CHAPTER III
MATERIALS AND METHODS

This study was conducted to evaluate the effects of garlic extract in STZ-induced diabetic rats. The major research question that was posed in this study whether hypoglycemia effect of garlic extract is true for STZ-rat model or not. If it is true, the further question is how it can effect on insulin level. To do so, tolbutamide was chosen as a comparative hypoglycemic agent. It is important to notify that **this part of experiment has been designed only for the comparison of the hypoglycemic effect between tolbutamide and garlic**. Therefore, the other parameters of cardiovascular function were not monitored in the diabetic with tolbutamide treated group. Actually, it has been a number of reports that tolbutamide treatment could caused coronary artery disease (Gerich,1989). Thus, in this study, it is better not to evaluate and compare the effect of tolbutamide on cardiovascular parameters as do in the other groups.

Besides, the effects of dyslipidemia and proteinuria were also observed in concomitant with the effects of garlic extract on cardiovascular system. The beneficial effects of garlic extract on cardiovascular system have been reported by our previous research group work. In this study, the effect of garlic extract on coronary vascular changes will be provided in details by using both scanning election microscope (SEM) and transmission electron microscope (TEM).

Chemical substances

The chemical substances that were used in this study were

1. Streptozotocin (STZ)
2. Chloroform
3. Tolbutamide
4. Sodium pentobarbital
5. Normal saline solution(NSS)
6. Heparin

7. Perfusate buffer (Krebs-Henseleit solution) pH 7.4

7.1 NaCl	118.00	mMol/L
7.2 KCl	4.70	mMol/L
7.3 $CaCl_2$	2.52	mMol/L
7.4 $MgSO_4$	1.66	mMol/L
7.5 $NaHCO_3$	24.88	mMol/L
7.6 KH_2PO_4	1.18	mMol/L
7.7 $C_6H_{12}O_6$	5.85	mMol/L
7.8 Bovine serum albumin	200	g/100 ml.

8. 3% Glutaraldehyde in phosphate buffer pH 7.4

Instruments

1. Electric Blender
2. Filter paper No.
3. Separating Funnel
4. Rota vapour RE 121
5. Small animal respirator(Harvard rodent ventilator model 683)
6. Polyethylene tube (Adams PE 50)
7. Pressure Transducer (Nikhon model TP 300 T)
8. Polygraphy (Nikhon RM 6000)
9. Electromagnetic flow probe (Nikhon model FB-020 T)
10. WOULFF bottle with three necks
11. Heat Exchanger
13. Thermoregulator (Grant)
14. Isotonic force transducer (Nikhon model TD-112 S)
15. Hemoglucometer (Reflolux-S) and Hemoglucostrip
16 Metabolic cage

Garlic Extraction

The procedures of garlic extraction were followed those described by Poolsanong (1984). Garlic bulbs purchased from the market were used for extraction. The dry outer scales were removed, then they were washed and dried. 100 g.of the

bulb was homogenized with 120 ml. of chloroform. The mixture was filtered by a four layers of fine muslin cloth and kept overnight in separating funnel. The chloroform fraction was then drawn off through filter-paper No.1 and chloroform was allowed to evaporate by Rota vapour RE 12 at 55°c. The resulting garlic extract had yellow color with pungent smell and the density about 1100 mg/ml. The extract was examined for allicin (diallyl disulfide) by mass-spectro gas chromatography (MSGC).

Animal Preparation

72 male Wistar Furth rats (Salaya Research Animal Center, Mahidol University) weighting 130-160 g. with age of 4-5 weeks were used. The animals were housed (6 rats per cage) in an air condition room about 25°c. They were fasted overnight before each induction STZ-injection. The animals were separated into four groups :-

1 . Controls group (n=18) : The animals had received intraperitoneal injection of normal saline solution (NSS).

2. Diabetic group (STZ-rats, n=18) : The animals were induced by a single intraperitoneal injection of 70 mg/kg.BW. of STZ (Sigma Chemical Co., St.Louis, MO, USA) which was dissolved in NSS. After 24-48 hours, blood glucose were monitored by hemoglucometer. Diabetic condition was confirmed by blood glucose concentration of 400 mg/dl or higher.

3. Diabetic with garlic extract treated group (STZ-G rats, n=18) : The animals were injected STZ same as STZ-rats and received daily oral feeding of garlic extract with the dose of 100 mg/kg.BW. starting at 1 day after the STZ-injection until the day of experiment.

4. Diabetic with tolbutamide treated group (STZ-T rats, n=18) : The animals were injected STZ same as STZ-rats and received daily oral feeding of tolbutamide with the dose of 0.25 g/kg.BW. starting at 1 day after the STZ-injection. As it was noted before that this group was used only for comparison of hypoglycemic effect. Therefore, at 8,16 and 20 weeks following STZ-injection, the blood sample was collected from the right carotid artery via heparinized polyethylene tube for further blood glucose and insulin analysis. (The experiment of isolated heart has not been

performed for this STZ-T group according to the reason explained previously in the beginning of this chapter).

All animals were allowed free access to water and standard chow throughout the study. In each group, they were used in the performing of isolated heart experiment at 8,16 and 20 weeks after the NSS or STZ injection. Such these group of 6 rats were referred as the three different aged group as concluded in the following diagram :

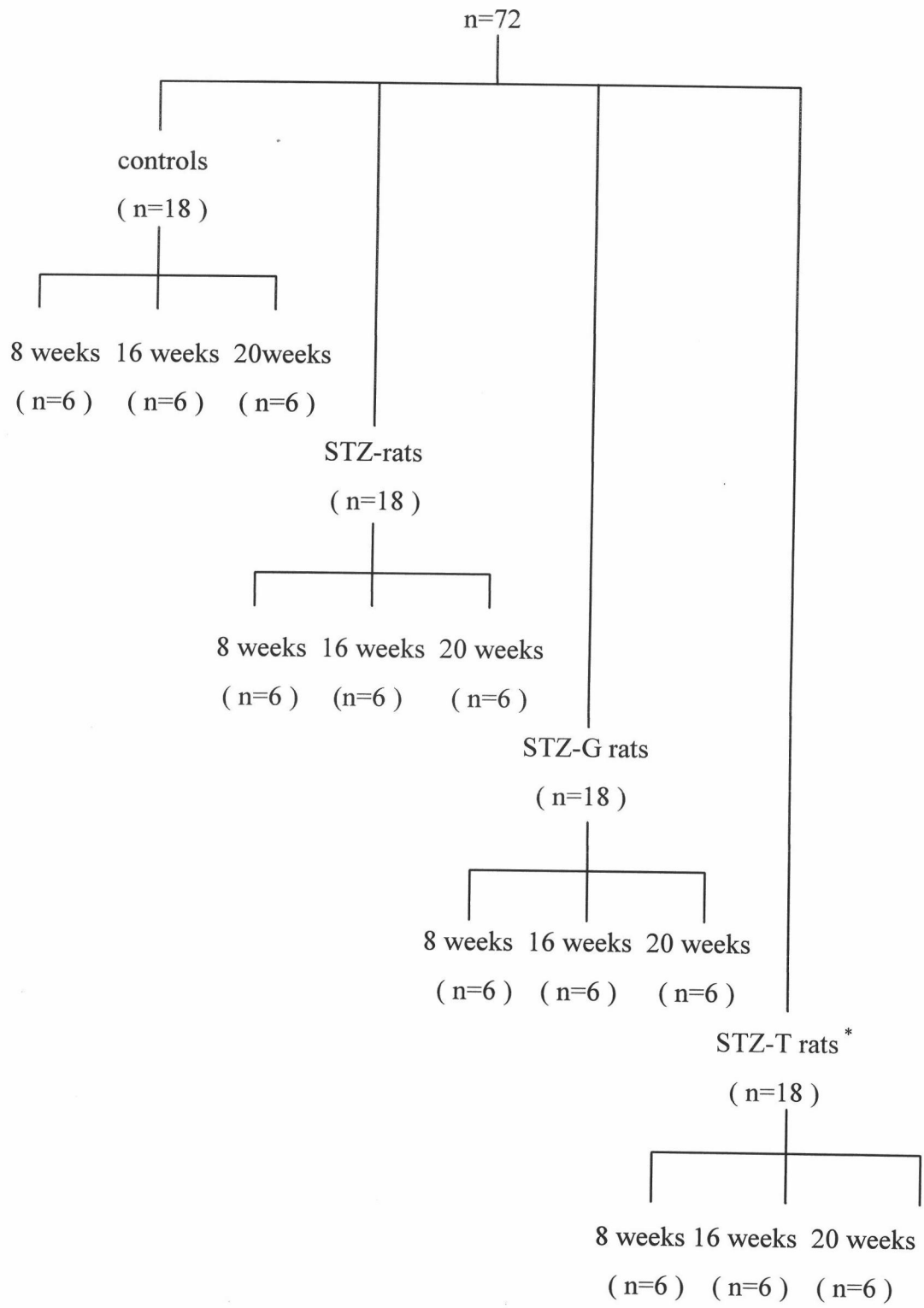

Fig.2 Diagram of Experimental Group

* As noted in the text, this STZ-T group was used only for the study of hypoglycemic effect.

In the present study, the monitored parameters were divided into three groups as followed :-

1. Cardiovascular parameters used to study the effect of garlic extract on STZ-rats were

1.1 Systemic arterial pressure

- Systolic blood pressure (SBP)
- Diastolic blood pressure (DBP)
- Mean arterial pressure (MAP)

1.2 Heart rate (HR)

1.3 Aortic flow rate (AFR)

1.4 Coronary flow rate (CFR)

1.5 Left ventricular isotonic contraction (LVIC)

1.6 Wet weight of heart

Note : Parameters 1.1,1.2 and 1.3 were recorded in the intact heart

Parameters 1.4 and 1.5 were investigated in the isolated heart with perfusate system

Parameter 1.6 was weighed at the end of experiment

2. To study the effect of diabetes and garlic extract on metabolic changes, the following parameters were monitored :

2.1 Blood glucose

2.2 Serum insulin

2.3 Cholesterol

2.4 Triglycerides

2.5 HDL cholesterol

2.6 Protein in urine

3. To study the effect of diabetes and garlic extract on coronary vascular structures and vascular wall thickness the scanning electron microscope (SEM) and transmission electron microscope (TEM) were used.

Methods

One day before the day of experiment, the animal was transferred to metabolic cage for recording of 24 hours urine volume. The collected urine sample was kept at 0^{o}c for further analysis of protein content. The animals were weighed and anaesthetized by intraperitoneal injection of 45 mg/kg.BW. of sodium pentobarbital. After tracheostomy, animals were ventilated with a small animal respirator (Harvard Rodent model 683). A catheter (PE-50 polyethylene tube) which filled with heparinized saline (0.5 unit/ml) was cannulated into the right common carotid artery toward the aortic arch. Systemic arterial pressure and heart rate were monitored via the catheter which was connected to a pressure transducer (Nikhon model TP-300 T) which recorded on polygraph (Nikhon RM 6000).

The opened chest was done to expose the heart. The pericardial sac was carefully removed. Three vessels, the right subclavian artery, the innominate artery and the arch of aorta were then loosely ligated (Fig.3). The aortic flow rate was measured using a 2 mm. diameter flow probe (Nikhon model FB-020 T) which was placed on the ascending aorta. Furthermore, the ligature of the right subclavian artery was tied and 150 units of heparin was injected into the right atrium. The right common carotid artery was then connected to the perfusate system which was kept at 37^{o}c and continuous bubbled with 95% O_2 and 5% CO_2 gas mixture. The constant pressure perfusate system was set at 60-70 mmHg (Fig.4). The right atrium was then quickly cut, blood samples were taken. The ligature on the aorta was then tied, directing the perfusate flow retrograde to the coronary circulation. The hearts were then carefully removed from the animals. After the hearts were allowed to equilibrate for 15 minutes, the coronary flow rate was measured as the volume of fluid that vent out from the cut right atrium per unit time. The left ventricular isotonic contraction (LVIC) was recorded through the wire hooked at the apex of left ventricle and connected to isotonic transducer (Nikhon model TD-122 S) with preload 5 grams (Fig.5).

At the end of each experiment, the hearts were disconnected from the perfusate system and weighed.

Vascular Morphology

All specimens were obtained from the heart of the experimental animals including 3 control rats, 3 STZ-rats and 3 STZ-G rats after 8, 16 and 20 weeks following STZ-injection. The specimens under investigation were excised from the heart walls which were 7 mm. From the proximity of the ascending aorta (Fig.6). The first portion was 1 mm. In thickness and fixed in fixative solution (3% glutaraldehyde in phosphate buffer, pH 7.4) for scanning electron microscope (SEM). The second specimen was taken from the left coronary artery, and was minced into small pieces. They were fixed in fixative solution for transmission electron microscope (TEM).

Preparation for Scanning Electron Microscope (SEM)

After rinsing in buffer and dehydration in a graded series of ethanol, critical point drying was carried out with CO_2 as the transition fluid. Specimens were secured, surface upwards, to the stud with double-side tape, surrounded with a conducting silver paint, and sputter-coated with gold. Examination and photography of the small arteries (50-70 mm in diameter) and arterioles (10-20 mm in diameter) at the 1000 and 1500 magnification, respectively. The thickness of vascular wall was randomly measured at 3 positions and numerical values were reported as means ± SD (As shown in Fig.7).

Preparation for Transmission Electron Microscope (TEM)

The specimen were prepared for TEM by post fixing with 1% osmium tetroxide in 0.1 M cacodylate buffer, then stained in saturated aqueous uranyl acetate solution, dehydrated in acetone and infiltrated using experiment. The ultrastructural details were carried out. In random electron micrographs, transverse sections of capillaries (5-10 mm in diameter) were selected at original magnification of 6000x, then each capillaries were photographed again at the magnification of 25000x. The widest and the narrowest parts of the basement membrane of each capillaries were recorded and as means ± SD (As shown in Fig.8).

Note: the morphological examination was operated by technician of Division of Electron Microscope, Department of Pathology, Faculty of Medicine, Chulalongkorn University.

Laboratory Analysis

Blood glucose concentration was determined by the glucose oxidase method (Trinder,1969). Serum insulin was detemined by radioimmunoassay. Serum concentration of cholesterol, triglycerides and HDL were assayed by enzymatic colorimetric method (Gordon et al.,1977; Meiattini et al.,1978; Fossati and Prencipe,1982). The urine protein concentration was determined by photometric colorimetric method.

Note: These analysis were performed at Bangkok R.I.A. Lab Co.,Ltd. That have the daily internal quality control and external quality control with Faculty of Medical Technology , Mahidol University.

Statistical Analysis

Results were expressed as means ± SD, and n is the number of animals. Statistical analysis was performed using the Student's unpaired t-test. Significance was accepted at p less than 0.05, 0.01 or 0.005.

$$\text{Hypoglycemic effect (\%)} = \frac{{}^{STZ}BG - {}^{STZ\text{-}G}BG}{{}^{STZ}BG - {}^{STZ\text{-}T}BG} \times 100$$

$$\text{Insulin effect (\%)} = \frac{{}^{STZ}\text{insulin} - {}^{STZ\text{-}G}\text{insulin}}{{}^{STZ}\text{insulin} - {}^{STZ\text{-}T}\text{insulin}} \times 100$$