CHAPTER III

MATERIALS AND METHODS

Animal Preparation

Experiments were carried out on twenty five adult male mongrel dogs, weighing 9-13 kilograms. The animals were deprived of food but not of water for 12 hours prior to study. On the day of the experiment, the animals were anesthetized with sodium pentobarbital (Nembutal) 30 mg/kg.bw. intravenously injection initially, and supplemented subsequent doses of 1-2 mg/kg.bw. when necessary to maintain anesthetic condition throughout the experiment.

The animals were tracheostomized, then endotracheal tube was inserted to free airways. Two femoral veins were cannulated with polyethylene tubes (PE 180) for infusion of the clearance solution and for venom injection in a series of experiment. The femoral artery was cannulated with polyethylene tube (PE 200) for blood collection and connected to the pressure transducer (PE 23 AA, Statham Instruments) that connected to recorder (Polygraph Model 79, Grass instruments Co.) which allowed continuous monitoring of arterial blood pressure and heart rate. Four legs were adhesive with surface electrode (E5SH standard disc) by Grass EC_2 TM cream and connected to polygraph input cables to record electrocardiogram. For recording respiratory rate, pneumograph belt was rounded the chest of animal and connected to volumetric pressure transducer (45R5 4A, Grass instruments Co.) that connected to recorder. Abdomen was explored in paracostal incision, carefully found out left ureter and cannulated with polyvinyl catheter (PV 190) for urine collection.

Before clearance studies, fluid replacement was done with 0.9% NaCl in volume 10 ml/kg.bw. Clearance study was started by injection solution containing p-aminohippuric acid (PAH) (Sigma chemical company). 1.2 gm% and inulin (In)

(Sigma chemical company) 5 gm% in normal saline solution that was administered 0.5 ml/kg.bw. Then the sustaining solution composed of PAH 1.2 gm% and inulin 0.5 gm% was infused with a constant peristaltic infusion pump (Eyela Model 3) at a rate of 1.5 ml/min for 60 minutes continuously to stabilize plasma inulin and PAH concentration.

Experimental Protocol

After equilibrium period, urine collection along with arterial blood sampling at midpoint of urine collection were done. Duration of urine collection period was 15 minutes, for 6 periods. Blood samples were taken of 4 ml. for each period, except the period that was determined for cardiac output, blood was taken for 20 ml. Blood collection was replaced with 0.9% NaCl and made volume equal to blood collection in each period.

The experiments were divided into 5 groups.

Group I : Six dogs were used as control animals. After an equilibration period of an hour, each animal was infused into the femoral vein with 0.9% NaCl at a rate of 1 ml/min throughout the experiment. After two periods of 30 minutes each the animal was injected with 3 ml of 0.9% NaCl. Four periods of 15 minutes each in the experiment clearance collections were obtained.

Group II : Four dogs were used in this group. After 30 minutes of the control period, each animal was injected with 3 ml of 0.9% NaCl and sustaining of this solution at the rate of 1 ml/min throughout the experiment. Two periods of 30 minutes each in the period of control were observed. Animal was injected with 250 μg/kg.bw. of lyophilized crude cobra venom (obtained from Queen Soavabha Memorial Institute, Thai red cross) in 3 ml of 0.9% NaCl via right femoral vein after 30 minutes of 0.9% NaCl infusion. After venom injection, blood and urine were collected every 15 minutes for 1 hour.

Group III : Six dogs were used in this group. The protocol was started by sample collection for control period, followed by pretreated with intravenous injection of

verapamil (M.W. = 491.1, Sigma chemical company) 0.2 mg/kg.bw. via the left femoral vein. After that, the sustaining solution was performed at the dose of 6 μg/kg.bw. in 0.9% NaCl at a rate of 1 ml/min throughout the experiment period. One 30 minutes in the pretreated with verapamil period was collected. Crude cobra venom was injected intravenously in a single dose of 250 μg/kg.bw. after verapamil pretreatment for 30 minutes. Four observation periods of 15 minutes each in the experimental collections were carried out.

Group IV : Five dogs were used in this group. A protocol identical to group I was followed, except that animal was injected with cardiotoxic fraction XIII of cobra venom (obtained from QSMI, Thai red cross) in 0.9% NaCl at the dose of 75% LD_{50} in mice (1.05 mg/kg.bw.) via right femoral vein. Blood and urine were collected every 15 minutes for 1 hour of the experimental period.

Group V : Four dogs were used in this group. Protocol was the same as group II. Cardiotoxic fraction XIII of cobra venom was used instead of crude cobra venom. After 30 minutes in the control period, animals were pretreated with verapamil 0.2 mg/kg.bw. and infused intravenously in the dose of 6 mg/kg.bw. diluted in 0.9% NaCl at a rate of 1 ml/min throughout the experimental period. Blood and urine were collected every 15 minutes for 1 hour after cardiotoxic fraction injection.

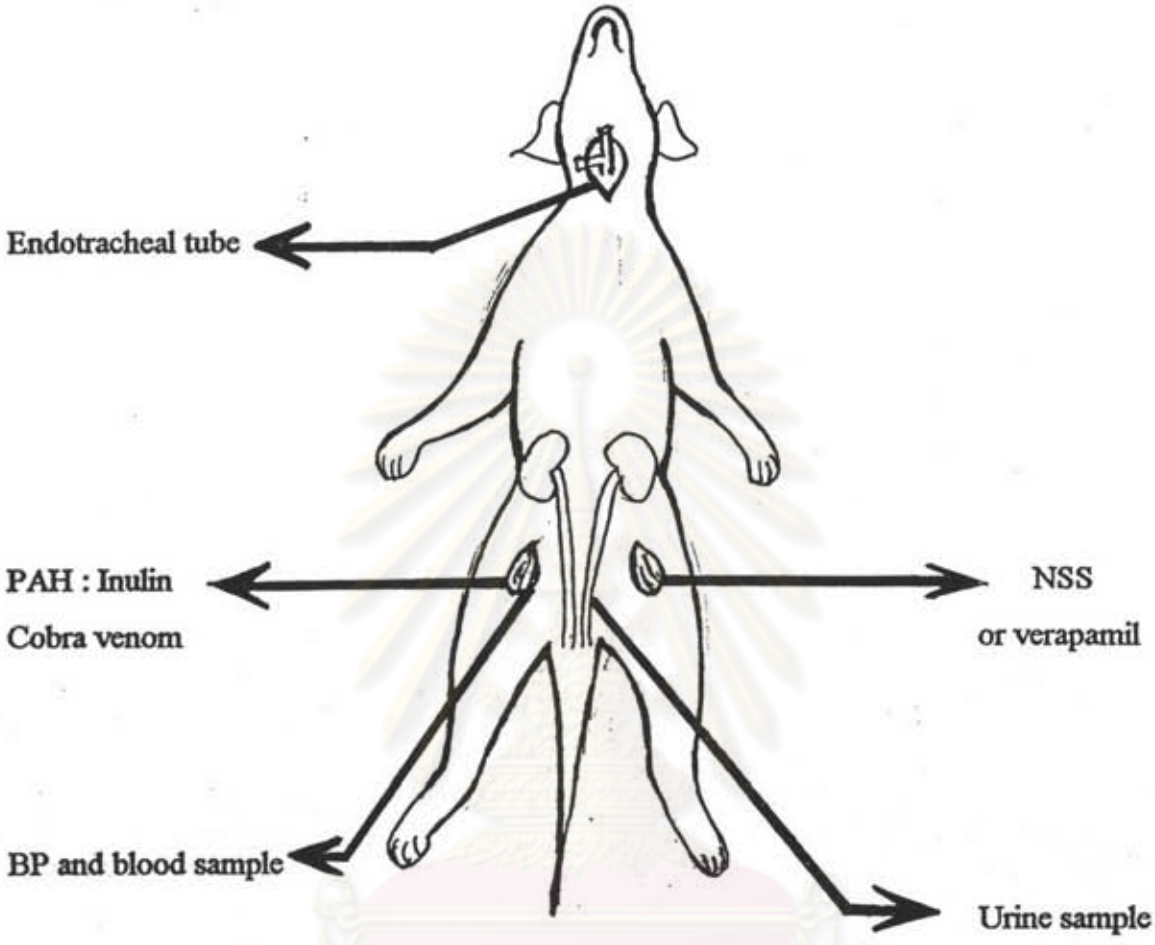

Figure A. Scheme of experiment

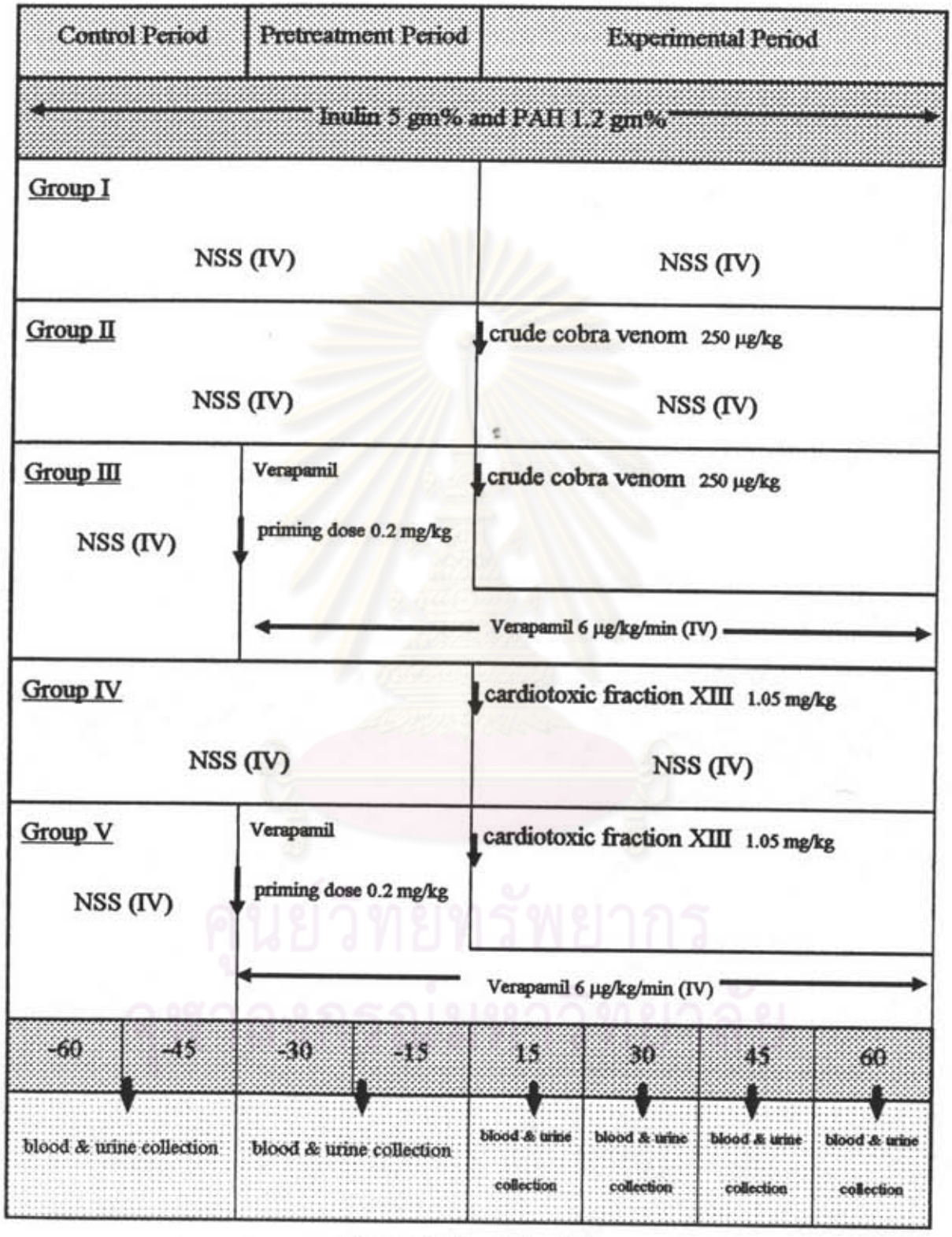

Figure B. Diagrammatic illustration of experimental protocols.

At the end of the experiment, the left kidney was excised stripped of surrounding fat and tissue stored it in ice-cold normal saline and weighing.

Isolation of Plasma Membrane from the Kidney

All steps of cell membrane preparation has been done at temperature 0-4°c. The renal capsule was removed, longitudinal dissected and washed 2-3 times in normal saline. Whole kidney was dissected and removed in ice-cold homogenizing medium; 250 mM sucrose, 5 mM EDTA, 0.01 mM dithiothreitol, 5 mM histidine, pH 7.5 and washed 2-3 times again. The tissue was chopped finely using scissors and added homogenization medium to give a final concentration of 2 gm tissue/15 ml. After that tissue was homogenated with Teflon motorized Potter Elvejham homogenizer by using five up and down stroke of pastle rotating at 1,000 rpm. The homogenate was centrifuged at 1,000×g for 10 minutes with automatic high refrigerated centrifuge (model IEC B22M applied with Rotor model 875) at 0-4°c to remove cell debris. The supernatant was centrifuged again at 15,000×g for 20 minutes to obtain cell membrane fraction. Removing supernatant then pellet was washed with ice-cold homogenizing medium 2 times and resuspended in 5-6 ml of the same solution .

Determination of Cardiac Output

Cardiac output (CO) was measured by dye dilution technique, using Evans blue dye (T-1824), as described by Chaiyabutr et al. (1980). A bolus does of T-1824 (0.5%) was injected into femoral vein. The series of blood samples were collected from the femoral artery immediately within 3-5 seconds after dye injection. Serial samples of arterial blood were collected 1 ml/second by means of peristaltic pump and fraction collection for a period of 10-15 seconds. Then the amount of dye in each blood was determined using by spectrophotometer. Cardiac output was calculated as described by Hamilton et al. (1948).

Determination of Blood and Urine Samples

Inulin concentration in plasma and urine were determined by the anthrone method which modified the method of Young and Raisz (1952). Determination of PAH concentration in plasma and urine were carried out by the method of Bratton and Marshall (1939) as described by Smith (1962).

The composition in the plasma and urine were measured as following : sodium and potassium concentration by flame photometer (Clinical flame Photometer 410C, Corning Ltd.), chloride concentration by chloridometer (Chloride Analyzer 925, Corning Ltd.), and osmolarity by the freezing point osmometer (The Advanced osmometer model 3D3).

The pH measurement in urine was determined by the electrometric techniques with glass electrode pH meter (Hanna Instrument 8520). Blood samples were determined pH and bicarbonate concentration by pH and blood gas analyzer (Ciba corning model 238).

Determination of Na^+-K^+ ATPase Activity

Na^+-K^+ ATPase activity was defined as the amount of inorganic phosphate liberated in the presence and absence of ouabain 1 mM, corrected for the spontaneous nonenzymatic breakdown of ATP. Total ATPase was determined in a 1 ml reaction mixture containing NaCl 130 mM, KCl 20 mM, $MgCl_2 \cdot H_2O$ 3 mM, ATP disodium salt 3 mM (Sigma Chemical Company), histidine buffer pH 7.5, 30 mM, and enzyme suspension enough to bring the final concentration to 0.25 mg/ml. The reaction was started by the addition of ATP in a constant temperature water bath at 37 °c for 15 minutes, and stopped by the addition of 0.2 ml of ice-cold 30% trichloroacetic acid. After centrifugation the inorganic phosphate liberated was measured by method applied from Fiske and SubbaRow technique, the optical density read at 650 nm in spectrophotometer (UV 1201, uv-vis spectrophotometer). The activity of the enzyme was expressed as micromoles of inorganic phosphate liberated per milligram of protein per hour. Determination of protein in the homogenates was carried out according to

the method of Lowry, Rosebrough, Farr, and Randall using bovine serum albumin (Sigma chemical Company) dissolved in normal saline as a standard.

Titration of Weak Acid with Strong Base Procedure:

1. 2.5 ml of urine sample was transferred to the flask and added 1 gm of potassium oxalate powder (M.W.=184.24 gm/mol) to precipitate the calcium which would otherwise interfere with the end point since calcium phosphate precipitated on neutralization of the urine.

2. 1 drop of 1% phenolphthalein indicator solution (M.W.=318.33 gm/mol) in alcohol was added and shook well for 2-3 minutes.

3. The direct titration of titratable acid (TA) was carried out by adding the standard 0.05 N sodium hydroxide solution from a burette, to the solution of sample acid containing a suitable indicator. The end point was marked by the first permanent pink color (pH 8.2). The volume of titrant required to reach the end point was read from the calibrated scale of the burette and calculated the normality and the urinary titratable acid excretion.

4. A back titration (residual titration) was useful if the sample should be a volatile base. Then a known volume of standard acid, in excess of that required for the stoichiometric reaction, 37% formaline solution 2 ml, was added in the above titrated mixture. The excess of acid ammonium (NH_4^+) was back-titrated with standard 0.05 N NaOH to the first permanent pink color. The volume of titrant require to reach the end point was read from the calibrated scale of the burette and calculated the normality of ammonium and the urinary ammonium excretion as following:

$$\text{Normality of TA or } NH_4^+ = \frac{\text{0.05N NaOH} \times \text{Volume of NaOH was used}}{\text{Volume of urine (2.5 ml)}}$$

$$\text{Urinary of TA or } NH_4^+ \text{ excretion} = \text{Normality of TA or } NH_4^+ \times \text{urine flow rate}$$

$$= \mu\text{Eq/min/kg.bw.}$$

Calculation

Mean arterial blood pressure (MAP) $= P_d + 1/3\ (P_s - P_d)$

Glomerular filtration rate (GFR) $= \frac{U_{In}V}{P_{In}V}$

Effective renal plasma flow (ERPF) $= \frac{U_{PAH}V}{P_{PAH}}$

Effective renal blood flow (ERBF) $= \frac{ERPF \times 100}{(100 - PCV)}$

Filtration fraction (FF) $= \frac{GFR \times 100}{ERPF}$

Renal vascular resistance (RVR) $= \frac{MAP}{ERBF}$

Filtered load of electrolyte $= GFR \times P_e$

Urinary electrolyte excretion $= U_e \times V$

Fractional excretion of electrolyte (FE_e) $= \frac{U_e \times V/P_e \times 100}{GFR}$

Urinary acid excretion (UAE) $= (U_{TA}V + U_{NH_4^+}V)$

Fractional water excretion $= V/GFR \times 100$

Osmolar clearance (C_{osm}) $= U_{osm}V/P_{osm}$

Free water clearance (C_{H_2O}) $= V - C_{osm}$

Statistical Analysis

All the data are presented as the mean±SEM. Statistical significance of differences between period in the same group was determined by the paired t-test. The unpaired t-test was used to estimate the statistical significance of the difference between group means. P-values less than 0.05 was considered significant.