10% trichloroacetic acid (TCA) containing BHT (50 ppm), heated at 90 °C for 30 minutes and cooled down to room temperature. The mixtures were centrifuged at 6,000 rpm for 10 minutes. The supernatants (80 $\mu$l) were mixed with 0.44 M $H_3PO_4$ (540 $\mu$l) and 0.6% thiobabituric acid (TBA) solution (180 $\mu$l) and then

were incubated at 90°C for 30 minutes to generate a pink-colored product called thiobarbituric acid reactive substances (TBARS). The solution was filtered through a syringe filter (polysulfone type membrane, pore size 0.45 µm, Whatman International, Maidstone, United Kingdom) and analyzed with a HPLC system. The TBARS were fractionated on the adsorption column (Water Spherosorb ODS2 type, 250×4.3 mm, 5 µm), eluted with mobile-phase solvent of 50 mM $KH_2PO_4$ : methanol at flow rate 1.0 ml/min and online detected at 532 nm. Data was recorded and analyzed with BDS software (BarSpec Ltd., Rehovot, Israel). A standard curve was constructed from the peak of the height of standard 1, 1, 3, 3-tetramethoxypropane (standard reagent for MDA) at different concentrations (0-10 µM). Plasma TBARS concentration was determined directly from the standard curve and reported as the MDA equivalent concentration. MDA concentration was expressed µM in serum and µM/mg in cardiac tissues (Mateos, Lecumberri, Ramos, Goya, & Bravo, 2005; S. Sivasinprasasn et al., 2015).

### 2.1.3 Chemical analysis

#### 1) Determination of plasma insulin level

Plasma insulin level was determined using a commercial ELISA kit (Merck Millipore, MA, USA). The insulin level was interpreted by calculation from the standard curve that was generated from the known-concentration standards (Noppamas Pipatpiboon, Hiranya Pintana, Wasana Pratchayasakul, Nipon Chattipakorn, & Siriporn C. Chattipakorn, 2013). The brief of the procedure includes the following: *(1)* diluted wash buffer 1:10 in 450 ml distilled water. *(2)* Washed well plate 5 times and add 10 $\mu$l Assay buffer to blank and sample well plate. *(3)* Added 10 $\mu$l matrix solution in blank, standard and QC in the well. *(4)*Added 10 $\mu$l insulin stardard 5 concentrations including 0.2, 0.5, 1, 2, 5 and 10. *(5)* Added 10 $\mu$l in each QC and sample in the well. *(6)* Added 80 $\mu$l detection antibody to the all of well plate, incubated at room temperature for 2 hours. *(7)* Washed the well plate and added 10 $\mu$l enzyme solution and incubated at room temperature for 30 minutes with shaker. *(8)* Washed 6 times and added 100 $\mu$l substrate, then, incubated at room temperature approximately 5-20 minutes. *(9)* Added 100 $\mu$l stop solution and measured O.D by microplate reader at wavelength 450 & 590

nm. The increasing of absorbance is directly captured by insulin proportion and can be derived by interpolation from a reference curve for determined insulin concentration.

**2) Determination of plasma glucose level**

Blood samples were collected from the tail tip into a NaF-coated tube after 5 hours of fasting. Blood was centrifuged at 6000 rpm for 10 minutes and plasma was collected. Plasma glucose was determined by using a colorimetric assay from a commercially available kit (ERBA diagnostics, Mannheim, Germany). Glucose was oxidized by glucose oxidase (GOD) into hydrogen peroxide ($H_2O_2$) and gluconic acid. $H_2O_2$ was degraded by peroxidase and formed a color-producing phenol. The color intensity of each sample was measured at 505 nm by a microplate reader. A standard curve was generated and the glucose level of each sample was interpreted by comparing its absorbance to the standard curve (Pipatpiboon, Pratchayasakul, Chattipakorn, & Chattipakorn, 2012a).

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{phenol 4AAP} \xrightarrow{\text{Peroxidase}} \text{Red dye} + 2H_2O$$

**3) Determination of total cholesterol (TC) level**

Total plasma cholesterol levels were determined by a commercial enzymatic colorimetric assay kit (ERBA diagnostics, Mannheim, Germany). The cholesterol ester was hydrolyzed by The cholesterol esterase (CHE) in the plasma into fatty acid and cholesterol. Cholesterol is oxidized by cholesterol oxidase (CHO) into cholest-4-en-3-one and $H_2O_2$. Cholesterol is degraded by peroxidase and forms a color-producing phenol (POD), which is pink colored, called quinoneimine and is measured at 500 nm by microplate reader (Pratchayasakul, Chattipakorn, & Chattipakorn, 2014a).

1. Cholesterol ester + $H_2O$ $\xrightarrow{CHE}$ Cholesterol + Fatty acids

2. Cholesterol + $O_2$ $\xrightarrow{CHO}$ Cholest-4-en-3-one +$H_2O_2$

3. $2H_2O_2$ + 4AAP + Phenol $\xrightarrow{POD}$ Quinoneimine + $4H_2O$

The brief of the procedure includes the following *(1)* prepared Reagen blank (Reagent 200 $\mu$l + Distilled water 2 $\mu$l), Standard (Reagent 200 $\mu$l+ Standard 2 $\mu$l), and Sample (Reagent 200 $\mu$l + Sample 2 $\mu$l) in the well plate. *(2)* Mixed and incubated for 10 minutes, at 37℃. *(3)* Measured absorbance of the sample $A_{sam}$ ans standard $A_{st}$ against reagent blank and then calculated TC concentrations level by following formula.

$$\text{Cholesterol (mg/dl)} = \frac{\Delta Asam}{\Delta Ast} \times Cst$$

$C_{st}$ = standard (calibrator) concentration

**4) Determination of plasma triglyceride (TG) level**

Plasma triglyceride level was determined by an enzymatic colorimetric assay using a commercial kit (ERBA diagnostics, Mannheim, Germany). As triglyceride is hydrolyzed into glycerol and free fatty acid (FFA), glycerol subsequently releases hydrogen peroxide ($H_2O_2$). $H_2O_2$ is degraded by peroxidase and generates the color complex which is called quinoneimine.

Triglycerides + $H_2O$ $\xrightarrow{LPL}$ glycerol + Free Fatty acids

Glycerol + ATP $\xrightarrow{GK}$ glycerol-3-phosphate + ADP

Glycerol-3- phosphate + $O_2$ $\xrightarrow{GPO}$ DAP + $H_2O_2$

$H_2O_2$ + 4 AAP + 3,5-DHBS $\xrightarrow{Peroxidase}$ Quinoneimine dye + $2H_2O$

The color intensity of each sample was measured at 505 nm by the microplate reader. A standard curve of triglyceride was generated and the concentration level of each sample

was interpreted by comparing its absorbance to the standard curve (Pratchayasakul, Chattipakorn, & Chattipakorn, 2014b). TC concentrations level was calculated by the following.

$$\text{Triglyceride levels (mg/dl)} = \frac{\Delta Asam}{\Delta Ast} \times Cst$$

$C_{st}$ = standard (calibrator) concentration

**5) Determination of HDL and LDL level**

The plasma HDL level was determined by an enzymatic colorimetric method using a HDL and LDL Cholesterol Quantification Kit (Biovision, USA) (Pintana, Apaijai, Pratchayasakul, Chattipakorn, & Chattipakorn, 2012; N. Pipatpiboon, H. Pintana, W. Pratchayasakul, N. Chattipakorn, & S. C. Chattipakorn, 2013; Pratchayasakul et al., 2015). Cholesterol esterase hydrolyzes cholesterol ester into free cholesterol. Cholesterol ester and free cholesterol was detected separately in the presence and absence of cholesterol esterase in the reaction. The plasma LDL was calculated following Friedewald's equation (Friedewald, Levy, & Fredrickson, 1972).

Plasma LDL = Plasma total cholesterol - Plasma HDL – TG/5

**6) Oral glucose tolerance test (OGTT) protocol**

OGTT was performed to measure the level of insulin sensitivity. After 12 hours of fasting, Glucose loading (2g/kg BW) was performed by gavage feeding. Blood from the tail tip was collected immediately before glucose feeding and at 15, 30, 60 and 120 minutes after feeding. Blood samples were centrifuged to collect the plasma. Plasma glucose levels during OGTT were used to determine insulin sensitivity (Nagarajan et al., 2013).



**7) Determination of insulin resistance (HOMA index)**

Homeostasis Model Assessment (HOMA) index was calculated from fasting plasma glucose and insulin levels to determine the degree of insulin resistance. A higher HOMA value indicated a higher degree of insulin resistance. HOMA index was calculated following this equation; (Pipatpiboon, Pratchayasakul, Chattipakorn, & Chattipakorn, 2012b).

$$\frac{\text{[fasting plasma insulin (μU/ml)] x [fasting plasma glucose (mmol/l)]}}{22.5}$$

**2.2 Statistical analysis**

Data were expressed as mean ± SEM. A one-way ANOVA followed by LSD significant different post-hoc was used to test the differences among groups. $P<0.05$ indicated a statistical significance.