CHAPTER III
MATERIALS AND METHODS

This experiment was designed to investigate the effects of garlic extract on coronary arteriolar responses to vasoactive agents in streptozotocin-induced diabetic rats (STZ-rats). Garlic extract in form of garlic oil was prepared by using chloroform extraction. Male Wistar rats were used in this study. These rats were separated into three groups: controls, STZ-rats and garlic-treated STZ-rats. And, in each group were also separated for study at 8, 12 and 16 weeks. In this study, the modified Langendroff method were used. The isolated arrested heart was prepared to investigate coronary arteriolar responses to acetylcholine (10^{-4}M), sodium nitroprusside (10^{-4}M), norepinephrine (10^{-4}M) and indomethacin (10^{-4}M). Responses to topical application of these agents were recorded with real time fluorescence microscopic system. And then were further off-line analysis using image processing program.

Chemical substances

Streptozotocin (STZ)

Garlic extract

Fluorescein isothiocyanate-labeled dextran, molecular weight 150,000 (FITC-Dx-150)

Acetylcholine (Ach)

Sodium nitroprusside (SNP)

Norepinephrine (NE)

Indomethacin

Heparin

Nembutal

Perfusate compositions	mM
NaCl	80.0
KCl	39.7
$CaCl_2$	2.5
KH_2PO_4	1.2
$MgSO_4.7H_2O$	1.2
$NaHCO_3$	25.0
Glucose	5.0
Bovine serum albumin	2 g/100 ml
pH = 7.4	
$O_2 : CO_2$ = 95% : 5%	

Garlic extract

The procedures of garlic extraction were followed those described by Poolsanong (1984). Garlic cloves purchased from the market were used for extraction. The dry outer scales of fresh garlic cloves were removed. Then, those cloves were extracted by using chloroform solution. Each 100 gram of the cloves were mixed and blended with 120 ml chloroform until the good mixture were obtained. After filtering out the residue, chloroform was extracted from this mixture by using Rota Vaporizer at 55°c. Finally, the filtrate which was the yellowish oily liquid was obtained. In this study, this oily garlic extract were prepared every two week, therefore, povidone, a kind of preservative agent, was not required. The allicin extract obtained can be examined by Gas Chromatography (Saifon Saritdikul, 1993).

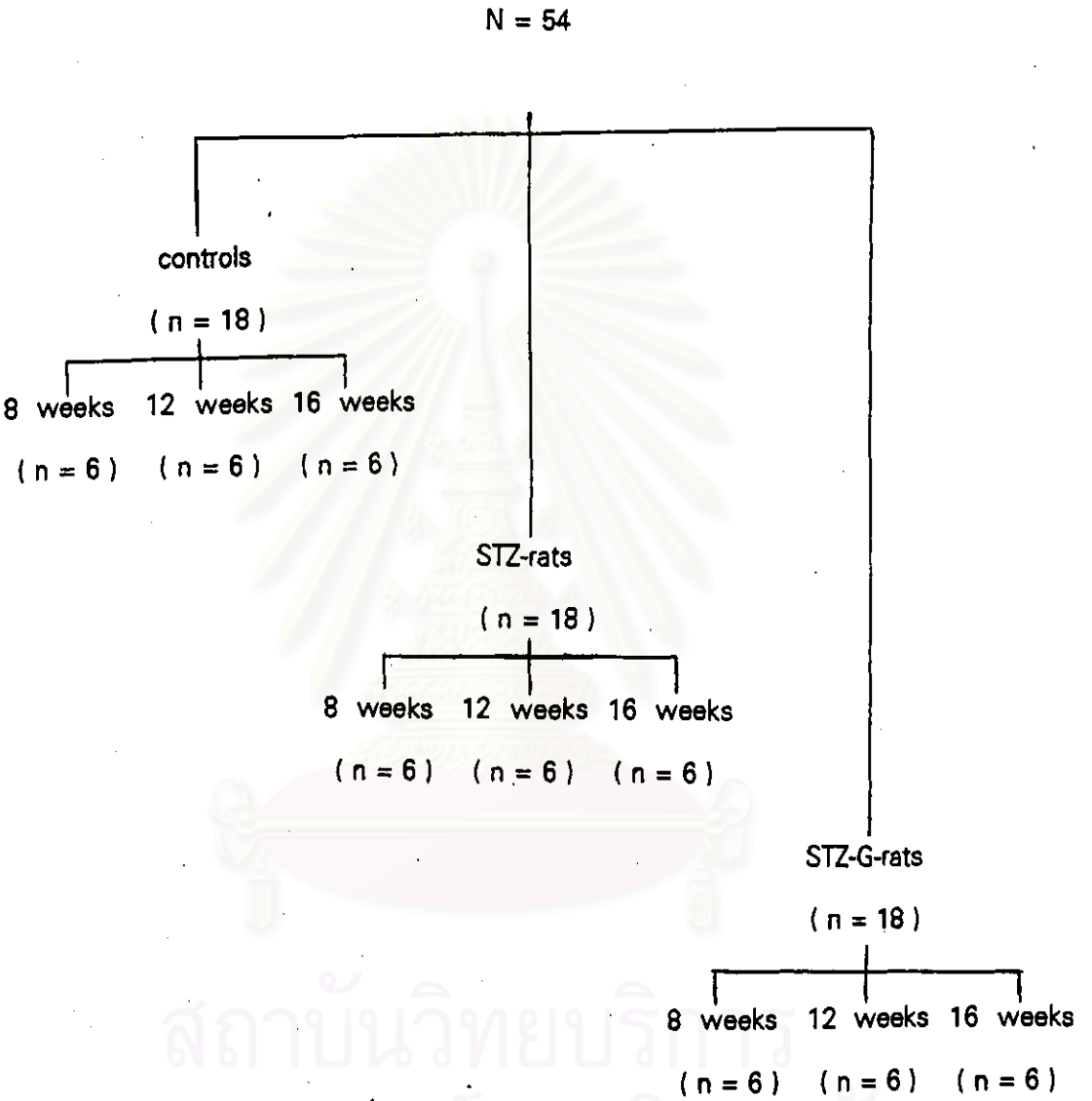

Fig. 3 Diagram of experimental animal groups.

Animal preparations

Male Wistar rats (n = 54) weighing between 150-160 gm with aged of 4-5 weeks were used in this experiment. All rats were fasted overnight before the diabetic induction. these rats were separated into three groups:

1) Control group (n = 18) : the animals were recieved normal saline solution by tail vein injection.

2) Diabetic group (n = 18) : the animals were recieved single tail vein injection of STZ 55 mg/kg of body weight.

3) Diabetic with garlic extract group (n = 18) : these animals were recieved the STZ injection as same as the diabetic group. Moreover, this group was treated daily with crude garlic extract via oral feeding with the dose of 100 mg/kg of body weight. This feeding was started one day after STZ injection until the experiment was performed. Nevertheless, in diabetic group was treated daily with normal saline solution in stead of garlic feeding.

Starting three days after STZ injection, the hyperglycemic state was confirmed by polyuria, polyphagia, polydypsia and blood glucose concentration of 400 mg/dl or higher. If the animals had not met the diabetic criteria of blood glucose concentration of $\geq$ 400 mg/dl, they were omitted in this investigation.

In each group, the animals were used in the performing of isolated heart experiment at 8,12 and 16 weeks after the normal saline or STZ injections. Such these groups of six rats were refered as the three different groups as showed in Figure 3.

Isolated rat heart preparation

Male Wistar Furth rats weighing about 200-400 g were used in this experiment. These animals were initially anesthetized with intraperitoneal injection (ip) of 45 mg/kg body weight of sodium pentobarbital. After tracheostomy, animals were respirated with a small animal respirator (Harvard rodent model 683). Then, a catheter (PE-50) was inserted into the

common carotid artery untill it reach the aortic arch. The common carotid arterial pressure (CAP) was recorded via this catheter by using pressure transducer (Nikhon model TP-300T) that connected to the polygraph (Nikhon RM 6000). The chest was opened with a medial sternotomy, the heart and great vessels of the heart were exposed. Then, the pericardial sac was carefully removed. Loose ligatures were placed around the right subclavian artery and arch of aorta. The heart was arrested and isolated by the modified Langendorff method (McDonagh et al., 1984). Before the isolation of the heart, the value of aortic flow rate was measured by the flow probe (Nikhon model FB-020T) which placed on the arch of aorta. After these measurements, the ligature of the right subclavian was tied and 150 units of heparin was injected into the right atrium. The catheter which cannulated the common carotid artery was connected to the perfusate system and the right atrium was then quickly cut open. The aortic ligature was quickly tied after which all perfusate flow was retrograde to the coronary circulation. The heart rapidly arrested upon perfusion because the perfusate contained 39.7 mMK^{+}. Cardiac arrest proved to be a good index that the perfusion circuit was complete and the heart was indeed receiving perfusate. After coronary perfusion was established the heart was carefully cut out of the thoracic cavity. It was placed on chamber for intravital fluorescence microscopy of the left ventricular epicardial microcirculation. At the end of each experiment, the hearts were disconnected from the perfusate system and weighted.

<u>Studies of dose reponses to vasoactive agents.</u>

Vessels selected for this study were third-order arterioles. In normal rats, the coronary arterioles were studied on dose response to acetylcholine, sodium nitroprusside, and norepinephrine at 10^{-7}M, 10^{-6}M, 10^{-5}M, and 10^{-4}M. Each vasoactive agent at different dose was topically applied on the same arterioles. This design to investigate that coronary arterioles in the isolated arrested heart have physiologic and pharmacologic properties as other studies.

And, the dose that cause maximum response was selected for future study about coronary arteriolar response to vasoactive agents.

Studies of coronary arteriolar responses to vasoactive agents

Vessels selected for this study were third-order arterioles ranging in size for 40-55 μm. After isolated heart proparation, about 10 min time peroid was allowed to reach a steady-state level of base-line tone of the arterioles. The base-line vasoactive substances. Between each application, the selected artorioles were washed three times with normal saline and a 10 min washout peroid was used to allow the vessel to return to its control diameter. The responses of coronary arterioles to 1 ml of 10^{-4} M acetylcholine (Ach), 1 ml of 10^{-4} M sodium nitroprusside (SNP), 1 ml of 10^{-4} M norepinephrine (NE), and 1 ml of 10^{-4} M indomethacin (Indo) were determined on the selected arteriole. In this study, intervals of actions of Ach, SNP, NE, and Indo were used about 2,2,3, and 3 min, respectively. The images of selected arterioles in responses to all vasoactive agents and their base-line diameter were recorded on the videotape entirely the experimental peroid. These images were used for further analized by using image processor system. The protocol used for studies of coronary arteriolar responses as showed in Figure 4.

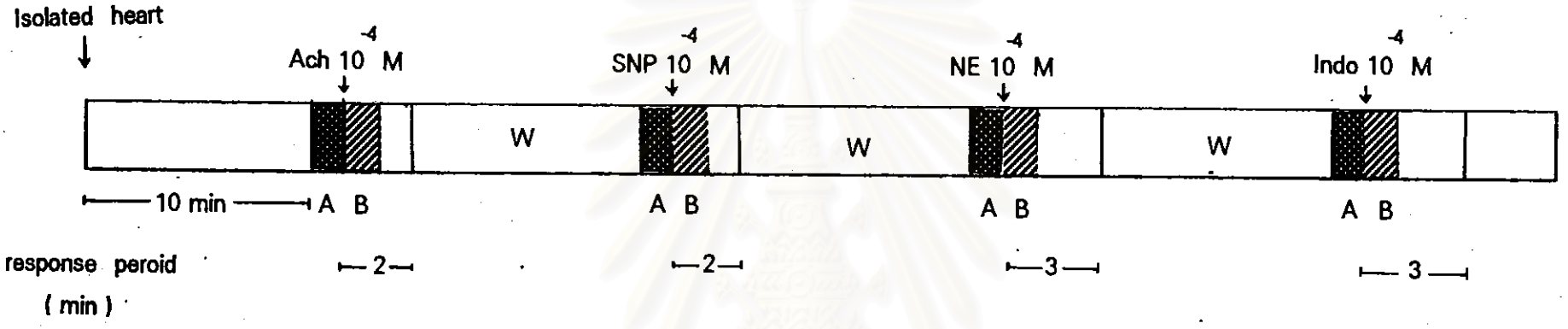

Fig. 4 The protocol used for studies of coronary arteriolar responses .

After isolated heart preparation , about 10 min time peroid was used to reach a steady-state level of base- line tone of selected arterioles . 1 ml of 10^{-4} M acetylcholine (Ach) was obtained at 2 min interval . After a 10 min washout peroid (W) and return to control diameter , 1 ml of 10^{-4} M sodium nitroprusside (SNP) was obtained at 2 min interval . After a 10 min washout peroid , then , contraction to 1 ml of 10^{-4} M norepinephrine (NE) was obtained . Finally , after 10 min washout peroid , 1 ml of 10^{-4} M indomethacin (Indo) was obtained . Images of selected arterioles before topical apllication of these vasoactive agents were recorded on the videotape for a basal diameter (A) . And , the images of these vessels were also recorded on the videotape about 1 min after topical apllication of each vasoactive agents (B) .

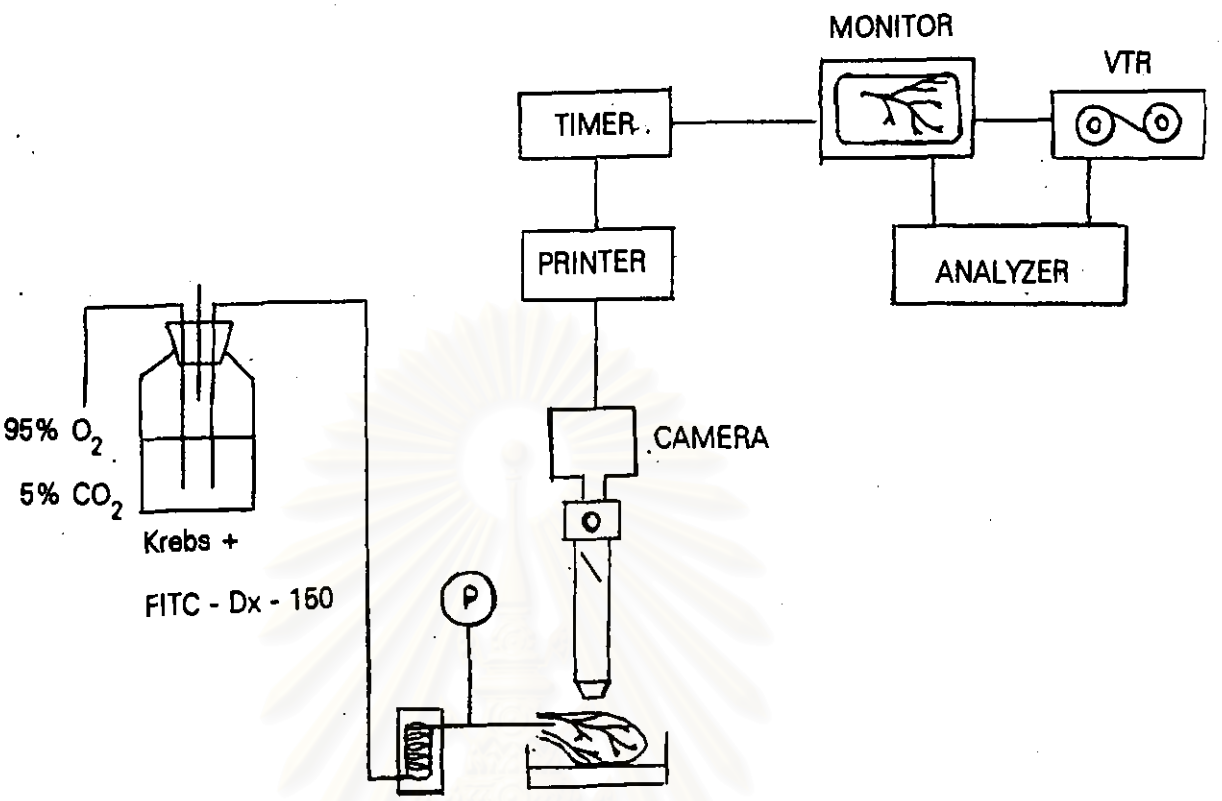

Fig. 5 Isolated heart preparation for direct visualization of coronary microcirculation. (McDonagh , 1983).

Fig. 6 Diameter of selected arterioles (distance between A to B) was evaluated by the software .

Direct visualization of the left ventricular epicardial microcirculation

Video images of the epicardial microcirculation of the left ventricle of isolated arrested hearts were obtained by epi-illumination fluorescent microscopy using fluorescein isothiocyanate-labeled dextran of 150,000 molecular weight (FITC-Dx-150). In this experiment, FITC-Dx-150 was added to the perfusate solution to make the final concentration equal to 5 mg/100 ml. After the FITC-Dx-150 reached to the heart, images of vessels could observe by epi-illumination fluorescence microscopy (Nikhon model optiphot-2). In this experiment, the epi-illumination system consisted of a 50 W mercury lamp with a 488 mm excitation filter and 515 mm emission barrier filter. The image of selected vessels could also observe on a black and white video monitor (Sony, GM-1411 QM) using a silicon intensifier target television camera(Nikon-SIT68) mounted on a fluorescence microscopy using a 20x long working distance objective (CF Achromat). Video images of microvessels were stored on videotape (Sony, SLV-X311) which connected to a video timer (UTG33) for time record. During experiment, microvessel images could print by using video graphic printer (Sony, UP-890CE). Diameter of microvessels were measured from the fluorescence video image of FITC-labeled dextran on the video monitor using digital image processor system. All instruments used for studies of coronary arteriolar functions as showed in Figure 5.

Determination of the arteriolar diameter

Videotapes of each experiment were played back and then the frame of third-order arteriole (40-55 μm) was paused for further digitized into x,y arrays of 512x512 window by using the software called "Gobal Image". The diameter of each selected arteriole (distance between A to B) as showed in Figure.6 was asscessed by the software indicated by number of pixels (n). Using videotape of standarded micrometer scale, the distance between two pixels was previously obtained which equals to 0.658 μm. Therefore, the diameter of each vessels was converted to micrometer by multiplied to 0.658 (= 0.658xn).

Drugs

Chemicals used in this investigstion were streptozotocin, acetylcholine chloride, norepinephrine bitartrate, indomethacin (all from Sigma); sodium nitroprusside (from Merck). Chemicals were prepared in distilled water except for indomethacin which was dissolved in distilled water containing $3x10^{-2}$ M Na_2CO_3. All concentrations are expressed as the final molar concentration and prepared immediately before use.

Data analysis

Data are expressed as means±SE. Statistical analysis of differences between groups was determined by Student's unpaired t-test. P values < 0.05 were considered as significance. In all experiments, n is the number of rats, or arterioles.