CHAPTER III

MATERIALS AND METHODS

3.1 Subjects

Male and female type 2 diabetic patients aged 35 years old and over from outpatient clinic at Public Health Center 66, Bangkok Metropolitan Administration were recruited to participate in this study. All subjects used only sulfonylureas and/or biguanide as oral hypoglycemic agent and they had fasting blood sugar (FBS) between 100-250 mg/dl (5.56-13.89 mmol/l). The subjects had blood pressure (BP) less than 160/100 mmHg. Their total cholesterol (TC) and triglyceride (TG) levels were less than 240 mg/dl (6.21 mmol/l) and 200 mg/dl (2.26 mmol/l), respectively. Their BMI were in between 18.5-29.9 kg/m^2. They were not currently on medication and any nutrition supplements or herbal products that affected the immune system or inflammation (e.g. immunosuppressive agents and anti-inflammatory agents). They were free from chronic diseases or conditions including liver disease, renal disease, cancer, immunodeficiency, infection, history of milk allergy, lactose intolerance, malnutrition, and surgery within 1 month before and during the study. In addition, they were not smoking and drinking alcohol regularly.

The experimental protocol was approved by the Ethics Committee for Researches Involving Human Subjects, the Bangkok Metropolitan Administration and was performed in accordance with the declaration of Helsinki. The written informed consent was obtained from each subject after the experimental protocol had been explained (**Appendix A**).

3.2 Experimental Design

This quasi-experimental study was conducted between March to June 2009. The subjects participated in a 10-week study with two consecutive periods: a 4-week pre-experimental period followed by a 6-week experimental period. The subjects were randomly assigned into 2 groups: a whey protein isolate (WPI) group and a control group. The subjects in the WPI group were supplemented with WPI (30 g per day) for 6 weeks while those in the control group did not receive any supplementation. For both groups, they were advised to consume diets that were suitable for diabetic patients. They were also asked to maintain their diets and levels of physical activity throughout the study. Before and after whey protein supplementation the levels of FBS, HbA1c, TC, TG, albumin, complete blood count (CBC), and inflammatory mediators (hs-CRP and IL-6) were determined in all subjects. Blood pressure (BP) and anthropometry were also recorded. Three-day food records were completed by the subjects. The subjects' compliance and adverse effects were assessed by interviewing and counting the remaining sachets of WPI.

3.3 Experimental Protocol

3.3.1 Pre-Experimental Period

The study started with a pre-experimental period over which the subjects received nutrition counseling and followed a diet suitable for diabetes management. Dietary advice involved a consideration of energy intake and the proportion of protein, fat, carbohydrate and other nutrients. On the first day of the study, each subject received dietary guideline to control blood glucose level and the booklet (**Appendix B**) with the details of dietary recommendation. The subjects were also

informed about the principle and the importance of the diets. During the pre-experimental period, all subjects were interviewed about personal information, health history on diabetes, dietary and physical activity behaviors, and nutritional status (**Appendix C**). Each subject was asked to maintain amount of energy intake and level of physical activity and required to take their regularly prescribed medications throughout the study.

3.3.2 Experimental Period

After 4 weeks of the pre-experimental period, fasting blood was obtained from each subject for determining baseline of inflammatory mediators (hs-CRP and IL-6) and biochemical parameters (FBS, HbA1c, TC, TG, albumin, and CBC). BP, body weight and height, waist circumference, hip circumference, triceps skinfold (TSF) thickness, and mid arm circumference (MAC) were also measured. A 3-day food record was done by the subjects prior to experimental period. In addition, all subjects were reminded to maintain their habitual diets and physical activity levels throughout the study. The subjects were randomly assigned into a WPI group or a control group.

The subjects in the control group were advised to maintain their regular diets while the subjects in the WPI group received 45 sachets of 30 g WPI/sachet for 6-week supplementation. They still consumed their regular diet and additionally took 1 sachet of 30 g WPI powder (Provon 290, Glanbia nutritionals Inc., USA; **Appendix D**) after breakfast. They were advised to take WPI in liquid form by mixing with water, milk, fruit juice, soup or other beverage and continue consuming the WPI until the end of week 6.

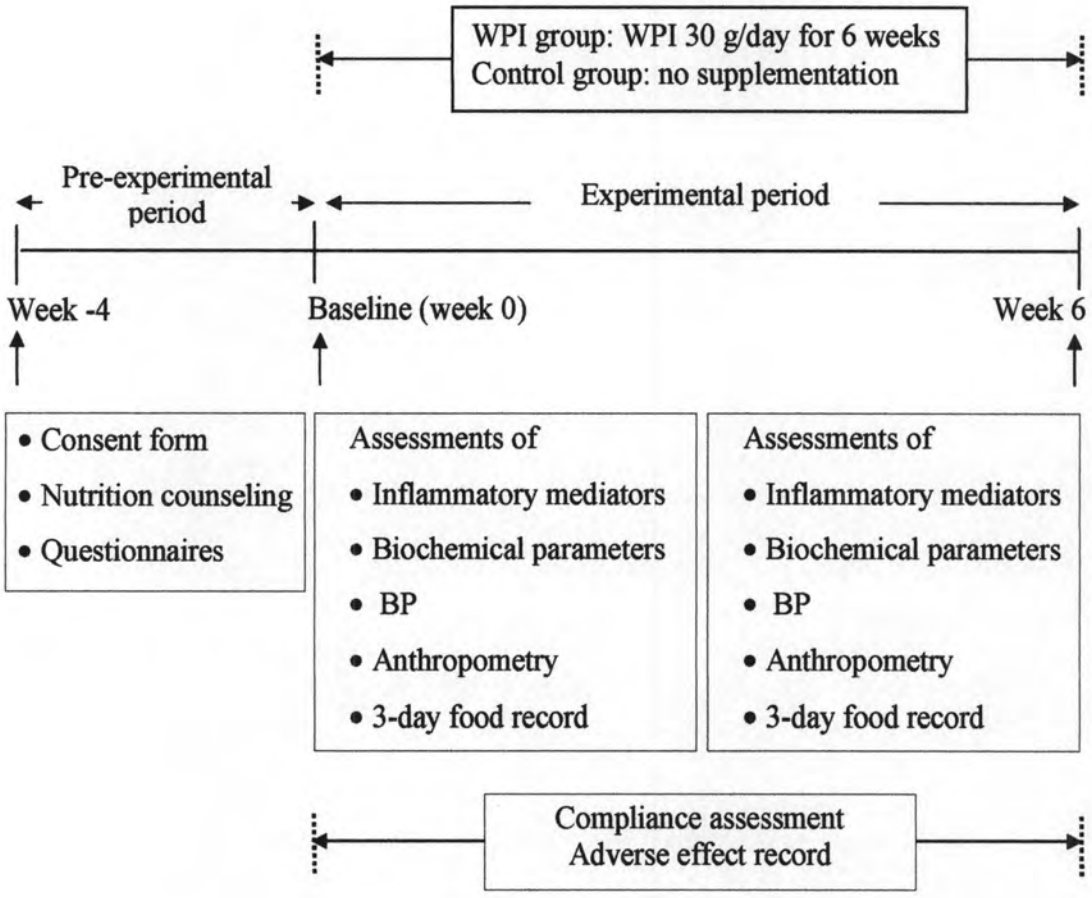

Figure 1 Experimental protocol

At week 6 of the experimental period, fasting blood was obtained from each subject for determining inflammatory mediators and biochemical parameters. Blood pressure and anthropometric measurements were taken. The experimental protocol of the study is presented in **Figure 1**.

3.4 Study Measurements and Data Collection

3.4.1 Dietary Intake Assessment

A three-day food record was used for assessment of energy distribution and nutrient intakes. The subjects were instructed how to record a 3-day dietary intake (1 weekend day and 2 weekdays). The example of dietary record and filling form were given to each subject (**Appendix C**). All items and portions of food consumed including name, method of preparation, and cooking were asked to record. The subjects estimated food portion size using standard household measuring cups and spoons.

Portion size measures were converted into grams of foods. The food records were analyzed for total energy intake and its distribution from protein, fat, and carbohydrate. The nutrient consumed was analyzed by the computerized program "Thai Nutrisurvey version 2.0 (2008)" modified for Thai food by Division of Nutrition, Department of Health and faculty of Tropical Medicine, Mahidol University. Average intakes of energy, protein, carbohydrate, fat, cholesterol, sugar, dietary fiber, and water were calculated.

3.4.2 Blood Pressure and Anthropometric Measurements

Blood pressure was measured in upper arm while the subject seated comfortably. BP was measured with sphygmomanometer (Hico Medical Co.Ltd., Tokyo, Japan) and stethoscope (Spirit Medical, UK). It was measured in terms of millimeters of mercury (mmHg).

Anthropometry in this study consisted of the measurements of body weight, height, waist circumference, hip circumference, TSF, and MAC. These measurements provided useful data for calculation of BMI, waist to hip ratio (WHR)

and mid-arm muscle circumference (MAMC). Body weight and height was performed by weight with height meter (402 KL Health O meter Professional, USA). BMI was calculated from weight and height as followed:

$$\text{BMI (kg/m}^2\text{)} = \frac{\text{weight (kg)}}{[\text{height (m)}]^2}$$

Each subject's waist was measured with a non-stretchable tape midway between the lowest rib and the iliac crest. The normal waist circumference was defined as <90 cm. in men and <80 cm. in women (Grundy et al., 2005). The hip circumference was measured at the widest part of the gluteal region. The measurement was performed two times in each patient, and the values were then averaged for final calculation. WHR were derived according to the following formula:

$$\text{WHR} = \frac{\text{waist circumference (cm)}}{\text{hip circumference (cm)}}$$

The normal WHR was interpreted as less than 0.9 in men and less than 0.8 in women (Centers for Disease Control and Prevention: CDC, 2009).

MAC was measured in centimeters halfway between the acromion process of the scapula and the olecranon process at the tip of the elbow with non-stretchable tape. TSF was measured at the marked midpoint (the same point of MAC) with a conventional skinfold caliper (Cambridge scientific industries Inc., Maryland). The MAMC was calculated as follows:

$$\text{MAMC} = \text{MAC (cm)} - [0.314 \times \text{TSF (cm)}]$$

Table 6 Clinical identification of the metabolic syndrome

Risk factor	Defining level
Abdominal obesity	Waist circumference
men	> 90 cm.
women	> 80 cm.
TG	≥ 150 mg/dl (1.70 mmol/l) or on drug treatment
HDL-C	
men	< 40 mg/dl (1.03 mmol/l)
women	< 50 mg/dl (1.30 mmol/l)
	or on drug treatment
BP	≥ 130/85 mmHg or on drug treatment
FBS	≥ 100 mg/dl (5.56 mmol/l) or on drug treatment

TG = triglyceride; HDL-C = high-density lipoprotein cholesterol; BP = blood pressure; FBS = fasting blood sugar; cm = centimeter; mg/dl = milligram/deciliter; mmol/l = millimole/liter; mmHg = millimeter of mercury
Source: Grundy et al., 2005

3.4.3 Metabolic Syndrome Assessment

Metabolic syndrome comprises of a cluster of abnormalities with insulin resistance and adiposity as its central features. Five diagnostic criteria were identified by the Adult Treatment Panel III (ATP III) in the executive summary of a report of the National Cholesterol Education Program (NCEP). The presence of any three of the five features was considered sufficient to diagnose metabolic syndrome (**Table 6**).

3.4.4 Physical Activity Assessment

The physical activity was assessed by the questionnaire that was modified from National Institute for Health and Clinical Excellence (NICE) guideline (NICE, 2006). The data were classified into four different levels of physical activity including inactive, moderately inactive, moderately active, and active.

3.5 Blood Sample Collection

At baseline and week 6 of the experimental period, 15 ml of 8-hour fasting venous blood was drawn from each subject. All blood samples were appropriately prepared for determination of inflammatory mediators (hs-CRP and IL-6) and biochemical parameters (FBS, HbA1c, TC, TG, albumin, and CBC). Blood in the amount of 8 ml was kept in clotted blood tube to determine hs-CRP, TC, TG, and albumin while 2 ml of blood was kept in sodium fluoride tube for FBS determination. In addition, 5 ml of blood was kept in EDTA containing tube for CBC and HbA1c determination, and this tube was processed for recovery of plasma by centrifugation at 5,000xg for 5 minutes in centrifugal machine (S-8 Boeco, Germany). Plasma was then aliquoted into microtubes and kept at -80 °C until IL-6 was assayed.

3.5.1 Biochemistry Determination

The FBS, HbA1c, TC, TG, and albumin were performed by colorimetry and turbidimetry using clinical chemistry analyzer (ABX-Pentra 400, Horiba, France) at laboratory unit, Public Health Laboratory Division, Bangkok Metropolitan Administration. The hs-CRP was determined by immunoturbidimetry method (Cobas Intrigra 400 plus, Roche diagnostics, Canada) at Bangkok Pathology-Laboratory Co. Ltd., Bangkok. The CBC was determined on automatic blood cell count machine (Pentra 60, Danish Medical Industry Ltd., Denmark) at laboratory unit, Public Health Center 66, Bangkok Metropolitan Administration.

3.5.2 Interleukin-6 Analysis

IL-6 concentration in plasma was detected and measured by the enzyme-linked immunosorbent assay (ELISA) technique (Quantikine Human IL-6 High Sensitivity ELISA Kit®, R&D Systems, USA). The analysis was performed following

the recommendation of the manufacturers (**Appendix E**). A monoclonal antibody specific for IL-6 was pre-coated onto a microplate. Standards and samples were pipetted into the wells, and any IL-6 present was bound with the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 was added to the wells. Following 6 washes to remove any unbound antibody-enzyme reagent, substrate solution was added to the wells. After an incubation period, amplifier solution was added to the wells, and the color developed in proportion to the amount of IL-6 bound in the initial step. The color development was stopped, and the intensity of the color was measured. The final step was determined the optical density using a microplate reader (Perkin Elmer Victor3V, USA).

3.6 Compliance and Adverse Effects of Whey Protein Supplementation

At 6-week of the experimental period, the subjects in WPI group returned the sachets of WPI. The sachets of WPI were counted and calculated for percentage of compliance as follow:

$$\% \text{ compliance} = \frac{\text{number of eaten sachets}}{45} \times 100$$

During 6-week of WPI supplementation, adverse effects were assessed by direct questions.

3.7 Statistical Analysis

Normal distribution of the variances was tested by Shapiro-Wilk, Skewness test, and Kurtosis test. Categorical data were expressed as number and percentage. The descriptive results of continuous variables were expressed as the

mean ± SEM. Chi-Square test was used for comparing frequency data within and between groups. Comparison of continuous data between the initial data and the follow-up data between and within groups were performed using independent t-test and paired t-test respectively. The relationships between some variables were determined by Pearson correlation test. The level of statistical significance was set at $p < 0.05$.