CHAPTER III

MATERIALS AND METHODS

The scientific questions posed in our research are:

1) Can we treat the vascular changes in diabetes by ACE-I ? (starting ACE-I after the development of vascular changes).

2) What is the major mechanism of ACE-I that can prevent the cardiovascular changes in diabetes, due to the decrease of arterial blood pressure or the direct inhibition of trophic effect of angiotensin II ?

To experimentally answer the first question, the diabetic rats were recieved cilazapril 10 mg/kg.BW., started 8 weeks after STZ injection (Sebastien et al. (1991). And to answer the second question, the titrated doses of cilazapril: 0.01, 0.05, 0.1, 1, and 10 mg/kg.BW. were used to varify for the dose of non-antihypertensive dose via oral feeding starting at 1 day after the STZ injection until the day of experiment, as the result showed in table 3.1 and Fig. 3.1. From this pilot results, the dose of 0.01 mg/kg.BW. will be used as the non-antihypertensive dose, througout this investigation. Especially, to answer the second question posed in this reseach this dose was used to clarify the mechanism(s) of cilazapril on STZ-induced diabetic model.

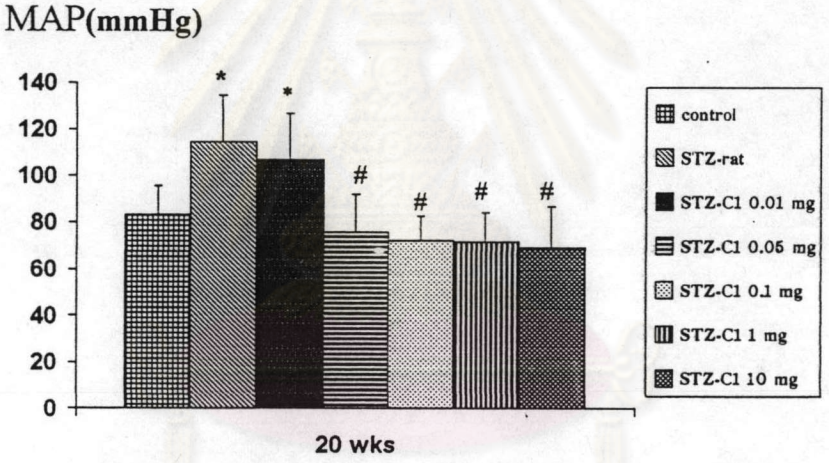
Table3.1 Cilazapril 0.01, 0.05, 0.1, 1, and 10 mg/kg.BW were used to defind the Mean ± SD of Control, STZ-rats, and Cilazapril treated STZ-rats dose of non-antihypertensive effect ,starting 1 day after STZ injection. Mean ± SD of Control, STZ-rats, and Cilazapril treated STZ-rats were monitored at 20 weeks of experimental period.

Groups		MAP (mmHg)
Controls	(n=5)	83.33±12.34
STZ-rats	(n=5)	114.44±15.64*
STZ-C1 0.01 mg	(n=5)	106.94±19.87*
STZ-C1 0.05 mg	(n=5)	75.83±16.29#
STZ-C1 0.1 mg	(n=5)	72.22±10.42#
STZ-C1 1 mg	(n=5)	71.67±12.47#
STZ-C1 10 mg	(n=5)	69.17±17.68#

* Statistical difference as compared to controls ($p<0.05$).

\# Statistical difference as compared to STZ-rats ($p<0.05$).

Figure 3.1 The doses of cilazapril 0.01,0.05,0.1,1,and 10 mg/kg.BW were used to defind the doses of non antihypertensive effect, starting 1 day after STZ injection. Mean $\pm$ SD of MAP (mmHg) monitored at 20 wks of experiments were showed for groups of controls, STZ-rats, and cilazapril treated STZ-rats.



* Statistical difference as compared to controls($p<0.05$).
# Statistical difference as compared to STZ-rats($p<0.05$).

Materials and methods

3.1 Chemical substances

Streptozotocin (STZ)

Cilazapril

Heparin

Sodium pentobarbital

Perfusate solution

	mM/L
NaCl	118.00
KCl	4.70
$CaCl_2$	2.52
$MgSO_4$	1.66
$NaHCO_3$	24.88
KH_2PO_4	1.18
$C_6H_{12}O_6$	5.85
Bovine serum albumin	2g/100ml

pH 7.4

$O_2 : CO_2 = 95\% : 5\%$

3.2 Animal preparations

Male Wistar Furth rats weighing about 100-150 g with age of 4-5 weeks were used in this study (N=45).

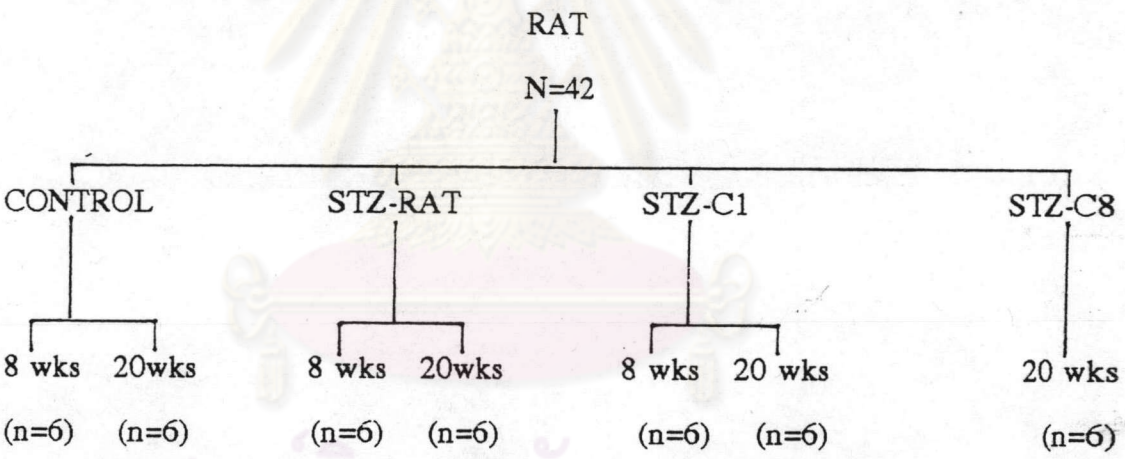
Animals were fasted overnight before the diabetic induction. The animal could be separated into four groups :

1) Control group (NSS) : the animals received intraperitoneal (i.p.) injection of normal saline solution (N=12)

2) Diabetic group (STZ) : the animals received single tail vien injection dose of STZ (50 mg/kg.BW.) (N=12)

3) Dibetic with cilazapril treatment starting at 1 day after STZ injection group (STZ-C1) : These animal received the STZ injections as same as the diabetic group. This group was treated daily with cilazapril 0.01 mg/kg.BW. (non-antihypertensive dose) ,1 and 10 mg/kg.BW. (antihypertensive dose) via oral feeding starting 1 day after STZ injection until the day of performing isolated heart experiment. (N=12)

4) Diabetic with cilazapril treatment starting at 8 weeks after STZ injection group (STZ-C8) : These animal were received the STZ injection. This group was treated daily with cilzapril 0.01, 1 and 10 mg/kg.BW. via oral feeding starting at 8 week after STZ injection until the day of experiment. (N=6)



Methods

On the day of isolated heart experiment, the animal was weighing and then anesthetized by intraperitoneal injection (i.p.) of 30 mg/kg.BW. of sodium pentobarbital after tracheostomy, animal were ventilated with a small animal respirator (Harvard Rodent model 683). Opening the chest was performed to expose the heart. The pericardial sac was carefully removed. Three vessels, the right subclavian artery, the innominate artery, and the ascending aorta were then loosely ligated. Fig 3.2. The aortic flow rate was measureed by the flow probe (Nikhon model FE- 020T) placed on the ascending aorta.

Before the isolation of the heart, the values of aortic flow rate were measured by the flow probe which placed on the ascending aorta. The common carotid arterial pressure (CAP) was recorded via the catheter (PE 180) that inserted into the common carotid artery by using pressure transducer (Nikhon model TP- 300T) that connected to the polygraph (Nikhon RM 6000). After these measurements, the ligature of the right subclavian artery was tied and 150 units of heparin was injected into the right atrium. The common carotid artery catherter was then connected to the perfusate system and the right atrium was then quickly cut open. The ligature on the ascending 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 Fig. 3.3.

The left ventricular isotonic contraction was recorded through the wire hooked at the apex of left ventricle and connected to isotonic transducer Fig. 3.4. With preloaded 5 grams, the patterns of contraction were recorded on the polygraph (Nikhon RM 6000).

At the end of each experiment, the heart were disconnected from the perfusate system and weighed. The top and the apex of each heart were cut off with the size of 2-mm. thickness as showed in Fig. 3.5 and then the heart was cut equally into four pieces. Each piece was about 3-4 mm thick. The second piece was used for further measurement of ventricular wall thickness and coronary wall thickness.

Morphological examination of ventricular wall thickness

The second piece of each heart that was collected by the method described in Fig. 3.5 Then soked with 10% formalin solution. All specimens were obtained totally from three controls, three STZ-rats, three cilazapril treated STZ-rats

at 1 day after STZ injection and three cilazapril treated STZ-rats at 8 week after STZ injection until 20 weeks.

The thickness of left ventricular wall (LV), right ventricular wall (RV) and interventricular septal wall (IVS) were measured by the micrometer of light microscope with 4x-objective. The measuring was performed at five positions of each wall, as showed in Fig. 3.6. Mean and SD of these five values of each wall were calculated and represented as the wall thickness of LV,RV and IVS.

Morphological examination of coronary wall thickness

The second piece of each heart that was collected by the method described in Fig. 3.5 was be fixed and prepared for scaning electron microscope examination by using the method described by Hearle et al., 1974.

Briefly, after rinsing in buffer and dehydration in a graded series of ethanal. Critical point drying was carried out with CO_2 as the transition fluid. Specimens were secured, surface upwards to study with double sided tape, surrounded with a conducting silver point, and sputter coated with gold. Examination and photography of the small arteries (50-70 μm in diameter), arterioles (10-20 μm in diameter), and capillaries (5-10μm in diameter) at the 1000, 1500, and 5000magnification, respectively. The thickness of vascular wall was randomly measured at 3 positions and numerical values were reported as means ± SD.

Statistical analysis

Data are expressed as mean ± SD. Oneway analysis of varience was performed to examine the difference of each parameter between STZ-rats, age matched controls, and cilazapril-treated STZ-rats.

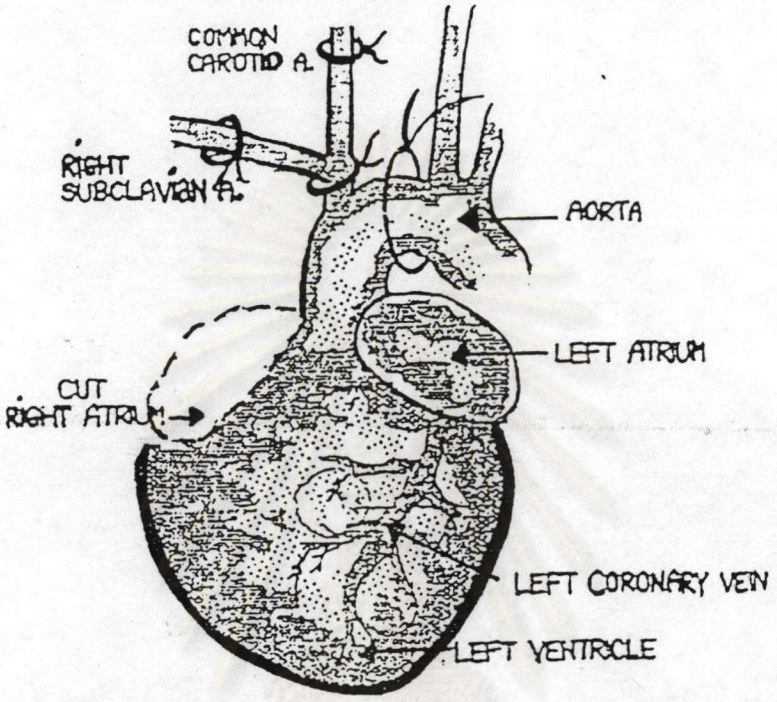


Figure 3.2 Cannulation procedure for perfusing the rat heart prior to isolation :

1. ligate the right subclavian artery
2. insert and secure catheter in common carotid artery
3. cut right atrium after beginning perfusion
4. ligate aorta immediately

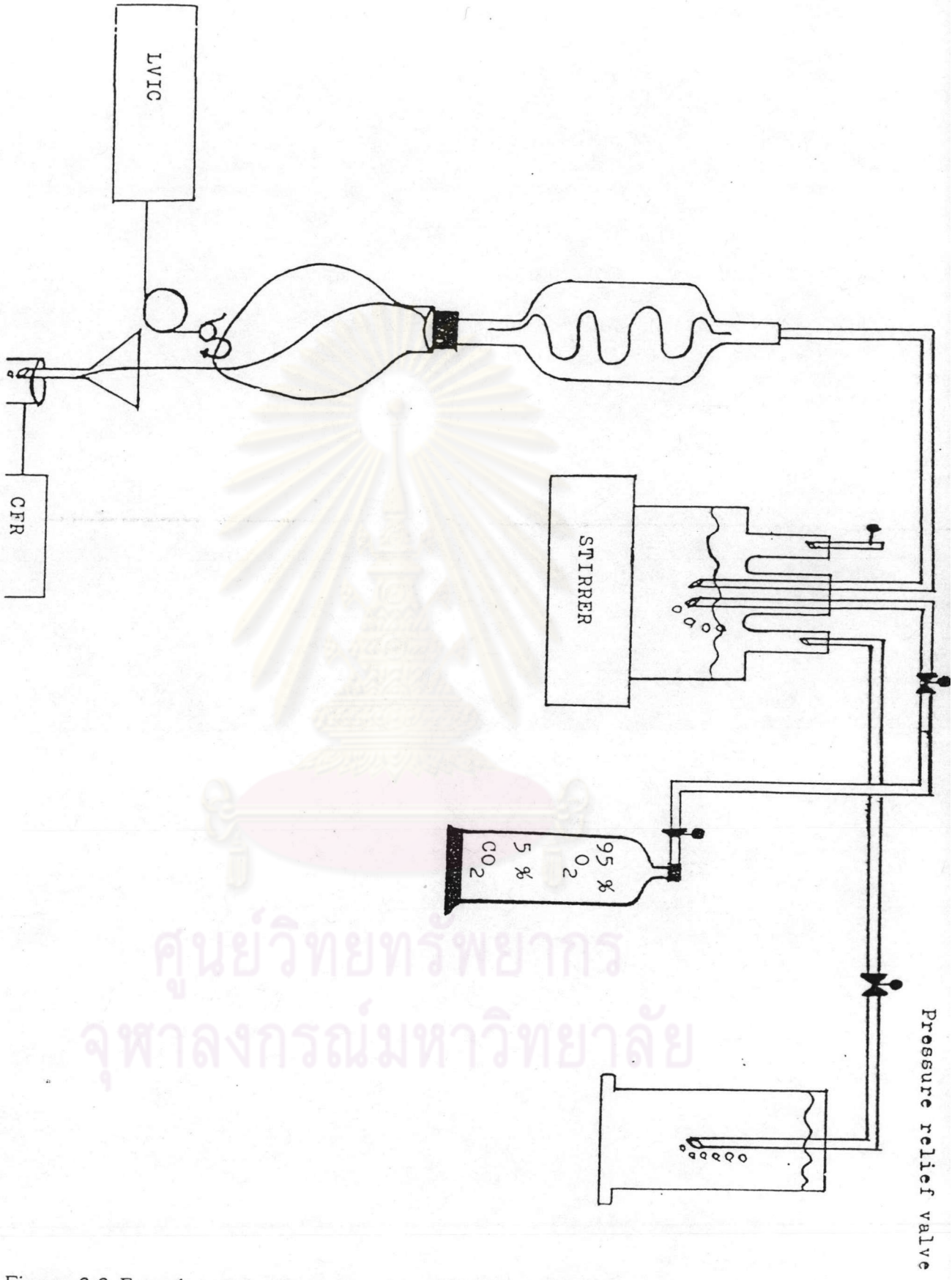


Figure 3.3 Experimental set-up for constant pressure perfusion of the isolated rat heart.

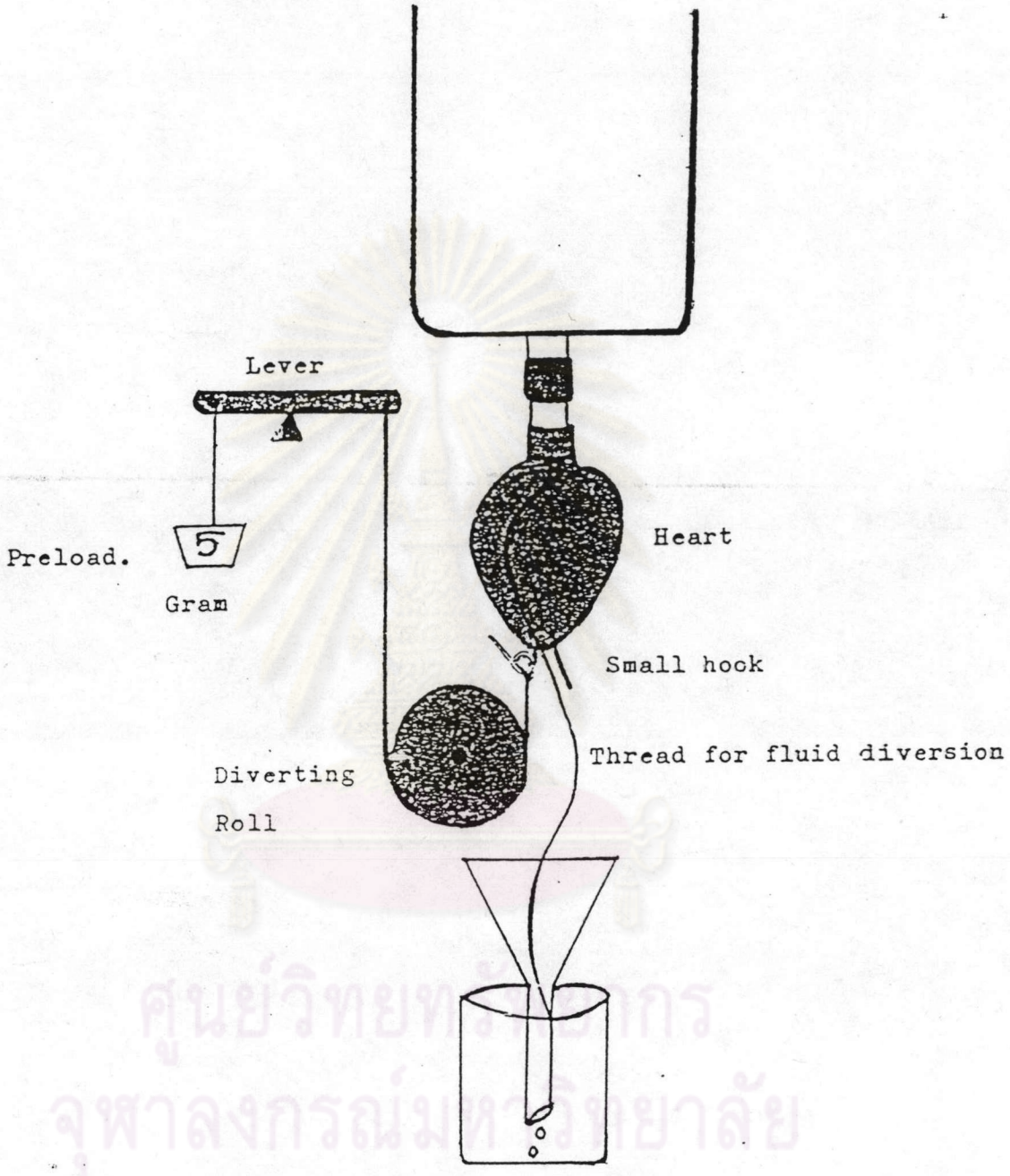


Figure 3.4 The force of contraction of each heart was measured with the preload of 5 gram.

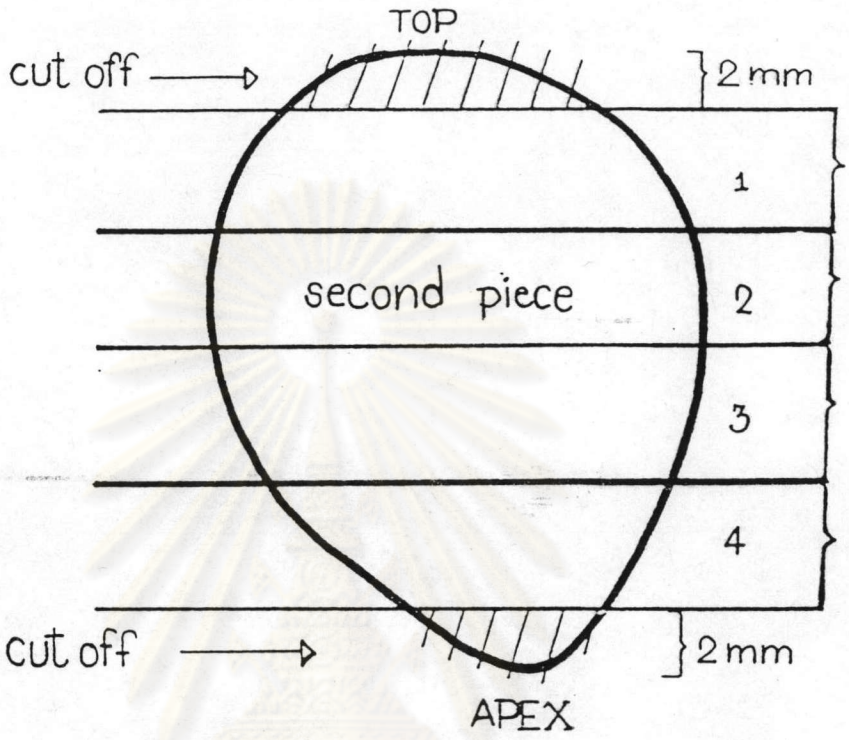


Figure 3.5 The heart was divided equally into four pieces. The thickness of each piece was about 3-4 mm. The second piece was then used for further measurement of venticular wall thickness and coronary wall thickness.

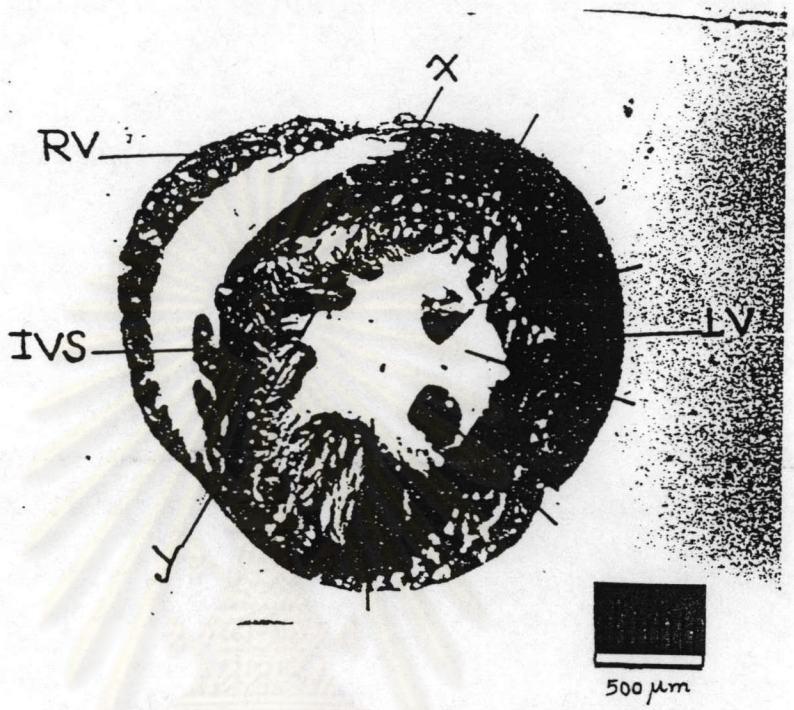


Figure 3.6 The example of five positions were randomly selected for measuring of wall thickness of left ventricle (LV).
The xy-line was located by connecting the points of RV and LV junctions. The walls of left ventricle and IVS were separated by this xy-line.