CHAPTER III

MATERIALS AND METHODS

1. Animal preparation

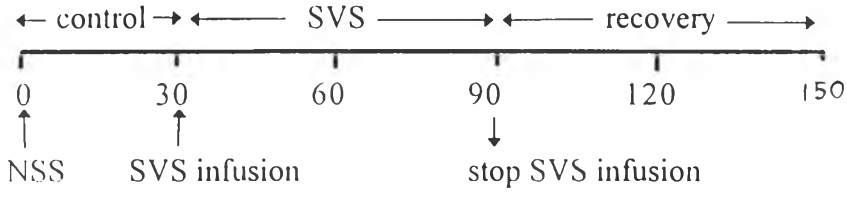
The experiments were performed in male Wistar rats, weighing between 250-300 g. The animals were maintained on normal rat food and tap water ad libitum until the experiment began. On the day before experiment, rat was anesthetized by intraperitoneal injection of sodium pentobarbiturate (Nembutal, Ceva Sanofi sante animale s.a., Paris, France) 45 mg/kg.BW. The supplemented subsequence dose of 2-3 mg was needed to maintain anesthesia throughout the experiment. Body temperature was continuously maintained at 37.5°C by a rectal probe which was connected to temperature controller and heating lamp.

2. Experimental procedures

2.1 Determination the effect of SVS on renal function.

After animal preparation, the rat was tracheostomized and both femeral arteries were cannulated with polyethylene tubes (PE50) for blood sample collection and blood pressure recording which was connected to pressure transducer (Statham PE23A) and recorder (Polygraph Model 79E, Grass Instruments Co.). Both femoral veins were cannulated for infusion of either normal saline (NSS) or various tested solution. Both ureters were cannulated to collect urine sample. During surgical preparation, NSS (1%/kg.BW/hr) was slowly infused by Harvard syringe pump to

replace water and fluid loss during surgery. Then, the solution containing 1% inulin and 0.2% p-aminohippuric acid (PAH) in NSS was intravenously infused with the rate of 1%kg.BW./hr. The animal was allowed to equilibrate for 45 min before experimental starting. The experiment was carried out to determine renal function and general circulation. The animals were received either NSS or SVS depending on experimental design. There were both intravenous infusion of SVS and SVS intubation. SVS were supplied by Dr. Dhuang Buddhasukh, Department of Chemistry, Faculty of Science, Chiang Mai University. Further purification was performed by methanol crystalization to obtain 95% purity of SVS in comparison to the standard SVS (Sigma Chemical Co,Ltd). Intravenous infusion of SVS (150 mg/ml NSS) was carried out by varying treatments from 100, 100 and 200 mg/kg.BW/hr. For the experiment of SVS infusion, five 30-min periods; one control, two SVS infusion periods and two recovery periods were undertaken. After the control period, SVS was primed and continued infusion for one hour in each treatment. Arterial blood sample (0.8 ml) was withdrawn at the mid period whereas urine was collected at the end of each period. Following blood withdrawal, the solution containing 6% albumin in NSS was mixed to the previous collected blood cells in equal amount of blood collection and was transfused to replace the amount of blood loss. Two further 30-min of recovery period were performed after stopping SVS administration. The protocol was accomplished by the following diagram.



For SVS intubation, 2 g/kg.BW of SVS dissolved in distilled water was fed by intragastric intubation without anesthesia. Intragastric intubation of SVS or NSS was separated into 2 consecutive 45-min period. Rat was free access to normal food and water in the cage. 6 hours after the first feeding, the animal was anesthetized by sodium pentobarbiturate, and then general animal preparation was performed like the group of SVS infusion. This dose of SVS was chosen since it was found from the pilot study that it was the dose that began to produce hypotension, natriuresis and diuresis . Six hours was chosen to perform in the present experiment since it was reported that SVS in blood reaches to the maximum level within 6-8 hours after ingestion in rats (Nakayama et al, 1986).

The renal function was evaluated by determining of glomerular filtration rate (GFR), effective renal blood flow (ERBF), effective renal plasma flow (ERPF), renal vascular resistance (RVR), filtration fraction (FF), fractional excretion of sodium, potassium and chloride (FE_{Na} ,FE_K , FE_{cl}), urinary excretion of sodium, potassium and chloride ($U_{Na}V$,U_KV, $U_{cl}V$). Plasma osmolality (P_{osm}), osmolar clearance (C_{osm}), free water clearance ($C\ H_2O$), plasma sodium, potassium ,chloride and glucose (P_{Na} ,P_K , P_{cl} and P_G) were also determined.

2.2 Histopathological study of renal tissure

After the end of experiment in 2.1, both kidneys were immediately excised and carefully stripped off the adhearing fat. The kidneys were transversed sectioned and washed by NSS. Kidney tissues were cut into small pieces around 0.5 cm thick, and then immersed in 10 % formalin solution. These renal tissues were processed by

paraffin technique and stained with hematoxylin and eosin. Histopathological changes were examined under the light microscope.

2.3 Determination of site and mechanism of natriuretic effect of SVS.

2.3.1. Lithium clearance (C_{Li})

C_{Li} method was used to determine proximal tubular reabsorption of Na^+ and H_2O as previously stated in chapter II. After surgical preparation, inulin solution (1 gm%) was intravenously infused at the rate of 1%kg.BW/hr throughout the experiment. Simultaneous infusion of LiCl solution (0.015 M) (Sigma Chemical Co., St. Louis, USA) was performed in 40 min later at the rate of 10 ml/kg.BW/hr. after given of a bolus dose of 0.2 ml of 0.07 M LiCl solution. By this procedure, plasma Li concentration could be maintained at the level of 0.2-0.4 mEq/L. Ten min following LiCl administration, the experiment was ready to perform. The experimental procedures were divided into five 30-min periods ; control, the first and second of SVS infusion period, the first and the second of recovery period. Urine sample and 0.5 ml of arterial blood were collected in each period for Na^+, Li^+ and inulin determinations. After each blood withdrawal, albumin solution (6% in NSS) with red blood cells collected from the previous period was perfused to replace blood loss. After the control period, 200 mg/kg.BW of SVS was intravenously infused as a priming dose and followed by continuous infusion at the rate of 200 mg/kg.BW/hr. for one hour. This dose of SVS was chosen because of the maximum natriuretic response as shown in the chapter IV. The magnitude of proximal Na^+ and H_2O reabsorption were shown by calculation according to Thomsen (1984, 1990).

2.3.2 Determination of renal Na^+, K^+ ATPase activity

In this experiment, the animals were divided into 2 groups, the control and SVS infusion group. After animal preparation, SVS (150 mg/ml) was infused at a priming dose of 200 mg/kg.BW, and followed by continuous infusion at the rate of 200 mg/kg.BW/hr. In the control group, NSS was administered instead of SVS. After 30 min of SVS or NSS administration, both kidneys were quickly removed and washed several times with ice-cold medium containing 0.1% deoxycholate, 30 mM L-histidine, 5 mM EDTA and 250 mM sucrose, pH 7.4. The removed kidneys were decapsulated and transversed section. Renal cortex from each half part of both kidneys was separated and the remaining half was pooled to determine both total renal and cortical Na^+, K^+ ATPase activity. Plasma membrane riched Na^+, K^+ ATPase was obtained by modify method of Lo et al. (1981) and Klein and Lo (1992). In brief, the renal tissue was homogenized (1/10 w/v, 10 strokes) and then centrifuged at 1,000 g for 10 min (High speed centrifuge model IE C-B 22M). The supernatant was saved, then the pellet was again resuspended with ice-cold medium and homogenized with 1/2 original volume five strokes. The homogenized tissue was centrifuged 1,000 g for 10 min. The repeated maneuver was again carried out as above. The pooled supernatant was then centrifuged 10,000 g for 10 min. The final supernatant was discarded but the remaining pellet at the bottom was resuspended with ice-cold medium. All steps were undertaken at 0-4 °C. The method was shown in the latter diagram. The final resuspension was used to measure protein concentration as described by Miller et al.(1959). The Na^+, K^+ ATPase activity. Mg^{2+} and Na^+, K^+ ATPase activity were also determined by diluting the suspension with ice-cold

medium (final protein concentration ~250-300 μg/ml). 0.1 ml of suspension was added by 0.9 ml of incubation medium containing 0.1 M NaCl, 20 mM KCl, 3 mM $MgCl_2$, 100 mM Tris, 1 mM EDTA, pH 7.4 with or without 1 mM ouabain. The samples were then incubated at 37 °C for 20 min after starting the reaction by addition of 0.1 ml of 30 mM ATP. The reaction was terminated by the application of 0.2 ml ice-cole 30% tricholoacetic acid (TCA). Inorganic phosphate (Pi) released was assayed by method of Bonting et al (1961). The Mg^{2+} ATPase activity was determined between reaction with and without ouabain. Na^+, K^+ ATPase activity was calculated as the difference in the activity of total ATPase and Mg^{2+} ATPase. Enzyme activity was expressed as μmole Pi released/ mg protein/hr.

2.3.3. Determination of renal mitochondrial activity

In this experiment, two groups of experiment were carried out as control and SVS infusion group. All animals were anesthetized with intraperitoneal injection of inactin (100 mg/kg.BW) instead of sodium pentobarbiturate. Since, it has been reported that sodium pentobarbiturate inhibits substrate utilization within site I but not site II of mitochondrial electron transport chain (Cohen, 1973 ; Bhayana, 1980) while inactin does not influence on both site (Malis and Bonventre, 1986). Furthermore, similar result on renal function were obtained in rat anesthetized with either inactin or sodium pentobarbiturate. After animal preparation, 200 mg/kg.BW of SVS was intravenously infused as a priming dose and followed by 200 mg/kg.BW/hr for 30 min. This time interval was chosen because of the highest response on renal function. In the control group, NSS was infused at the same rate (1%kg.BW./hr) as SVS group.

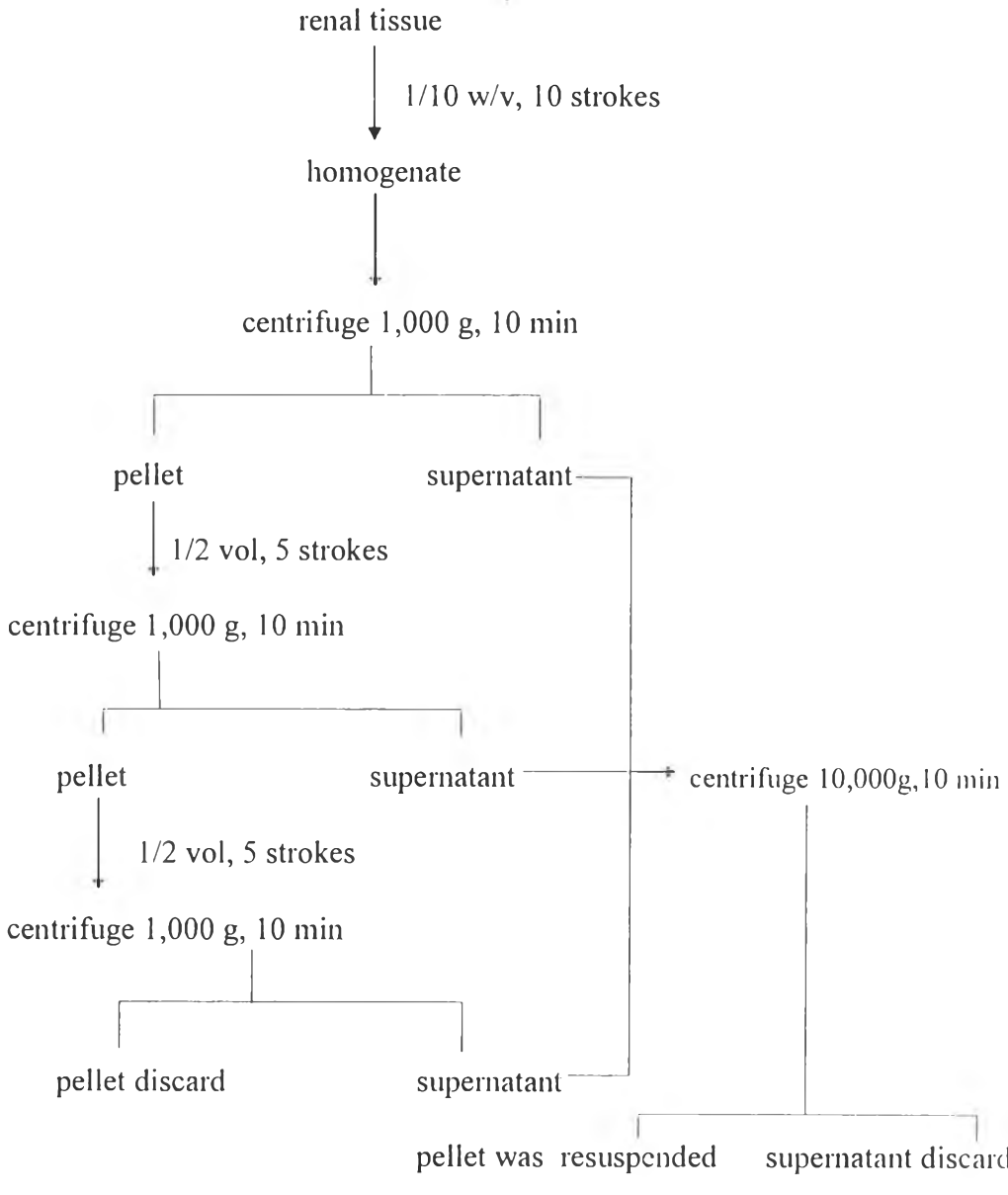


Figure 3.1 Diagram represents the method to separate renal Na^+, K^+ ATPase enzyme.

Renal mitochondrial preparation was manifested by modify method of Malis and Bonventre (1986). After stoping SVS or NSS infusion, both kidneys were quickly removed and washed several times with ice-cold medium containing 210 mM manitol,

70 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin (BSA), and buffered pH 7.4 with Tris-HCl. All steps were carried out at the temperature of 0-4°C. The renal capsule was removed. Renal tissue was cut into small pieces and mixed with the medium solution (1:20 w/v) and then homogenized 4 strokes. The homogenate was then centrifuged for 10 min at 700 g. The supernatant was decanted, and centrifuged again for 5 min at 10,000 g. The final mitochondrial pellet was resuspended with ice-cold medium to a final protein concentration of 25-30 mg/ml which was measured by the method of Miller (1959). Mitochondrial oxygen consumption (Vo_2) was determined in a closed chamber with Clark-type electrode (YSI model 5300) and continuously stirred by magnetic stirring bar at 25°C. The incubation medium used to measure mitochondrial respiration consisted of 120 mM KCl, 2 mM KH_2PO_4, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.2. Mitochondrial Vo_2 was recorded with recorder (Kipp & Zonen type BD 112 recorder). Respiratory substrate for site I was glutamate (5 mM) and malate (5 mM), and for site II was succinate (10 mM).

After the mitochondrial solution was pored into incubation medium, the substrate was added and state 4 respiration (substrate supporting respiration) was measured. ADP solution (600 nM) in the presence of glutamate plus malate or in the case of succinate (300 nM) was added to initiate state 3 respiration (ADP stimulated respiration). Mitochondrial activity in state 3 and 4 respiration, respiratory control ratio and ADP:O (P/O ratio) were determined.

Renal mitochondrial ATP synthetase activity was also evaluated. The preparing mitochondria was incubated in 0.8 ml of incubation medium containing 100

μM Tris-HCl and 10 μM $MgCl_2$ at pH 7.4. The reaction was started with 50 μM ATP and ran for 5 min at 30°C. The reaction was stopped by application of 1 ml of 10% TCA. The Pi released was measured in the same as Na^+, K^+ ATPase activity.

2.4 Measurements of plasma volume (PV) and blood volume (BV) .

This experiment was undertaken to evaluate whether hypotensive effect of SVS is due to the reduction of PV using dye dilution method. The animals were classified into 2 groups including control and SVS infusion group. Following animal preparation, both femoral artery and vein were cannulated for blood collection and SVS or NSS infusion. Arterial blood sample (0.3 ml) was withdrawn to collect plasma and measure hematocrit. The first plasma collection was used to be blank and mixed with standard Evan blue dye in determination of the Evan blue dye concentration. 200 mg/kg.BW of SVS (150 mg/ml) was intravenously infused and continuously introduced with the rate of 200 mg/kg.BW/hr throughout experiment. After starting SVS administration for 5 min, 0.3 ml of the Evan blue dye (T-1824, 1% anhydrous salt) was slowly infused through the other femoral vein. Following termination of dye infusion for 5 min, the subsequent blood sample (0.15 ml) was collected in every 5 min period up to 20 min. Plasma concentration of Evan blue dye was achived by standard curve of Evan blue concentration. PV was determined by modify method of Milnor (1980). BV was obtained by the equation ; $\frac{PV \times 100}{100 - Hct}$

2.5 Determinations of renal plasma threshold and tubular transport maximum of glucose (Tm_G) .

This experiment was carried out to define the mechanism of glucosuric influence of SVS infusion. After surgical preparation, inulin solution (1%) was intravenously infused at the rate of 1%/kg.BW/hr throughout experimental procedure. Following the beginning of inulin infusion for 25 min, glucose loading was performed. 10 gm% glucose in NSS was infused with the rate of 60 μL/min for 20 min. Plasma glucose was further elevated by increasing infusion rate to 72 μL/min. By this manoeuver, plasma glucose was raised to around 200 mg%. 10 min after changing rate of glucose loading, 0.3 ml of arterial blood was withdrawn and equal amount of 6% albumin in NSS was reinfused to replace volume loss. 15 min after changing rate of glucose infusion, urine sample was collected. Thereafter, plasma glucose was continuously raised every 15 min following urine collection by increasing glucose concentration to 15, 17.5, 20, 22.5, 25, 27.5 and 30 gm%.Plasma glucose concentration was augmented stepwise manner approximately 200-500 mg%. Arterial blood and urine sample were collected in every 10 and 15 min respectively after changing glucose concentration infusion. Renal plasma threshold for glucose was determined.

2.6 Determinations of glucose turnover rate (GTR) and plasma insulin concentration

This series of experiments was carried out to assess the mechanism of action of SVS infusion on glucose kinetics by determination of glucose turnover rate (GTR).

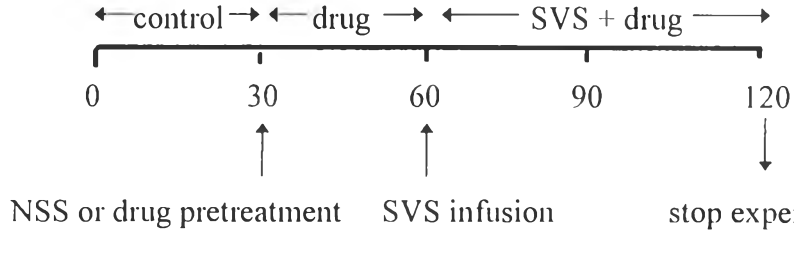
The principle of GTR has been accepted and used since 1959 (Steele, 1959 ; Katz et al., 1974 ; Umpleby and Sönksen, 1987). Sixteen animals were divided into 2 groups including control and SVS infusion groups. According to the experiment in 2.1, the results of SVS infusion at the rate of 200 mg/kg.BW/hr showed a high response for an increase in plasma glucose level. Therefore the rate of infusion, 1.33 ml/kg.BW/hr of SVS was chosen for the treatment in the series II of experiments. After SVS (200 mg/kg.BW) or NSS was intravenously introduced for 30 min in each group, 0.2 ml of the tracer solution containing 1 μCi (U-^{14}C)-glucose and 5μCi (3-^{3}H)-glucose was infused as a priming dose and followed by continuous infusion of the tracer solution containing of 1 μCi/ml of (U-^{14}C)-glucose and 5 μCi/ml of (3-^{3}H)-glucose with the constant rate of 40 μL/min. The continuous infusion of the tracer solution was undertaken for a period of 90 min. This procedure produced a constant of radiospecific activity of glucose in the plasma about 60 min after the infusion of infusion of isotope-labelled glucose. An arterial blood (0.5 ml) was withdrawn every 15 min at 30-90 min of isotope-labelled glucose infusion. Equal volume of 6% albumin solution together with the previous blood cells was reinfused back to replace blood withdrawal. GTR, glucose carbon recycling, glucose clearance and plasma glucose concentration were determined. Following 30 min of SVS or NSS infusion, arterial blood sample was withdrawn to determine plasma insulin and glucose concentration.

2.7 Determination the actions of SVS administration is mediated via norepinephrine, angiotensin II, arginine vasopressin, nitric oxide , prostaglandin or atropine.

The general protocols were the same as 2.1. The animals were divided into 9 groups according to which drugs was infused prior to SVS administration. Four 30-min periods were established, one control, one drug pretreatment, the first and second period of combination infusion of SVS and drugs. After the control period, each drug of the following ; norepinephrine (NE), prazosin, angiotensin II (A_{II}), arginine vasopressin(AVP),atropine, L-NAME, indomethacin, L-NAME+indomethacin was infused for 30 min. After that, 1.33 ml/kg.BW. of SVS (150 mg/ml) was injected as a priming dose and continuous infusion with the dose of 200 mg/kg.BW./hr in combination with the drug above. The procedures of arterial blood and urine sample collections were the same as 2.1.

Renal function was determined like 2.1. Plasma and urinary glucose concentration was also measured.

All of experiments were demonstrated as the following diagram.



Renal function was determined like 2.1. Plasma and urinary glucose concentration was also measured.

3.Analytical determinations

3.1. Determination of blood and urine compositions.

GFR and effective renal plasma flow (ERPF) were calculated from clearance of inulin and PAH respectively. Inulin concentration in plasma and urine was determined by the anthrone method as described by Young and Raisz (1952). Plasma and urine electrolyte were measured as following : Na^+ and K^+ concentration by Flame Photometer (Clinical Flame Photometer 410C ; Corning Ltd., Halstead Essex, Corning Ltd., Halstead Essex, England). Urine osmolality was determined by the freezing point osmometer (The advanced osmometer, Model 3D3, K.V., Science Ltd.). Since the plasma sample was too little to determine all parameters including plasma osmolality. Therefore, plasma osmolality was calculated by the equation as described by Ganong (1995).

plasma osmolality (mOsm/L) $= 2[Na^+] + 0.055[Glu] + 0.3[BUN]$

$[Na^+]$ = plasma concentration of sodium (mEq/L)

[Glu] = plasma concentration of glucose (mg%)

[BUN] = plasma concentration of urea nitrogen (mg%)

Plasma and urine glucose concentration could be obtained by enzymatic colorimetric test using glucose oxidase enzyme(Glucose liquicolor, Human Co., Ltd.). Plasma BUN was measured by modified method of Wybenga et al. (1971).

Plasma and urine Li^+ assay were accomplished by modify method of Amdisen (1967, 1971) using Flame Photometer like Na^+ and K^+.

3.2. Assays for glucose turnover rate (GTR)

Determination of GTR of (U-^{14}C)-glucose and 5μCi (3-^{3}H)-glucose according to Chaiyabutr and Buranakarl (1989) was accomplished.by the following procedures Plasma (150 μL) was deproteinized with 3 ml of 5% $ZnSO_4$ and 4.5% $Ba(OH)_2$, and then centrifuged. The supernatant was then passed through IRA anion exchange resin colume (Amberlite, IRA-400 c.p., Mallinckrodt, U.S.A.) packed in 3 ml plastic syringe to eliminate the contamination of labelled glucose by other organic acid such as lactate, pyruvate. The resin bed volume is 1 ml and the rate of flow was adjusted to 5 drops/min. 3 ml of deionized distilled water was added to the column to wash the remaining radioactive glucose. All of the elute was collected into 10 ml vial, and then evaporated on hot plate until dry. 0.5 ml of deionized distilled water was pored into the vial. 20 μL of the sample was used to measure glucose concentration and the remaining for determination of radioactivities of glucose using a liquid scintillation spectrometer (1214 Rackbeta, LKB Wallac, Sweden). GTR was calculated using values of the rate of infusion of (U-^{14}C)-glucose and (3-^{3}H)-glucose solution and specific activity of (U-^{14}C)-glucose and 5μCi (3-^{3}H)-glucose in arterial plasma at equilibrium by the assumption described by Umpleby and Sönksen (1987). Plasma insulin level was determined by radioimmunoassay at Ramathibodee hospital.

4.Calculations

4.1. Calcalation for general circulation and renal function

mean arterial blood pressure (MAP) = $DP + {}^{1}/_{3}(SP-DP)$

glomerular filtration rate (GFR) = $\frac{U_{In}V}{P_{in}}$ = C_{in}

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

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

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

Renal vascular resistance (RVR) = $\frac{MAP \times 1333 \times 60}{ERBF \times 1000}$ dyne-sec/cm^5

Tubular reabsorption of glucose (T_G) = $(GFR \times P_G) - (U_G V)$

Osmolar clearance (Cosm) = $\frac{U_{OSM}V}{P_{OSM}}$

Free water clearance (C_{H2O}) = V-Cosm

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

Clearance of substance (x) = $\frac{U_X V}{P_X}$

4.2. Calculation for C_{Li} method

Reabsorption of H_2O in proximal tubule = $C_{In} - C_{Li}$

Reabsorption of Na^+ in proximal tubule= $(C_{In} - C_{Li}) \times P_{Na}$

Reabsorption of H_2O in distal nephron = $C_{Li} - V$

Reabsorption of Na^+ in distal nephron = $(C_{Li} - C_{Na}) \times P_{Na}$

Delivery of Na^+ out of proximal tubule = $C_{Li} = C_{Naprox}$

The fractional excretion of Na^+ from proximal tubule = $C_{Li}/C_{In} = FE_{Li} = FE_{Naprox}$

Urinary excretion of Na^+ and H_2O expressed as fractional of amount delivered from proximal tubule = C_{Na}/ C_{Li} and V/C_{Li} respectively.

4.3. Calculation for measurment of mitochondrial activity

$$\text{Mitochondrial state 4 respiration} = \frac{Vo_2 \text{ in state 4}}{\text{mg mitochondrial protein}}$$

$$\text{Mitochondrial state 3 respiration} = \frac{Vo_2 \text{ in state 3}}{\text{mg mitochondrial protein}}$$

$$\text{Respiratory control ratio (RCR)} = \frac{\text{mitochondrial state 3 respiration}}{\text{mitochondrial state 4 respiration}}$$

$$\text{ADP : O or P/O ratio} = \frac{\text{nmol of ADP}}{\text{total } Vo_2 \text{ in state 3 respiration}}$$

4.4. Calculation for measurment glucose turnover rate

$$\text{GTR or rate of glucose appearance (Ra)} = \frac{\text{rate of (U-}^{14}\text{C)-glucose or (3-}^{3}\text{H)-glucose infusion}}{\text{specific activity of (U-}^{14}\text{C)-glucose or (3-}^{3}\text{H)-glucose}}$$

Specific activity of the radioactive was obtained by the specific radioactivity of each tracer in plasma at steady state (DPM/μmol glucose)

$$\%\text{glucose carbon recycling} = \frac{\{\text{GTR of (3-}^{3}\text{H)-glucose} - \{\text{GTR of (U-}^{14}\text{C)-glucose}\} \times 100}{\text{GTR of [3-}^{3}\text{H]-glucose}}$$

$$\text{Glucose metabolic clearance} = \frac{\text{GTR of (3-}^{3}\text{H)-glucose}}{\text{plasma glucose concentration}}$$

5.Statistics

All datas were expressed as mean±SEM. Statistical significant difference between values were compared using either paired or unpaired t-test. Wilcoxon signed rank test was also used. The values were considered to be statistically different at a level of $P<0.05$.