CHAPTER III

MATERIALS AND MEDTHODS

Materials

1. Animals

1.1 Sexually mature male Wistar albino rats of body weight between 200-250 g were purchased from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom.

Rats were housed two per cage under temperature controlled, 12-hour light/dark cycled with adlibitum access to diet and water throughout the study at the Faculty of Medicine, Srinakharinwirot University.

1.2 Adult female New Zealand White rabbits of body weight between 2.55 –4.35 kg were obtained from the Department of Animals husbandry, Faculty of Veterinary science, Chulalongkorn University.

The rabbits were housed individually in stainless steel cages in a room kept at of 20±2 °C with a 12-hour light-dark cycle in animal House of the Faculty of Medicine, Srinakharinwirot University.

All animals were acclimatized for at least seven days prior to the experiment and procedures involving the use of animals were performed in accordance to the health guidelines on care and use of laboratory animals from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom.

2. Chemicals

Chemical reagents were obtained from the following sources :

Acetylcholine hydrochloride (Ach)	Sigma, USA.
17β - estradiol	Sigma, USA.
Ethylenediaminetetraacetic acid (EDTA)	Merck, Germany.
Hydrochloric acid (HCL)	Merck, Germany.
L – ascorbic acid	Merck, Germany.
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Merck, Germany.
D (+) – Glucose monohydrate	Merck, Germany.
Diabasic sodium phosphate ($Na_2HPO_4.2H_2O$)	Merck, Germany.
Glutaraldehyde	Sigma, USA.
Magnesium sulfate tetrahydrate ($MgSO_4.7H_2O$)	Merck, Germany.
Monobasic sodium phosphate ($NaH_2PO_4.H_2O$)	Merck, Germany.
Noradrenaline (NA)	Sigma, USA.
Potassium chloride (KCl)	Merck, Germany.
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Germany.
Sodium choleate	Sigma, USA.
Sodium chloride (NaCl)	Merck, Germany.
Sodium hydroxide (NaOH)	Merck, Germany.
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Germany.
Sodium nitroprusside (SNP)	Sigma, USA.
Sodium pentobarbital (Nembutal®)	Vet Agritech, Thailand.

Pueraria mirifica

P. mirifica tuber powder, used in the study of high cholesterol fed rats, was obtained from Dr. Amphawan Apisariyakul at the department of Pharmacology, Faculty of Medicine, Chiang Mai University. *P. mirifica* tuber were collected from Amphur Mae Sod, Tak Province during March and April, 2000. *P. mirifica* tuber powder mixture was prepared weekly, by dissolving 6 g of the powder with 100 ml of double distilled water, mixed well, filtered out any remaining fiber with cloth filter and kept in refrigerator.

P. mirifica tuber powder, used in the study of ovariectomized rabbits, was purchased from Dr. Chaiyo Chaichanpitayud at the department of Pharmacognosy, Faculty of Pharmaceutical science, Chulalongkorn University. *P. mirifica* were cultivated at Ampur Mae Rim, Chiang mai Province in March, 2000. Dried *P. mirifica* tuber power 30 g was mixed with 100 ml of double distilled water as before preparation and kept in refrigerator for 1 week.

Experimental diets.

Rat chow.

Rats in the first groups were fed with normal rat chow and assigned as control group. The other groups were fed a diet containing 1 % cholesterol plus 2 % sodium choleate. All diets were purchased from C.P. company.

Rabbit chow.

Rabbits were fed with normal rabbit chow. All diet were purchased from C.P. company.

Preparation of reagents.

Kreb buffer solution.

Composition of the Krebs buffer was 24.0 mM $NaHCO_3$, 1.2 mM $MgSO_4.7H_2O$, 119.0 mM NaCl , 4.7 mM KCl , 1.2 mM KH_2PO_4 , 2.5 mM $CaCl_2.H_2O$, 5.0 mMGlucose and 0.026 mM. EDTA The solution was continuously bubbled with carbogen gas (95% O_2 + 5% CO_2) to obtain a pH 7.4 at 37 °C.

0.2 M Phosphate buffer (GOMORI), pH 7.4.

Stock solution monobasic sodium phosphate 27.6 g was made up to 1000 ml with deionized distilled water and stock solution dibasic sodium phosphate 53.6 g was made up to 1000 ml with deionized distilled water. Then, phosphate buffer pH 7.4 was prepared by stock solution monobasic sodium phosphate 19.0 ml mixed with stock solution dibasic sodium phosphate 81.0 ml.

2.5 % glutaraldehyde.

Prepared 2.5 ml of glutaraldehyde was dissolved in 98 ml of phosphate buffer pH 7.4.

3. Instruments

The following instruments were used :

Analytical balance	(Sartorius analytic, U.S.A.)
Autoclave	
Autopipets 10, 100, 1000 µl	(Gilson, France)
Centrifuges	(International equipments, Germany)
Carbogen gas	(TIG, Thailand)
Isometric force transducer	(AD Instruments, U.S.A.)
pH meter	(Beckman Instruments, U.S.A)
Recorder	
Organ bath	(Double ealled Harvard type)
Scanning electron microscope	(JSM-5410LV Model)
Surgical equipment	
Thermoregulating water pump	(DT Hetotherm, Japan)
Vial	
Vortex mixer	(Scientific Industriey, U.S.A)

Methods

1. Animal treatments

1.1 The study of high cholesterol- fed rats.

30 Rats were randomized to three groups. Each group comprised of 10 animals receiving one of the following daily treatments for 90 days.

Group 1 (Control group) : Rats were fed with normal rat chow and orally administered with double distilled water.

Group 2 (Cholesterol group) : Rats were fed with high cholesterol diet containing 1% cholesterol plus 2% sodium choleate and orally administered with double distilled water.

Group 3 (Cholesterol + *P. mirifica* group) : Rats were fed with high cholesterol diet containing 1% cholesterol plus 2% sodium choleate and orally administered with 100 mg/ kg/ day of *P. mirifica.*

Body weight of all rats were recorded every two weeks throughout the study period.

1.2 The study of ovariectomized rabbits.

Ovariectomy : Female New Zealand white rabbits age 6 months were anesthetized with amobarbital sodium (60 mg/kg ip). The bilateral ovaries were ligated and then removed. Animals were allowed to recover for 3 months with food and water ad libitum.

After 9 months, 20 rabbits were randomly assigned to four group. Each group comprised of 5 rabbits. receiving one of the following daily treatments for 90 days.

Group 1 (Intact Control group) : Normal rabbits were orally administered with double distilled water.

Group 2 (Ovariectomized group) : Ovariectomized rabbits were orally administered with double distilled water.

Group 3 (OVX+ Estrogen group) : Ovariectomized rabbits were orally administered with 4 mg/kg/day of 17β-estradiol.

Group 4 (OVX + *P. mirifica*) : Ovariectomized rabbits were orally administered with 100 mg/kg/day of *P. mirifica.*

Throughout the treatment period, body weight of all rabbits were measured were recored every week.

2. Sample collection

2.1 Blood

2.1.1. Rat : On the day which rats were scarified, blood samples were collected by cardiac puncture. Serum was separated by centrifugation at 3400 g for 10 min and kept at -20°C for the of blood biochemistry.

2.1.2. Rabbit : Blood samples of rabbits were obtained from the central ear vein at the beginning of the experiment and every 4 weeks thereafter. Blood were centrifuged at 3400 g for 10 min and the serum was frozen at -20°C for blood biochemistry analysis.

2.2 Preparation of isolated aorta.

2.2.1. Rats: At the end of the treatment, rats were anesthetized with sodium pentobarbital (Nembutal®) intraperitoneally. Blood samples were taken and aorta was isolated as shown in figure 3.1 and immediately placed in ice-cold Krebs buffer.

2.2.2 Rabbits : After 90 days of treatments, animals were premedicated with sodium pentobarbital (25 mg/kg body weight intramuscular injection) followed by general anesthesia with Nembutal® via the central ear vein (the initial dose was 40 mg and maintenance

dose was added during operation as required). Blood samples were collected and the animals were sacrificed with an overdose of sodium pentobarbital. The aorta was removed and placed in fresh cold Krebs buffer.

The removed aorta as carefully cleaned from adhering fat and connective tissue, then cut into rings of 3-5 mm thick, 4-8 rings per animal. Special care was taken to avoid contact to the luminal surface of the rings in order to preserve the endothelial structure. Rings of aorta were suspended horizontally between two stainless steel hooks for determination of vascular function in individual organ baths containing 25 ml of Krebs buffer at 37°C, bubbled with 95% O_2, 5% CO_2, within 1 hour of death. The aortic rings were connected to force transducers (MacLab 4e) and isometric tension was recorded on a Maclab recorder as shown in Fig 3.2. The ring were equilibrated for 60 min under a resting tension of 2 g to allow development of stable basal tone for the measurement of vascular function.

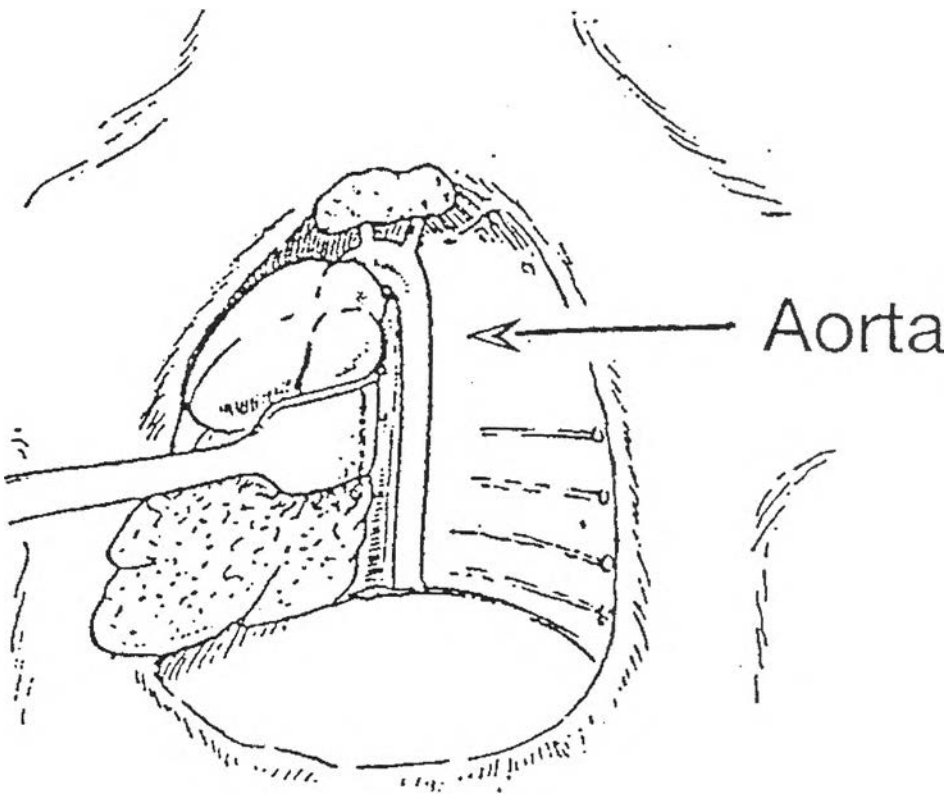

Fig 3.1 **Diagram of the isolated aorta from the male rat.**

3. Determination of blood biochemistry parameters.

Serum samples were determined for blood biochemistry parameter. The evaluation of total cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) (using commercial test kit of bioMerieux company, France) were performed by Faculty of Allied Health Sciences, Chulalongkorn University.

The analysis of HDL-cholesterol and LDL– cholesterol (using commercial test kit of The Roche company, Switzerland) in serum were performed by Professional Laboratory Management, Bangkok, Thailand.

4. Measurement of vascular function.

4.1 Determination of noradrenaline-induced aortic contraction.

The viability of the smooth muscle and endothelium was confirmed by obtaining a contractile response to $1x10^{-6}$ M of noradrenaline and subsequent relaxation response to $1x10^{-6}$ M of acetylcholine. Aortic rings from the control group were used in study if relaxation by $1x10^{-6}$ M acetylcholine reached at least 70 % of noradrenaline induced contraction plateau.

Thereafter, the vascular ring were repeatedly washed with Krebs buffer until the tension was returned to its baseline. A concentration-response curve to noradrenaline was obtained by the addition of increasing concentration of noradrenaline ($1x10^{-9}$ M to $1x10^{-4}$ M). Contraction was measured as the percentage increase from maximum noradrenaline- induced tension. The rings were then washed with Kerbs buffer for 30 min. During this period, buffer in the organ baths was replaced every 10 min, and the tension of vascular ring was adjusted until 2 g of preload was maintained.

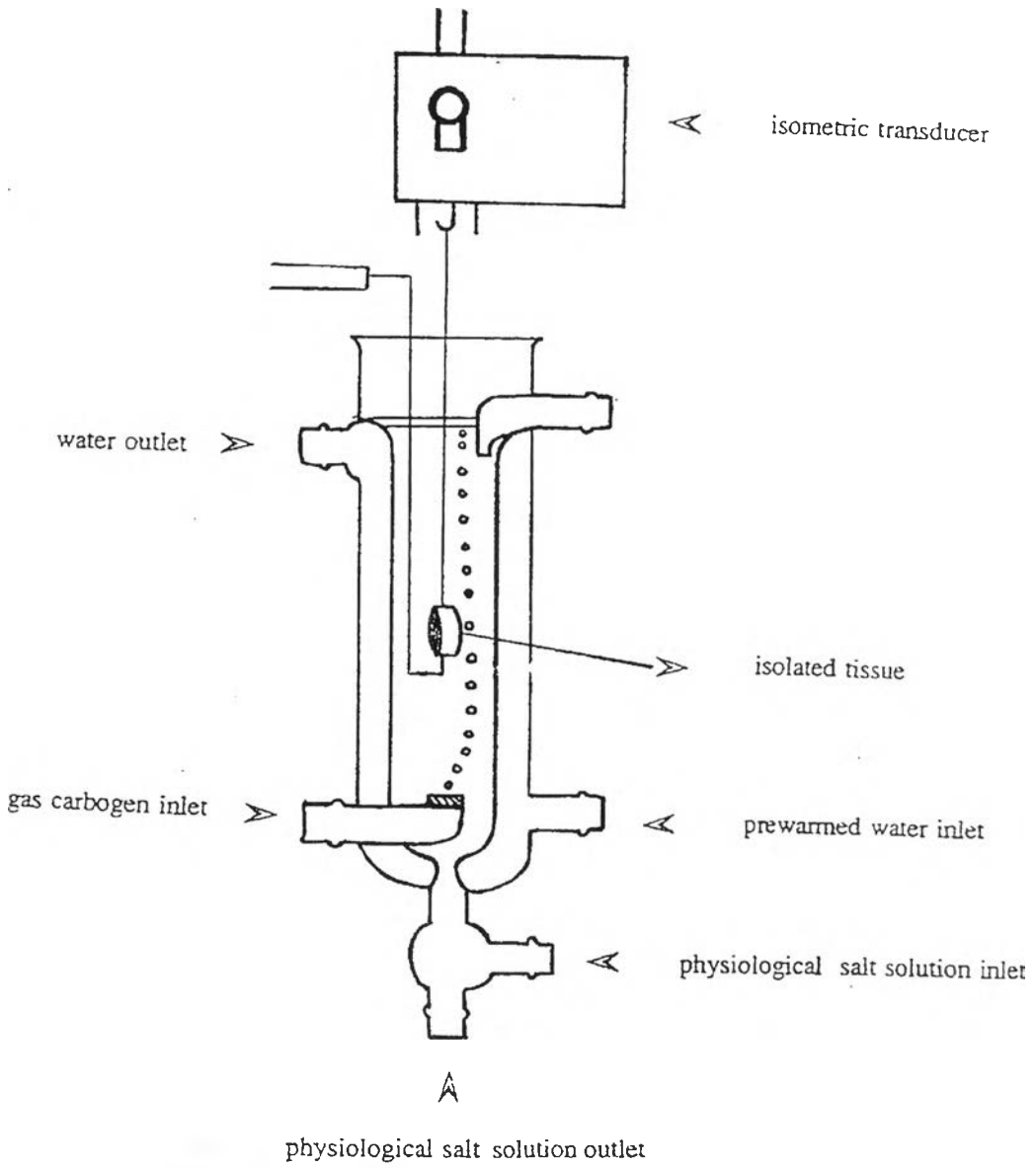

Fig 3.2 Schematic illustration of the experimental preparation used for the measurement of vascular function. The aortic ring was suspended in organ bath containing Krebs buffer and connected to an isometric force transducer.

4.2 Determination of acetylcholine-induced aortic relaxation.

The presence of a normal function endothelium was always verified by observing the relaxation response to acetylcholine. Endothelial control of vascular tone (endothelium-dependent relaxation) was assayed by the addition of acetylcholine.

The aorta rings were precontracted again with $1x10^{-6}$ M of noradrenaline. When the contractile response was stable, a concentration-response curve to acetylcholine ($1x10^{-9}$ M to $1x10^{-4}$ M) was obtained. After the cumulative response was stabilized, the rings were washed and allowed to equilibrate to baseline.

4.3 Determination of sodium nitroprusside-induced aortic relaxation.

Smooth muscle cell vasodilator function (endothelium-independent relaxation) was assayed using sodium nitroprusside.

After equilibration, the rings were contracted with $1x10^{-6}$ M noradrenaline. After a stable contraction was obtained, sodium nitroprusside were added to the bath in cumulative- concentration of $1x10^{-9}$ M to $1x10^{-4}$ M.

All relaxations were expressed as percentage of the initial contraction induced by $1x10^{-6}$ M of noradrenaline.

5. Morphological changes of the endothelium in rabbits.

At the end of the experiments, three to six representative aortic rings from each group were used for structural changes study. Blood was flushed off with phosphate buffer pH 7.4 and fixed in 2.5 % buffered glutaraldehyde for 12 hours. After that the rings were washed 3 time in phosphate buffer (10 minutes x 3). The prepared samples were kept for morphological changes at 4 °C in phosphate buffer.

Tissue were postfixed in buffer osmium tetroxide and dehydrated in grade series of ethanol for scanning electron microscopy study. The sample were critical point dried (CPD), coated with gold and investigated under scanning electron microscope (JSM-5410LV model). The procedures mentioned above were performed at the Scientific and Technological Research Equipment Center , Chulalongkorn University.

Statistics

All data were expressed as mean±standard error of means (S.E.M.) The effective concentrations for 50% of the maximal response (ED_{50}) were calculated for individual concentration-response curves and the mean of these values was reported as the negative logarithm of the molar concentration as initiated by Hafner et al, (1997).

Comparisons of group mean values were made by using one-way analysis of variance (ANOVA) and Scheffe F-test for multiple comparison was applied to identify differences among groups. Using 0.05 significant level of probability.