CHAPTER III

MATERIALS AND METHODS

Materials

1. Animals

32 female Wistar albino rats, body weight between 100-150 g were used. All of them were received from National Laboratory Animal Center (NLAC), Mahidol University, Salaya, Nakornchaisri, Nakornprathom.

All rats were housed at The Faculty of Veterinary Science, Chulalongkorn University and acclimatized for 1 week before the beginning of the experiments. They were housed under 12 hours light/dark cycle at room temperature, diet and water were fed *ad libitum*. They were fed with normal rat chow (CP 082) purchased from C.P. Company, Thailand.

All procedures involving the use of animal were performed in accordance to the health guidelines on care and use of laboratory animals from National Research Council and National Laboratory Animal Center. This research was approved by the Committee on Animal Care and Use, the Faculty of Veterinary Science, Chulalongkorn University.

2. Chemicals

These following lists are chemical reagents used in the experiments and their sources.

- Acetylcholine hydrochloride (Ach)	Sigma, USA
- Ascorbic acid	Sigma, USA
- Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Merck, Germany
- D (+)-glucose monohydrate	Merck, Germany
- Estradiol valerate (Progynon®)	Schering, Germany
- Formic acid	Merck, Germany
- Hydrochloric acid (HCl)	Sigma, USA
- Lanthanum chloride	Sigma, USA
- Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Merck, Germany
- Methanol	Merck, Germany
- N-(1-Naphthyl) ethylenediamine dihydrochloride (NNED)	Merck, Germany
- Nitric acid	Sigma, USA
- Noradrenaline (NA)	Sigma, USA
- Potassium chloride (KCl)	Merck, Germany
- Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Germany
- Sodium chloride (NaCl)	Sigma, USA
- Sodium citrate	Merck, Germany
- Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Germany
- Sodium nitroprusside (SNP)	Sigma, USA
- Sulfanilamide (p-Aminobenzenesulfonamide)	Merck, Germany
- Tiletamine + Zolazepam (Zoletil®)	Virbac, Germany

Pueraria mirifica

Tuberous root extracts of *P. mirifica*, Good Manufacturing Practice (GMP) grade, were obtained in tablet (180 mg/tablet) and manufactured by Kow Laor Co.,Ltd., Samutprakarn, Thailand.

3. Instruments

These following instruments were used in the experiments.

- Analytical balance (Sartorius analytic, USA)
- Atomic absorption spectrophotometer (Variant, Australia)
- Autopipette (Gilson, France)
- Carbogen gas tank and regulator (Thailand Industrial Gas; Thailand)
- Centrifuge (International equipments, Germany)
- Isolated organ bath (double walled Harvard type) (AD Instruments, USA)
- Isometric force processor (PowerLab, USA)
- Isometric force recorder (PowerLab, USA)
- Isometric force transducer (AD Instruments, USA)
- Microplate reader (Tecan A-5082, USA)
- Microtubes (Axygen, USA)
- Minor surgical equipments (Mueller Instrumente, Germany)
- Muffle furnace (Ney Vulcan 3-1750, USA)
- pH meter (Beckman Instruments, USA)
- Vortex mixer (Scientific Industry, USA)
- Water bath (with thermoregulating water pump) (DT Hetotherm, Japan)

Methods

1. Animal treatments

All rats were randomly divided into 4 groups, 8 rats for each group. Group 1, 2 and 3 were operated to remove both of bilateral ovaries, ovariectomy (OVX). Group 4 was undergone laparotomy operation (sham group). All of operated rats were allowed to recover for 4 weeks before the beginning of experiments.

Ovariectomy (OVX)

Female Wistar albino rats were anesthetized with tiletamine + zolazepam (Zoletil®) at a dosage of 40 mg/kg by intraperitoneal injection. Aseptic surgical procedures were performed under anesthetic conditions. Flank region skin and abdominal wall were opened parallel to the last rib, then bilateral ovaries were ligated with absorbable suture materials (chromic catgut 4-0). The ligated ovaries were removed subsequently. The opened skin and abdominal wall were closed with chromic catgut 4-0 and povidone iodine was used as an antiseptic.

After recovery period, each group of rats was received these following treatments. Body weight was measured every week throughout the treatment period.

Group 1: (OVX + *P. mirifica*)

Ovariectomized rats were orally administered with *P. mirifica* at a dosage of 100 mg/kg/day for 42 days.

Group 2: (OVX + Estrogen)

Ovariectomized rats were subcutaneous injected with estradiol valerate (Progenon®) at a dosage of 300 µg/kg once a week, for 42 days.

Group 3: (OVX group)

Ovariectomized rats were orally administered with distilled water for 42 days.

Group 4: (Sham group)

Non-ovariectomized rats were orally administered with distilled water for 42 days.

2. Experimental protocols

2.1 Effects of *P. mirifica* on vascular functions and pathogenicities of thoracic aortas

2.1.1 Preparation of isolated thoracic aortas

At the end of treatments, rats were anesthetized with tiletamine + zolazepam (Zoletil®; 40 mg/kg, IP) and euthanized by thoracic wall opening along the costo-chondral junction line. Blood samples were taken and each of descending thoracic aorta was isolated and immediately placed in ice-cold modified Krebs-Henseleit buffer solution with carbogen gas bubbled.

The isolated aorta was carefully cleaned from adhering fat and connective tissue and gently washed the aortic lumen with pasture pipette, then cut into rings of 5 mm length. Special cares and gently procedures were taken to avoid contact to luminal surface of rings in order to preserve the endothelial structure. Aortic rings were mounted and suspended horizontally between two stainless steel hooks in individual organ baths which containing 25 ml of modified Krebs-Henseleit buffer solution at 37 °C, bubbled with carbogen gas (95% O_2 + 5% CO_2). For determination of vascular functions, one stainless steel hook was fixed and another one was connected to isometric force transducer (AD Instruments). The isometric tension was detected, processed and recorded by PowerLab processor and recorder. The rings were maintained and equilibrated under a resting tension of 1 g for 45-60 minutes to allow development of stable basal tone for the vascular functions measurements.

2.1.2 Measurements of vascular functions

After incubation period, the vascular functions measurements were performed. The contraction and relaxation of aortic rings were detected as the change of isometric tension. The tension was recorded and processed by Chart 5 for Windows program (AD Instruments).

2.1.2.1 Determination of noradrenaline-induced aortic contraction

The viability test was performed to ensure the stabilization of vascular smooth muscle and endothelial cells. The aortic rings which response after induce at least 50% of 10^{-6} M acetylcholine-induced relaxation in 10^{-6} M noradrenaline pre-contracted were used in subsequent studies. Viable aortic rings were repeatedly washed with modified Krebs-Henseleit buffer solution until the tension was returned to baseline.

A concentration-response curve to noradrenaline was obtained by the addition of noradrenaline in cumulative concentration of 10^{-9} M to 10^{-4} M. Vascular contraction was measured as the percentage increase from baseline compared with maximum noradrenaline-induced tension.

After that, the rings were washed with modified Krebs-Henseleit buffer solution for 30-45 minutes. During this period, buffer solution in organ bath was replaced every 10-15 minutes and resting tension was adjusted to baseline.

2.1.2.2 Determination of acetylcholine-induced aortic relaxation (Endothelium-dependent vascular relaxation)

Endothelium-dependent vascular relaxation was assayed by the addition of acetylcholine in cumulative concentration.

Noradrenaline (10^{-6} M) was used to pre-contract the aortic rings. When the contractile response was stable or reached the steady maximal response, a concentration-response curve to acetylcholine was obtained by the addition of acetylcholine in cumulative concentration of 10^{-9} M to 10^{-4} M.

Vascular relaxations were expressed as percent of relaxation of the contraction induced by NA. Reversal of tone back to baseline value was taken as 100% relaxation.

After the study finished, the rings were washed and resting tension was adjusted to baseline.

2.1.2.3 Determination of sodium nitroprusside-induced aortic relaxation (Endothelium-independent vascular relaxation)

Vascular smooth muscle vasodilation (endothelium-independent vascular relaxation) was assayed by the addition of sodium nitroprusside in cumulative concentration.

After equilibrated, the rings were pre-contracted with 10^{-6} M noradrenaline. When the contractile response was stable or reached the steady maximal response, a concentration-response curve to sodium nitroprusside (cumulative concentration; 10^{-9} M to 10^{-4} M) was obtained.

Vascular relaxations were expressed as percent of relaxation of the contraction induced by NA. Reversal of tone back to baseline value was taken as 100% relaxation.

2.1.3 Histopathological study of thoracic aortas

The individual isolated aorta was cut into a piece of 5 mm length and then fixed in 10% phosphate buffer formalin for 24 hours. The fixed tissues were blocked in paraffin-based bedding and cut with ultra-sectioning microtome into the pieces of 5-6 microns thickening. The tissues were fixed on glass slides and then dyed with Hematoxylin-Eosin (H&E) stain. Microscopic examination was performed to study the pathogenicities of thoracic aorta.

2.2 Effects of *P. mirifica* on bone mass and pathogenicities of femoral bones

2.2.1 Preparation of femoral bones

At the end of treatment, rats were anesthetized with tiletamine + zolazepam (Zoletil®; 40 mg/kg, IP) and euthanized by thoracic wall opening. After blood samples and thoracic aorta isolation were taken, both of femoral bones were dissected and isolated. The adhering muscles and tendons of bones were cleaned off by gauze rubbing. All of right femurs were kept in sealed plastic bags under temperature of 0 °C. The left femurs were fixed in 10% phosphate buffer formalin.

2.2.2 Measurements of relative bone weight, relative ash weight and bone calcium

The frozen (right) femurs were thawed at room temperature for 1 hour. The bones were dried at 180 °C for 2 hours and weighted. After that, they were ashed by muffle furnace at 600 °C for 16 hours and weighted. Relative bone weight and relative ash weight were calculated in comparison with body weight of individual rats. Bone ashes were then dissolved in 10 ml of 10% nitric acid and 10,000 ppm of lanthanum chloride was added. Bone calcium determination was performed by flame ionization-atomic absorption spectrophotometer (FI-AAS).

2.2.3 Histopathological study of femoral bones

The left femoral bones were fixed in 10% phosphate buffer formalin for 1 week, then cut in longitudinal section and fixed again in decalcifying agent for 2 weeks. The decalcified bones were blocked in paraffin-based bedding and cut with ultra-sectioning microtome in to the pieces of 5-6 microns thickening. The tissues

were fixed on glass slides and then dyed with Hematoxylin-Eosin (H&E) stain. Pathogenicities of femoral bones were studied under microscopic examination.

2.3 Effects of *P. mirifica* on blood biochemistry parameters

2.3.1 Blood sample collection

Blood samples of rats were obtained at the sacrificial day. Samples were collected by cardiac puncture and heparinized saline was used as an anticoagulant. Plasma was separated by centrifugation at 3,000 g for 15 minutes and then kept at -20 °C for determination of biochemistry parameters.

2.3.2 Determination of blood biochemistry parameters

2.3.2.1 Determination of nitric oxide (NO) level

Protein precipitated plasma samples were determined for nitric oxide (NO) level with colorimetric non-enzymatic assay (Tsikas, 2006). Cadmium (Cd) beads were used in conversion of nitrate to nitrite. Griess reagents, color reagent 1 and color reagent 2, were added respectively. Absorbances of colorized samples (violet-red) were measured with microplate reader at 540 nm of wavelength.

2.3.2.2 Determination of alkaline phosphatase (ALP) level

The evaluations of alkaline phosphatase (ALP) in plasma samples were performed by The Faculty of Veterinary Science, Chulalongkorn University. Commercial test kits of bioMerieux Company, France were used.

2.3.2.3 Determination of lipid profiles

The analysis of total-cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride in plasma samples were assayed by The Faculty of Allied Health Sciences, Chulalongkorn University. Commercial test kits of The Roche Company, Switzerland were used in these evaluations.

Statistics

All data were presented as mean ± standard error of mean (S.E.M.). Comparisons of group mean values were performed by using one-way analysis of variance (one-way ANOVA) and multiple comparisons were used to identify differences among groups. 95% significant level of probability ($p < 0.05$) was used.