CHAPTER II

MATERIALS AND METHODS

Animal care

The experiments were performed in male Wistar rats, weighting between 100-120 g. The animals were obtained from the National Animal Center, Salaya Campus, Mahidol University. All animals were housed individually in rodent cages in the animal room where the temperature was maintained approximately at 22±2 ºC with 12:12 hours dark-light cycle (light period: 06.00-18.00). The animals were acclimatized at least 1 week before the beginning of the experiment.

Animal treatment procedure

The animals were matched as closely as possible for body weight (BW) and then randomly divided into 8 groups (10 per group):

Group 1: Normal diet control (NDC)

Group 2: Normal diet and curcuminoids treatment at dose of 30 mg/kg BW (ND_{30})

Group 3: Normal diet and curcuminoids treatment at dose of 60 mg/kg BW (ND_{60})

Group 4: Normal diet and curcuminoids treatment at dose of 90 mg/kg BW (ND_{90})

Group 5: High-fat diet control (HDC)

Group 6: High-fat diet and curcuminoids treatment at dose of 30 mg/kg BW (HD_{30})

Group 7: High-fat diet and curcuminoids treatment at dose of 60 mg/kg BW (HD_{60})

Group 8: High-fat diet and curcuminoids treatment at dose of 90 mg/kg BW (HD_{90})

The NDC group consumed a standard rat chow (C.P. Mice Feed Food No. 082; energy content 3.8 kcal/g) containing fat 20% of total energy in the diet (%E) while the HDC group received a high fat diet containing fat 60% E over the 12 weeks of experimental period. The ND_{30}, ND_{60} and ND_{90} groups received a standard rat chow and curcuminoids at doses of 30, 60 and 90 mg/kg BW/day, respectively. Similarly, the animals in HD_{30}, HD_{60} and HD_{90} groups received high fat diets and curcuminoids at doses of 30, 60 and 90 mg/kg BW/day, respectively for 12 weeks. The composition of diets is shown in Table 3.

Commercial curcuminoids [Fluka; purity $\geq$ 95% (TLC)] was used in the present study. Curcuminoids was dissolved in 0.5% tragacanth (Sigma) suspension, and 2% curcuminoids solution was daily administered at different doses (30, 60 and 90 mg/kg BW/day) by oral gavage. The NDC and HDC groups were received the same dose of 0.5% tragacanth suspension as the vehicle by oral gavage everyday. The animals had free access to food and water and body weight and food intake were daily recorded throughout the 12 weeks. Spectral analysis of heart rate variability (HRV) was performed to determine cardiac autonomic nervous system activity at 0 and 12 weeks. After 12 weeks of dietary intervention, the animals were sacrificed, blood and tissue samples were collected for further analysis.

Table 3 Composition of experiment diets

	Normal diet (ND)		High Fat diet (HD)	
	g	**%kcal**	**g**	**%kcal**
CHO	495.30	51.99	365	13.96
Fat	83.70	19.76	310	59.16
Protein	269.00	28.23	250	26.88
Vitamin and Minerals	65.40	0.0	70	0.0
Fiber	34.30	0.0	30	0.0

Diets ingredients and nutrient analyses were modified from Srinivasan *et al.* (2005). Energy per gram (kcal/g): CHO 4; fat 9; protein 4.

Measurement of Heart rate variability (HRV)

Cardiac autonomic nervous activity was evaluated by spectral analysis of RR interval variability. Bipolar electrocardiograghy was performed at weeks 0 and 12. The animals were anesthetized with ether for lead insertion and then left in consciousness for at least 5 min. before recording the electrocardiogram (ECG). Conscious bipolar ECG was recorded continuously for a 10 min. by Power Lab with chart 5.0. During recording of the ECGs, the animals were calm and in a familiar environment with unnecessary noise and movement prohibited. The ECG data were analyzed using the program MATLAB. From the ECG, normal sinus beats were automatically identified with human rechecked. The RR interval was determined using the peaks of QRS complex and stored as interval tachogram. From section of tachogram of at least 300 interval values, standard deviation of all RR intervals (SDNN) and root mean square of successive difference (RMSSD) were calculated. Power spectra of RR interval variability were obtained by use of a Fast Fourier Transform (FFT) algorithm. Three major oscillatory components are usually detectable (Langager *et al.*, 2007). The high frequency (HF; about 0.6-3 Hz and varying with respiration) component is associated with parasympathetic activity. The low frequency (LF; about 0.2-0.6 Hz) component is correspondent to the slow waves of arterial pressure and is associated with sympathetic activity. The power below 0.2 Hz is considered to be very low frequency (VLF) which is completely unknown in it origin. Each spectral component was calculated as integrals under the respective part of the power spectral density function and was presented in absolute unit (ms^2). To minimize the effect of changes in total power on the LF and HF components, LF and HF were expressed as normalized units (LFnu and HFnu) by divided it by the total

power minus VLF. LF/HF ratio is considered an index of cardiac sympathetic/para-sympathetic tone balance.

Determination of insulin resistance (HOMA index)

Insulin resistance was assessed by the Homeostasis Model Assessment (HOMA) (Matthews *et al*., 1985; Bonora *et al*., 1998). HOMA is a mathematical model describing the degree of insulin resistance starting from fasting plasma insulin and glucose concentrations. The accuracy and precision of the HOMA method have been compared with independent estimates of insulin resistance.

HOMA index is calculated as follows:

$$\text{HOMA} = \frac{[\text{fasting insulin level } (\mu\text{U/ml}) \times \text{fasting glucose level (mmol/l)}]}{22.5}$$

Animal sacrifice

At the end of the experiment, the animals were anesthetized with intraperitoneal injection of pentobarbital sodium (80 mg/kg BW) following overnight fasting. After being completely anesthetized, the abdominal cavity was opened following median line of the abdomen up to the thoracic cavity. Blood was rapidly collected from the right atria and placed in 2 eppendrof tubes. One tube contained anticoagulant (sodium fluoride (NaF) for measurment of plasma glucose concentration and the other contained EDTA for plasma insulin, free fatty acid and triglyceride concentrations. The plasma was separated by centrifugation. One lobe of liver was then excised and frozen in liquid nitrogen immediately. Thereafter, both hindlegs were skinned and samples of the soleus and red portion of gastrognemius muscles were surgically

removed from each leg. On removal, each muscle sample was trimmed of connective tissue and adherent adipose tissue and frozen in liquid nitrogen. The plasma and tissue samples were stored at -70 ^{0}C for subsequent analyses. After tissue dissection, visceral fat which included retroperitoneal, epididymal and perirenal fat (Borst and Conover, 2005) were removed and weighed.

Biochemical analysis

Determination of glucose: The plasma glucose concentration was determined by enzymatic colorimetric method using commercial kits (Biotech, Bangkok, Thailand). The principle of the method is based on the fact that glucose in the samples is oxidized by the glucose oxidase into the gluconate and hydrogen peroxide (H_2O_2). The amount of H_2O_2 formed is proportional to the glucose content of the sample. In the presence of peroxides, H_2O_2 reacts with a substrate chromogen to form p-chlorophenol (red-chinonimin). The absorbance of the reaction mixture is read by a spectrophotometer (UV-1700, UV-visible spectrophotometer, Shimadzu) at 500 nm against the blank. A standard curve of glucose concentration was constructed by plotting the absorbance against the respective concentrations of standard glucose. The glucose concentration in each unknown sample was read out by interpolation of its absorbance on the standard curve.

***Determination of triglyceride*:** The plasma triglyceride or glycerol concentration was analyzed using a commercial enzymatic colorimetric kit (Fluitest TG, Biocon Diagnostik, Germany). The principle of the method is that the triglyceride in the samples is hydrolyzed by the lipoprotein lipase into glycerol and free fatty acids. In

the presence of ATP and glycerolkinase, glycerol forms glycerol-3-phosphate which is further oxidized by glycerol-3-P-oxidase into DAP and H_2O_2. The enzyme peroxidase in the substrate-chromogen solution catalyses the reaction between H_2O_2, p-chlorophenol and 4-aminoantipyrine to form the color complex (quinonimine). The absorbance of the reaction mixture was read by a spectrophotometer (UV-1700, UV-visible spectrophotometer, Shimadzu) at 546 nm against the blank. A standard curve of glycerol concentration was constructed by plotting the absorbance against the respective concentrations of standard glycerol. The triglyceride or glycerol concentration in each unknown sample was read out by interpolation of its absorbance on standard curve.

***Determination of free fatty acid*:** The plasma free fatty acid (non-esterified fatty acid) concentration was analyzed using a commercial enzymatic kits (NEFA C, Wako pure chemical, Japan). The principle of the method is that the free fatty acid in sample is converted to Acyl-CoA, AMP and pyrophosphoric acid (PPi) by the action of Acyl-CoA synthase (AOS) under coexistence with coenzyme A (CoA) and adenosine 5' triphosphate disodium salt (ATP). Then, the acyl-CoA is oxidized and yields 2, 3-transenoyl-CoA and hydrogen peroxide by the action of Acyl-CoA oxidase (ACOD). In the presence of peroxidase (POA), the hydrogen peroxide formed yields a blue purple pigment by quantitative oxidation condensation with 3-methyl-N-ethy-N-(β-hydrooxyethyl)-aniline (MEHA) and 4-aminoantipyrine. The absorbance of the reaction mixture was read by a spectrophotometer (UV-1700, UV-visible spectrophotometer, Shimadzu) at 550 nm against the blank. A standard curve of free fatty acid concentrations was constructed by plotting the absorbance against the

respective concentrations of standard free fatty acid. The free fatty acid concentrations in each unknown sample were read out by interpolation of its absorbance on the standard curve.

***Determination of insulin*:** The plasma insulin concentrations were determined using a Sandwich ELISA (Rat/Mouse Insulin ELISA Kit, LINCO Research, USA). The principle of the method is based sequentially on 1) capture of insulin molecules from samples to the wells of a microtiter plate coated by pre-titered amount of a monoclonal mouse anti-rat-insulin antibodies and the binding of biotinylated polyclonal antibodies to the capture insulin, 2) wash away of unbound materials from samples, 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by mornitoring horseradish peroxidase activities in the presence of the substrate 3, 3', 5, 5'- tetramethylbenzidine. The enzyme activity is then measured spectrophotometrically by the increased absorbance at 450 nm, corrected from the absorbance at 590 nm, after acidification of formed products. Since that increase in absorbance is directly proportion to the amount of capture insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standard of known concentration of rat insulin.

***Determination of tissue triglyceride content*:** Tissue homogenates were prepared for triglyceride assay by a modification of the method of Frayn and Maycock (1980). A 100-200 mg portion of liver and muscle was minced and put into

a glass tube containing 3 ml of chloroform-isopropanol 7:11 (v/v). The homogenate was left at room temperature for at least 16 hours. Then, a 1 ml of homogenate was pipetted into a glass tube and evaporated to dryness at 40^{o} C for 16 hours. The dried residue was dissolved and mixed in 10% bovine serum albumin. The triglyceride concentration was analyzed with a commercial enzymatic colorimetric (Fluitest TG, Biocon Diagnostik, Germany).

Statistical Analysis

All values are presented as means ± SE. One way analysis of variance (ANOVA) test were used to compared the mean values among the experimental groups. The Fisher post hoc test was use for the significance of difference between experiment groups. When comparing differences within group, the Student's *t*-test was performed. Simple correlation analysis was used to determine the relationship between the plasma parameters, visceral and HRV measurements. A level of $p<0.05$ was taken to be the threshold of statistical significance.