CHAPTER III

MATERIALS AND METHODS

Animals.

Fourteen adult male mixed-breed dogs, weighing between 11-19 kilograms were used. Dogs were housed individually in cages throughout the observation period. Water was available ad libitum, and food was offerred once a day.

Experimental procedures.

Fourteen dogs were randomly selected and allocated to three groups; five dogs were assigned to group 1 as the control group. Other five dogs were assigned to group 2 and were fed with ethylene glycol(EG) ($C_2H_6O_2$, MW 62.07, Merck) 1.5 ml/kg.bw. The remains were assigned to group 3 and were fed with EG 3.0 ml/kg.bw.

The solutions were fed via nasogastric tubes (water in group 1 and ethylene glycol in group 2 and group 3). An hour before nasogastric intubation, dogs were given chlorpromazine 1 mg/kg, per os to prevent vomiting. A day after EG ingestion, dogs were anesthetized by intravenous injection of pentobarbital sodium (Nembutal, Sanofi Animal Health) at the dose of 25 mg/kg.bw., placed in left lateral recumbency, and then an endotracheal tubes were inserted. The right jugular vein was surgically exposed at the mid cervical part. A Swan-Ganz catheter (number 6F) connected with a pressure transducer (P23ID, Gould Electronics) was catheterized into the vein. The balloon of the Swan-Ganz catheter was inflated with 1.0 ml of air and then passed
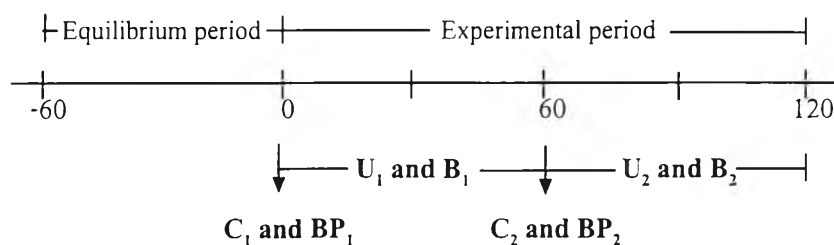
into the pulmonary artery. The location of the catheter tip was determined by recording the intravascular pressure and observing blood pressure patterns. The catheter was advanced until it passed and wedged in the pulmonary vasculature, and then it was slightly withdrawn. Finally, the balloon was deflated.

The left femoral artery was cannulated with polyethylene tube (PE240, Intramedic) for blood collection and connected with a pressure transducer (P23ID, Gould Electronics). Both pressure transducers were linked to the polygraph recorder (Grass Model 79, Grass Instruments Co.) which allowed the continuous monitoring of blood pressure and heart rate.

The right ureter and the right renal vein were approached by right paracostal incision. The right ureter was cannulated with polyethylene tube (PE 160, Intramedic) for urine collection. The right renal vein was cannulated with an intravenous catheter (Insyte No.23, Becton Dickinson and Company) connected to polyvinyl catheter for collecting renal venous blood.

Before clearance studies, 0.9% NaCl 10 ml/kg.bw was given as the fluid replacement. The primed injection of the solution containing 5.0 gm% inulin (In) (Sigma Chemical Company) and 1.2 gm% PAH(MW 194.2, Sigma Chemical Company) in normal saline solution at the dose of 0.5 ml/kg.bw was given for the clearance study. Then, the sustaining solution composed of 0.5 gm% inulin and 0.12 gm% PAH was infused by a peristaltic pump (Model 3, Eyela) at the rate of 1.5 ml/min for 60 minutes to stabilize the plasma inulin and PAH concentration prior to blood sample collections.

After equilibrium period, urine was collected twice at 60 min apart, and five milliliters of the arterial blood sample were collected at the mid-period of urine collection. Before each period of urine collection, 0.5 milliliter of the arterial blood from the femoral artery, the central venous blood from the pulmonary artery, and the renal venous blood from the renal vein were collected for blood gas analyses. The oxygen uptake was measured and used to calculate the cardiac output as shown in the diagram.

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|-Equilibrium period -|----------- Experimental period ----------|

-+--------+--------+--------+--------+--------+--
-60      0                60               120

         |----- U₁ and B₁ ----|---- U₂ and B₂----|

      C₁ and BP₁              C₂ and BP₂
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BP	=	Blood pressure	U	=	Urine
C	=	Cardiac output	B	=	Blood

At the end of the experiment, the right kidney was isolated, and immediately flushed with ice-cold 0.9% NaCl, weighed, and chilled with 0.9% NaCl for measuring the lipid peroxide concentration and the xanthine oxidase activity.

Determination of cardiac output.

Cardiac output (CO) was measured by the Fick method, using oxygen as described by Greddes (1984). Venous blood flowing through the lungs from the right heart to the left heart picked up oxygen. If the amount of oxygen uptake per minute was known, blood flow through the lungs could be calculated. The oxygen uptake was measured using a spirometer. The partial pressure of oxygen in pulmonary artery and femoral artery were also measured using blood gas analysis (Model 238, Ciba Corning). The haemoglobin concentration was measured using haematology cell counter (Model 150, Baker instruments).

The cardiac output was expressed by the following expression:

$$\text{Cardiac Output} = \frac{\text{Oxygen uptake}}{\text{arterial - venous oxygen concentration}}$$

Determination of lipid peroxide concentration.

Lipid peroxides were determined by Thiobarbituric acid reaction as described by Ohkawa et al. (1979).

After chilling in ice-cold 0.9% NaCl, kidney cortex was isolated and tissue homogenates was prepared in a ratio of 1 gm. of wet tissue to 9 ml. of 1.15% KCl by using a homogenizer (Model K4424, Glas-Col). The kidney homogenate (0.5 ml.) was added with 0.2 ml. of 8.1% sodium dodecyl sulfate(MW 288.4, Sigma Chemical Company) and 1.5 ml. of 20% acetic acid solution, adjusted to pH 3.5 with 1 M NaOH, and 1.5 ml. of 0.8% aqueous solution of Thiobarbituric acid (MW 144.1, Sigma Chemical Company). The mixture was made up to 4.0 ml. with distilled water, and then heated in water bath at 95°C for 60 minutes. After cooling in tab water, 1.0 ml. of distilled water and 5.0 ml. of the mixture of n-butanol and pyridine,15:1(v/v), were added, and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was drawn, and its absorbance was measured at the wave length of 532 nanometer .The external standard was used 1,1,3,3-tetramethoxypropane (TMP, MW 164.2, Sigma Chemical Company). The level of lipid peroxide was expressed as nmol of malondialdehyde (MDA) per gram kidney protein.

Determination of xanthine oxidase activity.

Xanthine oxidase activity was measured by the method of Hashimoto (1974). Kidney cortex was washed in ice cold 0.25 M sucrose solution and blotted on a filter paper. Kidney homogenate was prepared in a ratio of 1 gm. of wet tissue to 9 ml. of 0.25 M sucrose by using a homogenizer (Model K4424, Glas-Col).

The kidney homogenate was dialysed against 200 volume of 0.25 M sucrose solution at 5°C. After the overnight dialysis, the fresh homogenate was used to measure the xanthine oxidase activity.

The enzyme activity was measured by the formation of uric acid from xanthine (MW 152.1, Sigma Chemical Company) as indicated through the increase in absorption at 292 nm spectrophotometrically. Potassium oxonate(MW 195.2, Sigma Chemical Company) was added to inhibit uricase enzyme activity which converted the uric acid to allantoin. The 10% kidney homogenate, 0.2 milliliter, was incubated for 30 min at 30°C in 3 ml. of the medium containing 12 μmole xanthine and 0.3 μmole K-oxonate in phosphate buffer (pH 7.5). The reaction was stopped by the addition of 0.1 ml. of 100% (w/v) TCA, and the mixture was centrifuged at 6,000 rpm for 30 minute. Clear supernatant was measured against blank at 292 nm. The blank was similarly treated without xanthine. One unit of xanthine oxidase was defined as the amount of the enzyme which catalyzed the formation of 1 μmole of uric acid from xanthine per minute at 30°C.

Determination of kidney protein.

Kidney protein was determined by Lowry's method. Homogenate samples from the preparations for measuring lipid peroxide and xanthine oxidase activity were diluted with 50 volume of 0.9% NaCl. The diluted sample (150 μl) was added with 3 ml. of the fresh reagent (consisting of 2% Na_2CO_3 in 0.1 M NaOH, 50 ml.; 1% $CuSO_4 \cdot 5H_2O$, 0.5 ml.; and 2% sodium tartate, 0.5 ml.) incubated for 10 min at room temperature, and then added 300 μl of 1 N Folin&Ciocalteu's phenol reagent. After 30 min, the mixture was then measured at 650 nm against blank in which 0.9% NaCl was used to replace diluted sample. The standard was 100 mg% bovine serum albumin in 0.9% NaCl.

Analyses of blood and urine samples.

The inulin concentration in plasma and urine was measured by the Anthrone procedure, described by Young and Raisz (1952). The PAH concentration in plasma and urine was determined by the method of Bratton and Marshall (1939) as described by Smith (1962).

The electrolytes in plasma and urine were measured as follows; the sodium and potassium concentrations, using a flame photometer (Chemical Flame Photometer 410C, Corning Ltd.), the chloride concentration, using a chloridometer (Chloride Analyser 925, Corning Ltd.).

The osmolality of plasma and urine was measured by the freezing point osmometer (Model 3D3,The Advanced Osmometer).

The acid-base status in blood and urine was measured by the pH and blood gas analyzer (Model 238, Ciba corning), and the glass electrode pH meter (Model 420A, Orion).

The urea nitrogen in plasma and urine was determined after combining the urea to 2,3-butanedione-2-oxime in acid. The formed complex reacted with thio-semicarbazide to give a red-violet color.

The glucose in plasma and urine was determined after the enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacted under the catalysis of peroxidase with phenol and 4-aminophenazone to give a red-violet quinoneimine color as an indicator.

Determination of urine titratable acid.

The acid in urine was titrated using strong base as follows:

1. Urine sample (2.5 ml.) was placed in the flask and added 1 gm. of potassium oxalate powder 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 in alcohol was added and shook well for 2-3 min.

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

4. The back titration was carried out by adding 2 milliliters of 37% formalin (saturated in calcium carbonate) . The excess of acid ammonium (NH_4^+) was back-titrated with the standard 0.05 N sodium hydroxide to the first permanent pink color. The volume of titrant was read from the calibrated scale of the burette and the normality of ammonium and the urinary ammonium excretion were calculated.

5. The calculating formulas:

Normality of TA or NH_4^+ $= \dfrac{0.05NaOH \times \text{Volume of NaOH}}{\text{Volume of urine}}$

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

(μEq/min/kg.bw.)

Calculation.

Mean arterial blood pressure (MAP) = Diastolic pressure + 1/3 pulse pressure

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

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

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

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

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

Filtered load of electrolyte $= GFR \times P_e$

Urinary electrolyte excretion $= U_e \times V$

Fractional excretion of electrolyte (FE$_e$) $= \dfrac{U_e/P_e}{U_{In}/P_{In}}$

Anion gap $= (Na^+ + K^+) - (Cl^- + HCO_3^-)$

Net acid excretion (NAE) $= $ titratable acid $+ NH_4^+ - $ urinary HCO_3^-

Osmolal gap $= $ measured plasma osmolality - calculated plasma osmolality

Calculated osmolality $= 2(Na^+ + K^+) + (glucose/18) + (BUN/2.8)$

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

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

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

Stroke-work index (SWI) $= \dfrac{(MAP - PCW)(SV)0.0136}{BSA}$ g-m/m^2

Pulmonary vascular resistance (PVR) $= \dfrac{(Pulmonary\ mean\ pressure)(13.6/10)(980)}{(CO)(1000/60)}$

Total peripheral resistance (TPR) $= \dfrac{(MAP)(13.6/10)(980)}{(CO)(1000/60)}$

Renal oxygen uptake $= ERBF(\text{renal A-V oxygen difference})$

Statistical analysis.

All data are presented as the mean±SD. Statistical significance of difference between group was determined by the one-way ANOVA and Duncan's new multiple rank test. The P-value less than 0.05 was considered significant.