CHAPTER III

MATERIALS AND METHODS

1. Materials

- Aerosol resistance pipette tip : 200 µl (Molecular Bio-Pro)
- Autoclave tape (3M,USA)
- Aluminum foil (Diamond® ,USA)
- Beakers : 50 ml, 1,000 ml. (Pyrex® ,USA)
- Coplin staining jars
- Culture plates (Nunc, USA)
- Cylinder (Pyrex®, England)
- Disposable gloves
- Glass pipettes : 1 ml, 5 ml, 10 ml. (Witeg, Germany)
- Heparinized Vacutainer 5 ml (Vacutainer®,USA)
- Humidified chamber
- Microscope glass cover slips (Chance, England)
- Needle (Vacutainer System PrecisionGlide ™, UK)
- Plastic cover slips (ApopTag®)
- Pasteur pipettes
- Parafilm (American National Can™ ,USA)
- Reagent bottles : 250 ml, 1,000 ml (Duran®, Germany)
- Slide film (Eritchome 400, Kodak)
- Sterile polypropylene centrifuge tube : 15ml, 50 ml. (Nunc™,USA)
- Slide (Super Frost, Germany)
- Slide box
- Sterile membrane filters (Whatman ®, Japan)

- Slotted microscope slide staining dish
- Tube rack
- Tissue Culture flasks (Nunc™ , USA)
- Syringe 5 ml

2. Equipments

- Autoclave (HICLAVE™, HIRAYAMA)
- CO_2-Incubator (REVCO ULTIMA)
- Dark room
- Differential counter
- Fluorescence microscope (Olympus), Andrology Unit, Department of Obstertrics and Gynecology, Faculty of Medicine, Chulalongkorn University.
- Freezer-20 °C
- Fume hood (Model 252, NEWLAB®)
- Hemocytometer (Boeco, Germany)
- Incubator (Heraeus)
- Bioharzard lamina-flow hood (Gelman Science)
- Light microscope (Olympus, Japan)
- Low-speed centrifuge (Beckman)
- Autopipette
- pH meter (Eutech Cybernetics)
- Refrigerator 4 °C (SANYO)
- Timer
- Thermometer

3. Reagents

3.1 General reagents

- Absolute ethanol (Merk, Germany)
- Acetic acid (Merk, Germany)
- Fetal Bovine Serum (Gibco BRT, Germany)
- Formaldehyde
- Gentamycin (GOH)
- Hank Buffered Salt Solution (HBSS) Powder (Gibco BRL)
- Histopaque®-1077 (Sigma, USA)
- Heparin (LEO)
- Hoeschts 33258 Dye solution
- Hydrochloric acid : (Merk, Germany)
- L-Glutamine (Gibco BRL)
- Paraformaldehyde powder (Sigma, Germany)
- Potassium chloride
- Potassium hydrogen phosphate
- RPMI 1640 (Gibco BRL)
- Sodium chloride
- Sodium hydroxide (Merk)
- di-Sodium hydrogen phosphate monobasic
- Sodium bicarbonate
- Clorox

3.2 Reagent Kit

- Apoptag® Fluorescein kits (Intergen, Canada)

3.3 Enzyme

- Terminal deoxynucleotidyl transferase (TdT) enzyme (Intergen, Canada)

4. Animals

Male Wistar rats weighing 220-250 g were obtained from the National Center of Scientific Use of Animals (Mahidol University, Salaya, Nakhonpathom). Rats were fed the clean-normal rat chow and water at least 3 days before the experiment in the laboratory with controlled temperature between 23-25 °C and light 14 hours a day (from 6.00 a.m. - 8.00 p.m.). All rats used once only were divided into 3 main groups as follow:

1. UUO rats (n = 72)
2. Sham rats (n = 48)
3. Control rats (n = 5)

5. Parameters to study

- blood urea nitrogen, serum creatinine
- number of circulating lymphocyte
- percentage of circulating lymphocyte cell death
- apoptotic index of circulating lymphocyte

6. Experimental procedure

The experiments were divided into 2 studies:

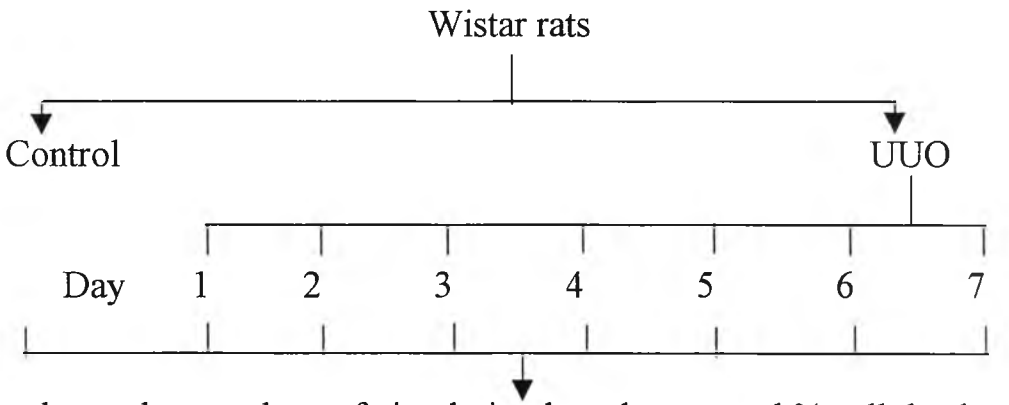
Study 1: Studying the number of circulating lymphocytes after 1- day to 7-day of UUO

Animals were classified into 2 main groups as follow:

1. Control group (normal rats) (n = 5)
2. UUO groups were subdivided into 7 groups according to the experimental duration, i.e. UUO for 1 day to 7 days (n = 28-35).

The process led to UUO was: After 3 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 %). On the next day, the animals were operated on under light anesthesia with sodium pentobarbital (60 $mg.kg^{-1}$). The left ureter was ligated with 4/0 silk suture at 1/3 point from renal pelvis through a small abdominal incision. The incision was then closed, and the animals were further treated as the experimental protocol. On the certain experimental due-day, the UUO rats were re-anesthesized and blood samples were collected from the abdominal aorta to measure the number of lymphocytes.

Experimental design : study 1



Blood sample : numbers of circulating lymphocyte and % cell death

Study 2: Studying the apoptosis of circulating lymphocyte

The animals were divided into 2 main groups:

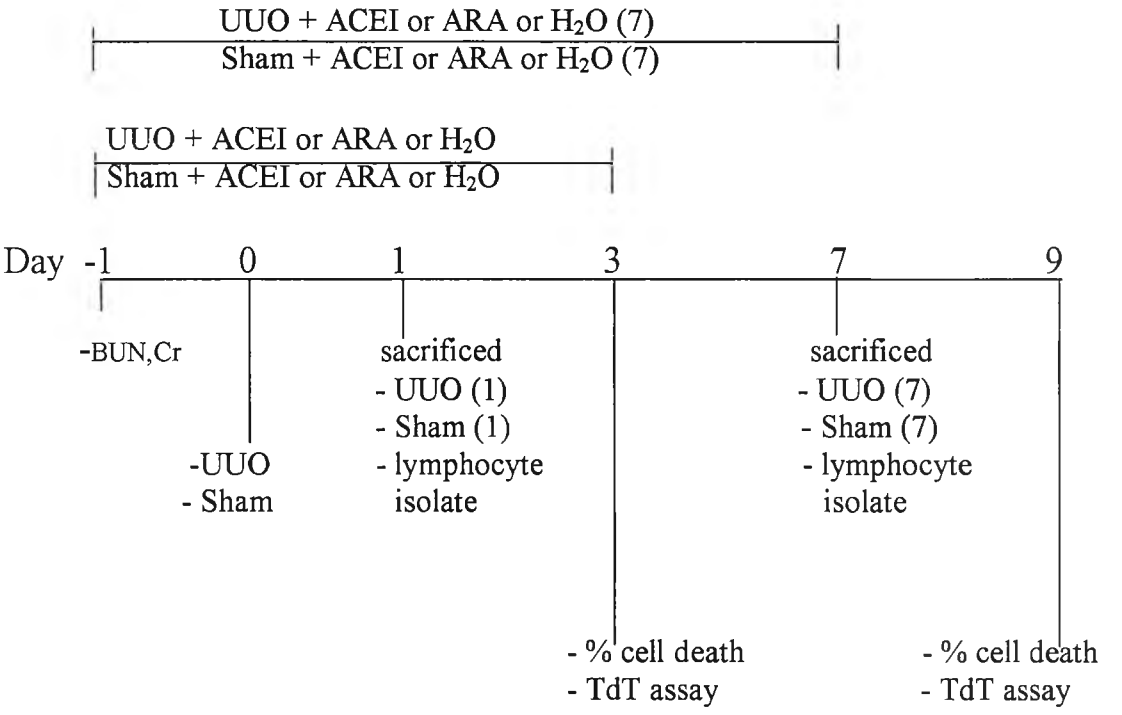
1. UUO group (n = 48)
2. Sham group (n = 48)

Sham groups comprised of the rats that were operated and only wiped ureter through a small abdominal incision which was then closed.

The rats in UUO and sham groups were fed the same normal rat chow, but with three different kinds of drinking solution as follow:

1. water (only distilled water)
2. water + ACEI (Enalapril 200 mg/L; 5 mg/kg/d)[175]
3. water + ARA (Losartan 500 mg/L; 10 mg/kg/d)[175]

Experimental design : study 2

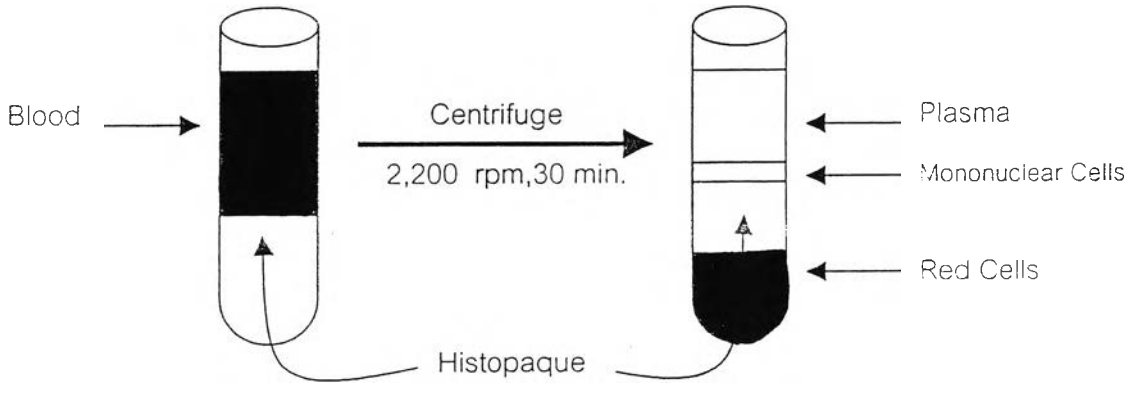


ACEI or ARA were provided one day before the operation and continuously for 1 day or 7 days after the operation (n = 8 rats/group). On each experimental due day, the animals of respective groups were operated under anesthesia and the blood samples were collected through an

abdominal incision from abdominal aorta to trace BUN and Cr as well as to analyze mononuclear lymphocytes[24] as follow:

Lymphocytes Isolation

1. Pipette 5 ml Histopaque-1077 at room temperature into each 15 ml polypropylene centrifugue tube.
2. Mix the blood 1:1 with Hank Buffered Salt Solution (HBSS) added 2 μl Heparin at temperature.
3. Transfer 9 ml of the blood / HBSS mixture on to the top of the histopaque tube. Be careful not to mix the two parts together, cap the tube tightly.
4. Centrifuge at 2,200 rpm for 30 minutes at room temperature.
5. Using a pipette to remove the top plasma layer from the tube. Discard this layer into a container of Clorox.
6. Use a swirling motion with the pipette, collect the next layer (white ring of lymphocyte) from the tube. The next layer is the residual histopaque. Try not to collect much of this layer and do not collect the red cell layer which sits at the bottom of tube. (See the following diagram)



7. Immediately transfer the lymphocytes ring into a new sterile polypropylene 15 ml centrifuge tube. Add 12.5 ml HBSS (+2 µl/ml Heparin + 1% Fetal Bovine Serum to the tube. Cap tightly, then invert the tube 3 time to mix.
8. Centrifuge at 1,000 rpm for 10 minutes at room temperature.
9. Decant the supernatant from the centrifuge tube into a Clorox container. Flip to softly disperse the lymphocyte pellet. Add 10 ml HBSS (+2 µl/ml Heparin + 1% Fetal Bovine Serum) and mix by pipetting up and down 3 times.
10. Centrifuge at 1,000 rpm for 10 minutes at room temperature.
11. Decant the supernatant into the Clorox container and mix the cells. Add complete RPMI medium (RPMI 1640 media + 5.2% Fetal Bovine Serum + L-Glutamine 20.28µl/ml + Gentamicin 0.97µl/ml) to 5 ml. Mix by pipetting up and down 3 times.
12. Put an aliquot of cell suspension on the counting chamber and count the cells. Calculate the total cell counts and add complete RPMI medium to obtain the suspension of cells at the concentration of 4×10^5 cells/ml for culture. Transfer 6 ml of this cell suspension into 2 culture plates.
13. Incubate all the culture plates in CO_2 incubator at 37 °C, 97% humidity, 0.35% CO_2. The cells will be harvested at 48 hours.
14. Stain 50 µl of the cell with 0.4 % trypan blue. Then, the cells were viewed under light microscope. The appearance of cells stained with dark blue was counted as death cells and calculate percentage of cell death.

Studying apoptosis of circulating lymphocyte

After calculating the percentage of cell death, the lymphocytes were analyzed for apoptotic cells by means of fixing cells with ApopTag. as follow:

Fixing cells with ApopTag.

1. Mix cells in the culture plates by pipetting up and down 3 times to resuspend the cells more evenly.
2. Transfer 3.0 ml of the 4 x 10^5 cell/ml into a new polypropylene centrifuge tube. (1,200,000 cells)
3. Centrifuge at 1,000 rpm for 10 minutes at 0 °C.
4. Aspirate out all supernatant and flip to softly disperse the lymphocyte pellet.
5. Add 2 ml of PBS at 0 °C. into each tube. Mix cells by pipetting up and down 3 times.
6. Centrifuge at 1,000 rpm for 10 minutes at 0 °C.
7. Aspirate out all supernatant and flip to softly disperse the lymphocyte pellet.
8. Repeat step 5 to 7
9. Resuspend the cells in 250 µl PBS/1% Paraformaldehyde fixative. Let sit for at least 10 minutes at room temperature and proceed to the slidemaking protocol. For storing, add PBS/ 1 % Paraformaldehyde fixative to 5 ml volume and store in the refrigerator 4 °C until use (within 2 weeks).

ApopTag. slidemaking protocol

1. Clean microscope slides by rinsing with double distilled, deionized water and dry.
2. Centrifuge the paraformaldehyde fixed sample at 1,000 rpm for 10 minutes at room temperature.
3. Carefully aspirate the supernatant out without disturbing cell pellets. Flip to softly disperse the lymphocyte pellets.
4. Add 100 µl PBS/1% Paraformaldehyde fixative to resuspend cells. Flip to softly disperse the lymphocyte pellets.
5. Drop 20 µl of this cell suspension onto a clean slide.
6. Allow the slides to air dry for 1 hour in a fume hood or laminar flow carbinet.
7. Fix the cells by placing the slides in absolute ethanol overnight at -20°C.
8. Transfer slides from absolute ethanol. Allow slides to air dry for 1 hour in a fume hood or laminar flow cabinet, and mark around the area of the specimen.
9. Wash slides for 5 minutes in PBS at room temperature for 3 times.
10. Allow slides to air dry, put one drop (13.5 µl) of equilibration buffer (Apop Tag Kit) to the area marked on the slide. Cover the marked area with plastic coverslip (Figure 7) and incubate the cells at room temperature for 5 minutes on bench top.

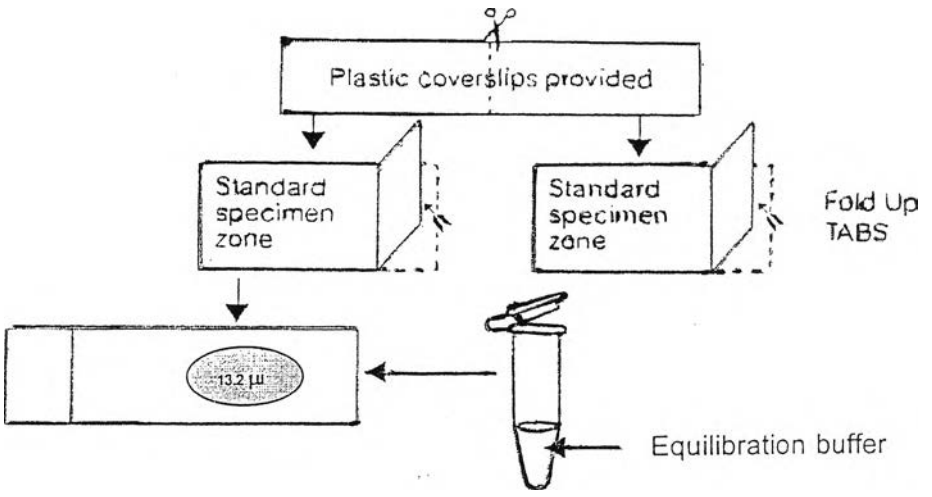


Figure 7 Demonstrate the slide marking and covered with plastic coverslip.[24]

11. After incubation, carefully remove the coverslip and dry around the marker area with absorbent. (Note : the positive control slide was treated with DNase I, and incubated in CO_2 incubator 1 hour before going to step 12.)
12. Add 13.2 µl of TdT mixture prepared freshly or within 6 hours to the specimen area. Cover with a plastic coverslip and incubate in CO_2 incubator at 37 °C, 97% humidity, 0.35 % CO_2 for 1 hour. (Note : The negative control slide was created by omitting TdT enzyme)
13. After incubation, carefully remove the coverslip and dry off around the marker area with absorbent.
14. Place the slides into the Coplin staining jar containing a warm stop wash buffer, incubate at 37 °C for 30 minutes.
15. Wash slides for 5 minutes in PBS at room temperature 3 times.

16. Allow the slides to air dry in a fume hood or laminar flow cabinet.
17. Add 13.2 µl Fluorescein Isothio-Cyanate-Dextran (FITC) mixture prepared freshly or within 6 hours onto the specimen area. Cover with a plastic coverslip, incubate for 45 minutes in a humidified chamber place in a dark room at room temperature. (Note : Avoid exposure to light as much as possible)
18. After incubation, carefully remove the coverslip and dry off around the sample area. Wash slides 3 times for 5 minutes in PBS in the dark room.
19. Rinse the slides 1 minute with Hoechst dry solution, then rinse the slides in PBS for 1 minute in the dark room.
20. Allow the slides to air dry in a fume hood or laminar flow carbinet. Add 10 µl DAPI-Antifade solution to the specimen area and cover with a standard glass coverslip and mount. Develop the slides in the dark room for 10 minutes before viewing by fluorescence microscope. If storage of the slides are required, put some rubber cement to edges of the coverslip and store at -20 °C in a dark box.
21. The slides were viewed on a fluorescence microscope for monitor apoptotic cells. The lymphocyte cells were determined by counting the total lymphocytes 1,000 cells per slide, and counted the apoptotic cells in the same field under the blue filter. The apoptotic index were calculated.

7. Statistical analysis

The results of all parameters are expressed as the mean ± SEM. The comparison of the sampling groups was determined by Analysis of Variance (ANOVA) using the Statistical Packages for Social Science (SPSS) in which the statistical significance was defined as $p < 0.05$.