CHAPTER III

MATERIALS AND METHODS

1. Experimental Animals

The study was performed in male Wistar rats weighing 220 to 250 grams and were obtained from the National Center of Scientific Use of Animals (Mahidol University, Salaya, Nakhonpathom). The animals were housed in a well ventilated room in which the temperature was 23-25°C with an automatic lighting schedule, which provided darkness from 8 p.m. to 6 a.m. The animals were given free access to standard laboratory chow and water as follow the experimental protocol. All rats were used once only and were kept in metabolic cages for 24 hours to collect urine before the experiment and before sacrificed. Urine volume was measured, and the samples were stored at -80 °C until use.

2. Chemicals

2.1. Chemical Agents

Sodium pentobarbital (Nembutal®) was purchased from Sanofi, France. Enalapril® (20 mg) was obtained from Biolab, Thailand. Losartan® (50 mg) was obtained from M&H, USA. Absolute ethanol, 95% ethanol, xylene, dioxane, di-sodium hydrogen phosphate (Na_2HPO_4), potassium di-hydrogen phosphate (KH_2PO_4), Trisma® acid, Trisma® base, triton X-100, 30% hydrogen peroxide and gelatin were purchased from

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Merk, Germany. Paraformaldehyde was purchased from Sigma, USA. Hematoxylin solution (progressive stain) was purchased from C.V. Laboratories, Thailand. ABC-streptavidin-horseradish peroxidase complex was purchased from Vector, USA. Paraffin pour embedding medium was purchased from St. Louis, USA. Nitric oxide (NO_2^- and NO_3^-) assay kit (Catalog No. DE 1500) was purchased from R&D system, USA.

2.2. Antibodies

Mouse monoclonal antibody against NOS II (iNOS, Catalog No. N39020-150) and NOS III (eNOS, Catalog No. N30020-150) were purchased from BD transduction, Japan. Normal swine serum (Code No. X0901, Lot. 110) and biotinylate swine anti-goat-mouse-rabbit immunoglobulin (Multi-Link, Code No. E0453, Lot. 021) were purchased from Dako, Denmark.

3. Experimental Procedure

Animals were divided into 2 main groups as follow:

1. Sham group (S) (n = 8)
2. Unilateral Ureteral Obstruction (UUO) groups (n = 48)

3.1. Sham and UUO Operation

After three days to familiar with the new housing, the animals were weighed and collected blood sample from the tail for measuring BUN in order to assess kidney function (less than 30 mg %). After 24-hour urine

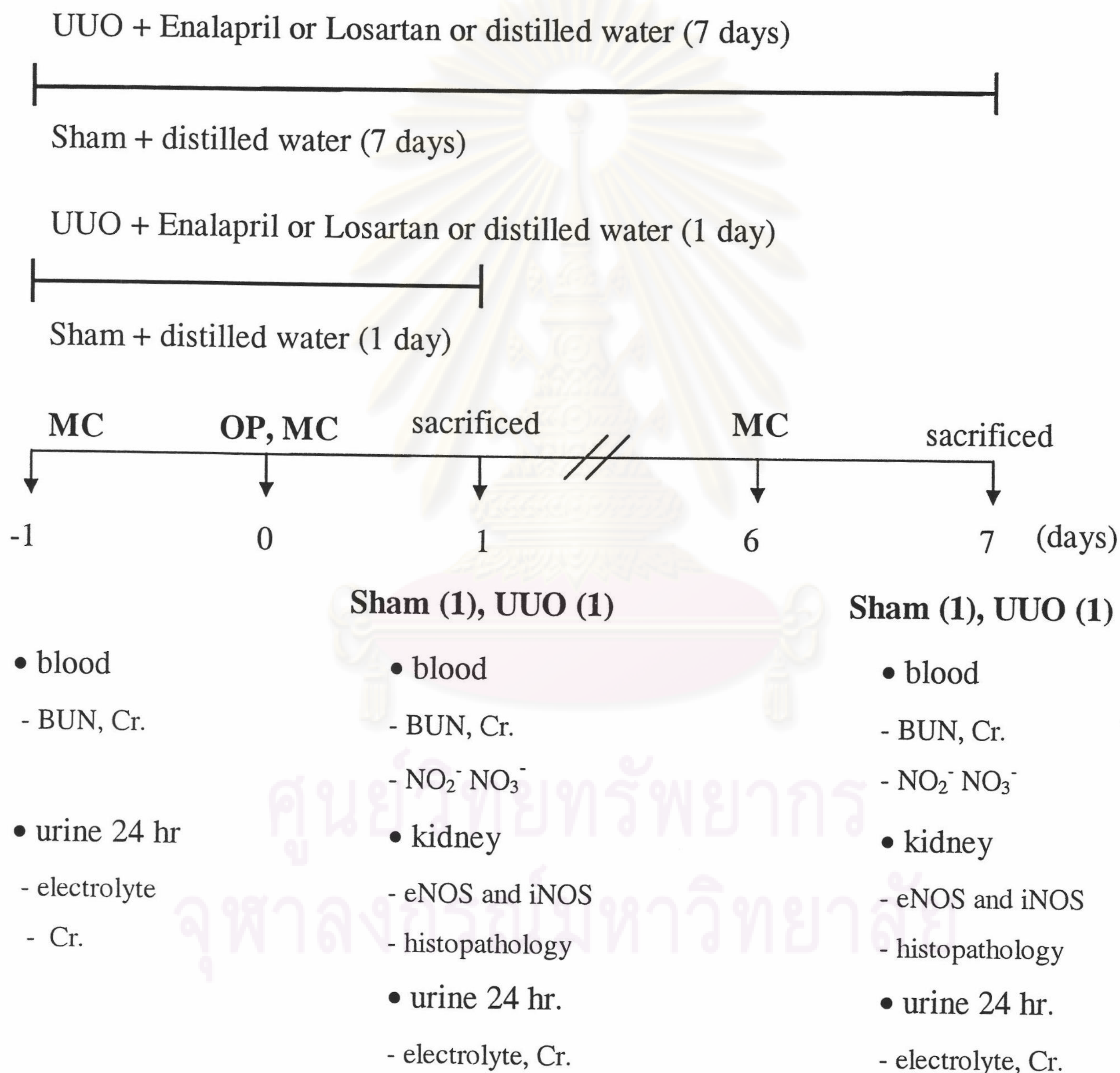
collection, the rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (60 mg/kg body weight). Using an aseptic technique, a midabdominal incision was made to expose the kidneys. The left ureter was ligated with 4/0 silk suture at 1/3 point from renal pelvis. The incision was then closed. The sham group comprised of rats that were operated and only wiped left ureter. All rats were allowed to wake up and return to a clean cage with free access to food but UUO groups were three different kinds of drinking solution as follow:

1. Water (only distilled water)
2. Water+ACEI (Enalapril® 200 mg/L drinking water) (Klahr and Morrissey, 1997; Ishidoya et al., 1995)
3. Water+ARA (Losartan® 500 mg/L drinking water) (Fujinaka et al., 2000)

The volume of drinking water was approximately 30 ml/rat/day. ACEI or ARA were provided one day before the operation and continuously for 1 day or 7 days after UUO. The sham group received an equal amount of only distilled water daily (n = 8 rats/group). Twenty-four hour urine was collected before the due date. On each experimental due date, the animals of respective groups were re-operated under anesthesia. Blood samples were collected from the aorta through an abdominal incision and centrifuged at 3500 rpm. Serum was stored at -80 °C until use for BUN, Cr, and electrolyte measurements, as well as NO metabolite assay (NO_2^- and NO_3^-). The kidneys were removed and fixed in 4% paraformaldehyde overnight. Renal tissues were embedded in paraffin,

cut into 4-5 μm thick sections for eNOS, iNOS protein expression and histological evaluation.

3.2. Experimental Design



(MC = metabolic cage and OP = operation)

3.3. Immunohistochemical Study

Paraffin-embedded kidney sections were cut at 4 μm. in thickness. Slides were deparaffinized in xylene and alcohol, with endogenous peroxidase activity being quenched in 3% hydrogen peroxide for 10 minutes. The non-specific binding of the antibody was blocked by incubating tissue sections with 5% normal swine serum in PBS-A (PBS + 1% BSA + 0.3% triton X-100) for 30 minutes at room temperature. Then, the section was incubated in primary antibody for eNOS or iNOS in concentrations of 1:100 and 1:200, respectively (diluted in PBS-A) over one hour at room temperature.

After incubation, the sections were rinsed 3 x 10 minutes with PBS-B (PBS + 0.25% BSA + triton X-100) and incubated with biotinylated swine anti-goat-mouse-rabbit immunoglobulin (Multi-Link) diluted 1:50 in PBS-B for 60 minutes at room temperature. After incubation, tissue sections were rinsed 2 x 10 minutes with PBS-B and then 1 x 10 minutes in PBS. The tissue sections were reacted with ABC-streptavidin horseradish peroxidase complex (diluted in PBS) for 60 minutes at room temperature. The sections were then rinsed 2 x 10 minutes in PBS and 10 minutes in 0.05 M Tris-HCl buffer (pH 7.6). The sections were reacted for peroxidative activity in a solution containing 0.025% 3, 3'-diaminobenzidine (DAB) and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) for 30 minutes. Then, sections were washed 2 x 5 minutes with distilled water. Finally, they were counterstained with haematoxylin and were coverslipped with permount.

Areas of staining were identified, and the intensity of staining was scored from 0 to 3 (0 = no staining, 1 = weak positive, 2 = moderate staining, and 3 = strongly positive staining) (Hegarty et al., 2001). All slides were viewed and scored by three blinded observers.

3.4. Nitric Oxide (NO_2^- and NO_3^-) Assay

Since most of NO is oxidized to nitrate (NO_3^-) and nitrite (NO_2^-), the concentration of these anions have been used as a quantitative measure of NO production (R&D system, USA). The assay involves the conversion of NO_3^- to NO_2^- by nitrate reductase. All regents, and working standard were prepared as directed in the handout. Serum samples were diluted 2-fold into Reaction Buffer (1x). The reaction is followed by a calorimetric detection of NO_2^- as on azo dye product of the Griess reaction. The Griess reaction is based on the two step diazotization reaction in which acidified NO_2^- produces a nitrosating agent which reacts with sulfosalicylic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm (all NO_2^- and NO_3^- assays were run in duplicate).

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Calculation of results:

A standard curve was created by reducing the data using computer software (Anthos 2010 software version 1.7 with printer, Anthos labec instruments, Austria) capable of generating a linear curve-fit. As an alternative, a standard curve was constructed by plotting the mean absorbance of each standard on the Y-axis against the endogenous nitrite or

total nitrite concentration on the X-axis. The concentration of nitrite corresponding to the mean absorbance from the nitrite standard curve was calculated. The concentration of nitrate in the sample was determined as follow:

1. Measure the endogenous nitrite concentration (X μmol/L) using the nitrite assay.
2. Measure the total nitrite concentration (Y μmol/L) after the conversion of nitrate to nitrite using the nitrate reduction assay procedure.
3. Determine the nitrate concentration in the sample by subtracting the endogenous nitrite concentration from the total nitrite concentration.

$$\text{Nitrate concentration} = Y - X \ \mu\text{mol/L}$$

3.5. Morphologic Evaluation of Kidney

Renal tissue injury was assessed in tissue sections stained by the Periodic Acid-Schiff (PAS) reaction and Masson's trichrome technique. Sections were scored in a blinded semiquantitative manner. The numerical scores indicate the following: 0 = normal structure, 1 = areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving less than 25% of cortical tubules, 2 = similar changes involving greater than 25% but less than 50% of cortical tubules, 3 = similar changes involving greater than 50% but less than 75% of cortical tubules, 4 = similar changed involving greater than 75% of cortical tubules, and 5 = complete cortical necrosis (Walker et al., 2000).

3.6. Calculation for Assessment of Renal Function

Creatinine clearance (C_{Cr}) $= \dfrac{U_{Cr} \times V}{P_{Cr}}$

Urinary electrolyte excretion $= U_e \times V$

Fractional electrolyte excretion (FE_e) $= \dfrac{U_e V/_{Pe} \times 100}{CCr}$

4. Statistical Analysis

All data were expressed as mean ± S.E. The results of blood and urine parameters were compared by using ANOVA. Probability values of less than 0.05 were considered to be statistically significant. The intensity scores of renal eNOS protein expression and renal pathological scores were present in descriptive statistics by measures of central tendency (Mode).

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