CHAPTER III

MATERIALS AND METHODS

1. Animal preparation

Male Sprague-Dawley rats weighing 180-220 gram, from The National Animal Center of Mahidol University, were used. All rats were rest 3 days before the diabetic induction or the injection of citrate buffer. The rats were divided randomly into two groups as control and diabetic groups. In the diabetic groups, the rats were induced diabetes mellitus with streptozotocin (STZ) in citrate buffer, pH 4.5 at a dose of 55 mg/kg BW by a tail vein injection. The control rats were injected with the citrate buffer without STZ as a placebo. The blood glucose concentration higher than 200 mg/dl was verified for hyperglycemic state. Fasting blood samples were obtained from the tail vein two days after the injections for measuring glucose concentration by using glucometer (Advance Glucometer, Boehringer Mannheim, Germany). The animals did not exhibit hyperglycemia at 48 hours after STZ injection would be excluded from the study.

After the hyperglycemic verification, the control and diabetic rats were divided randomly into four subgroups as the following:

Group 1 (CON): The control rats were given with tap water.

Group 2 (CON-AA): The control rats were supplemented with L-ascorbic acid in drinking water.

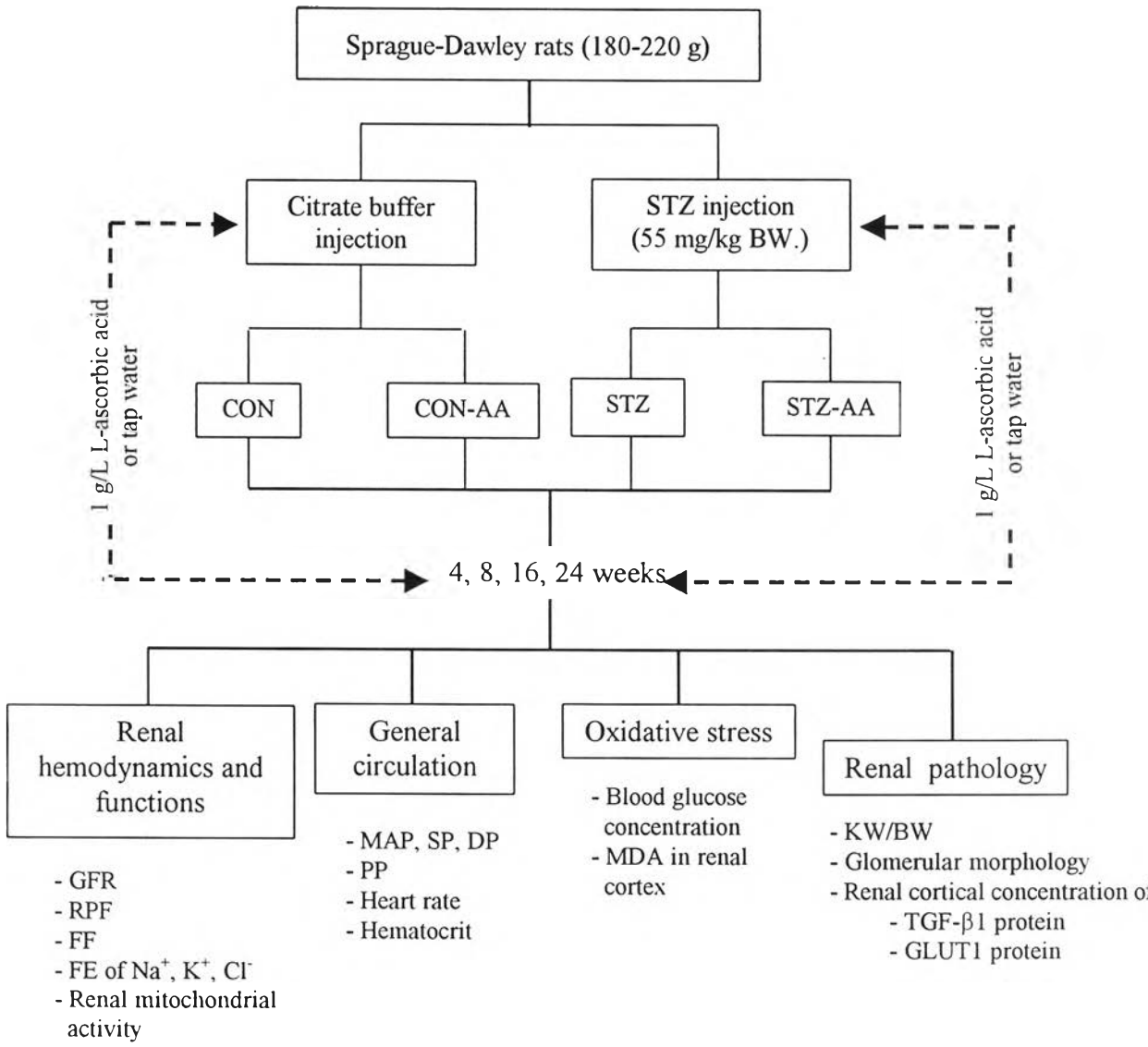
Group 3 (STZ): The diabetic rats were given with tap water.

Group 4 (STZ-AA): The diabetic rats were supplemented with L-ascorbic acid in drinking water.

All animals were fed with standard rat chow and tap water or L-ascorbic acid solution *ad libitum* throughout the experimental period.

Ninety-six rats of either controls or diabetic rats using for the renal function studies were performed in the experimental periods at week 4, 8, 16 and 24 after the supplementation with AA. For the studies of renal mitochondrial activity, thirty-two animals of either controls or diabetic rats were performed at week 8 and 24.

Experimental design



2. L-ascorbic acid supplementation

L-ascorbic acid solution (BDH, VWR International Ltd., England) was prepared freshly by dissolving in tap water at the concentration of 1 g/L (Dai et al., 1995; Jariyapongsakul et al., 2002) and was supplemented to the rats in both **CON-AA** and **STZ-AA** groups after confirming the hyperglycemic state. The solution was supplemented *ad libitum* to the rats and was changed daily. Instead of L-ascorbic acid solution, tap water was given *ad libitum* to the rats in both **CON** and **STZ** groups.

3. Experimental procedures

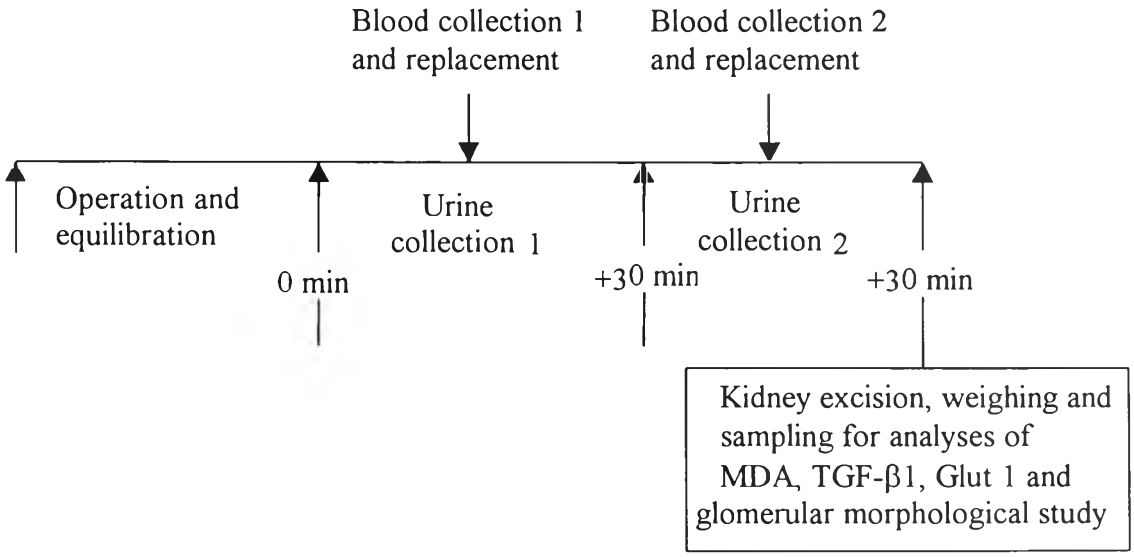
3.1 Determination of the effect of L-ascorbic acid supplementation on renal hemodynamics and functions in STZ-induced diabetic rats

Studies of renal hemodynamics and function were performed at week 4, 8, 16 and 24 after the supplementation of AA in either control or STZ rats. At the specified week of the experimental period, the level of blood glucose concentrations of animals were measured by a glucometer (Accue-CHEK Advantage, Roche). The animal was fasted for 9-10 hours before the experiment. On the day of the experiment, the rat was weighed and anesthetized with pentobarbital sodium (60 mg/kg BW). A tracheostomy was performed to insert a tracheal tube to open the air way and to remove some secretion. Right common carotid artery was canulated to collect blood samples and recorded blood pressure with a computerized polygraph (McLab System, ADInstruments). Femoral vein was cannulated with polyethylene tube for infusion of normal saline or the solution of inulin and para-aminohippuric acid (PAH).

Both ureters were canulated for urine sample collections by the operation at *linea alba*. Normal saline was infused at the rate of 10 ml/kg BW/hr by a syringe pump (Harvard syringe pump 21) to replace the body fluid loss during the surgery. After the surgery, the mixture of 1 g/dl inulin and 0.2 g/dl PAH in normal saline was infused at the rate of 10 ml/kg BW/hr instead of normal saline throughout the experiments. The animal was allowed to equilibrate for 45 minutes. After the equilibration, urine samples were collected two periods of 30 minutes each consecutively. The rate of the urine flow was measured. To study the renal hemodynamics, clearances of inulin and PAH were determined to represent the glomerular filtration rate (GFR) and effective renal plasma flow (ERPF), respectively.

Blood sample was collected at the mid-point of urine collections. Total volume of 0.8 ml of replacing blood was performed immediately to the rat after each blood sampling. To prepare the replacing blood, red cells from another rat were washed 3 times and mixed with 6% bovine serum albumin in normal saline to access the same total volume (Chaiyabutr et al., 1985; Suanarunsawat et al., 1999). The blood samples were determined for the values of hematocrit by a microhematocrit centrifuge (model Z230H,BHG HERMLE) and measured by micro-capillary reader (I.E.C. Cat No. 2201, DAMON/IEC DIVISION).

The experiment of renal function study in issue 3.1 was performed by the following diagram:



The renal hemodynamics and functions were evaluated by determining of glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal blood flow (ERBF), renal vascular resistance (RVR), filtration fraction (FF), fractional excretion of sodium (FE_{Na}), fractional excretion of potassium (FE_K), fractional excretion of chloride (FE_{Cl}), urinary excretion of sodium (UV_{Na}), urinary excretion of potassium (UV_K), urinary excretion of chloride (UV_{Cl}) and urine flow rate (V).

Urine and plasma samples were analyzed for the concentration of inulin by anthrone method and for the concentration of PAH by the modified method of Smith (1962) to determine GFR and ERPF, respectively. The concentrations of electrolytes in both urine and plasma for sodium, potassium and chloride ions were determined to calculate the fractional excretion of the electrolytes. The concentrations of sodium and potassium were measured by flame photometer (Flame photometer 410C, Ciba Corning, Inc.) and the concentration of chloride by chloridometer (Chloride analyzer 925, Ciba Corning Inc.).

Calculation for the measurements of renal hemodynamics and function

The calculated values are obtained using the following equations:

$$\mathbf{GFR} = \mathbf{C_{in}} = \mathbf{U_{in}\, V/P_{in}}$$

C_{in} = clearance of inulin (ml/min)

V = urine flow rate (ml/min)

U_{in} = urinary inulin concentration (mg/ml)

P_{in} = plasma inulin concentration (mg/ml)

$$\mathbf{ERPF} = \mathbf{C_{PAH}}$$

$$= \mathbf{U_{PAH}\ V/P_{PAH}}$$

ERPF = effective renal plasma flow (ml/min)

C_{PAH} = clearance of PAH (ml/min)

V = urine flow rate (ml/min)

U_{PAH} = urinary PAH concentration (mg/ml)

P_{PAH} = plasma PAH concentration (mg/ml)

$$\mathbf{ERBF} = \frac{\mathbf{RPF}}{\mathbf{1-(Hct/100)}}$$

ERBF = effective renal blood flow (ml/min)

Hct = hematocrit value (%)

$$\mathbf{FF} = \frac{\mathbf{GFR\ x100}}{\mathbf{ERPF}}$$

FF = filtration fraction (%)

$$\mathbf{V/GFR} = \frac{\mathbf{V\ x100}}{\mathbf{GFR}}$$

V/GFR = fractional excretion of urine

$$\mathbf{FE} = \frac{\mathbf{(U_E\ V/P_E)x100}}{\mathbf{GFR}}$$

FE = fractional excretion of electrolytes (%)

U_E = concentration of urinary electrolytes (μEq/μl)

P_E = concentration of plasma electrolytes (μEq/μl)

$$\mathbf{RVR} = \frac{\mathbf{MAP}}{\mathbf{ERBF}}$$

RVR = renal vascular resistance (mmHg/ml/min/g KW)

MAP = mean arterial pressure (mmHg)

ERBF = effective renal blood flow (ml/min/g KW)

At the end of the experiments, both kidneys were immediately excised, removed the adhering fat and weighed.

3.2 Determination of the effect of L-ascorbic acid supplementation on renal mitochondrial activity in STZ-induced diabetic rats

In renal mitochondrial activity study, thirty-two rats were divided into four groups (CON, CON-AA, STZ and STZ-AA) of the experimental periods (week 8 and 24). The animals were performed streptozotocin-induced diabetes mellitus followed the process of animal preparation that was previously mentioned. The mitochondrial activity was examined by using the method of Malis and Bonventre (1986).

Preparation of mitochondrial homogenate

At week 8 and 24 of the experimental periods, the rats were anesthetized with pentobarbital sodium (60 mg/kg BW). The kidneys were immediately isolated, removed the adhering fat and cut into small pieces in ice-cold homogenizing buffer. After washing the renal tissue 3 times with the ice-cold homogenizing buffer, the renal tissue was homogenized in ice-cold homogenizing buffer (1:10 w/v) with a homogenizer (Model K4224, Glas-Col). The homogenized tissue was centrifuged at 700 g, 4°C for 10 minute. The supernatant was centrifuged at 10,000 g, 4°C for 5 minutes (Suanarunsawat, et al., 1999). The mitochondrial pellet was resuspended with the ice-cold homogenizing buffer. The procedure was carried out in ice-cold condition throughout the whole process.

To correct the values of oxygen consumption, the mitochondrial homogenate was determined total protein by Lowry's method following the procedure in 3.3.

Chemicals for renal mitochondrial activity

Homogenizing medium was composed of 210 mM mannitol, 70 mM sucrose, 1 mM EGTA and 0.5% bovine serum albumin. The medium pH 7.4 was adjusted with Tris-HCl.

Incubation medium was consisted of 120 mM KCl, 2mM KH_2PO_4, 1mM $MgCl_2$ and was adjusted pH 7.2 with 5 mM Tris-HCl.

The respiratory substrates for site I were 5 mM glutamate and 5mM malate; and 10 mM succinate for site II.

To initiate the state 3 respiration, 600 nM of ADP was added in the presence of glutamate and malate or 300 nM in the case of succinate as the substrate.

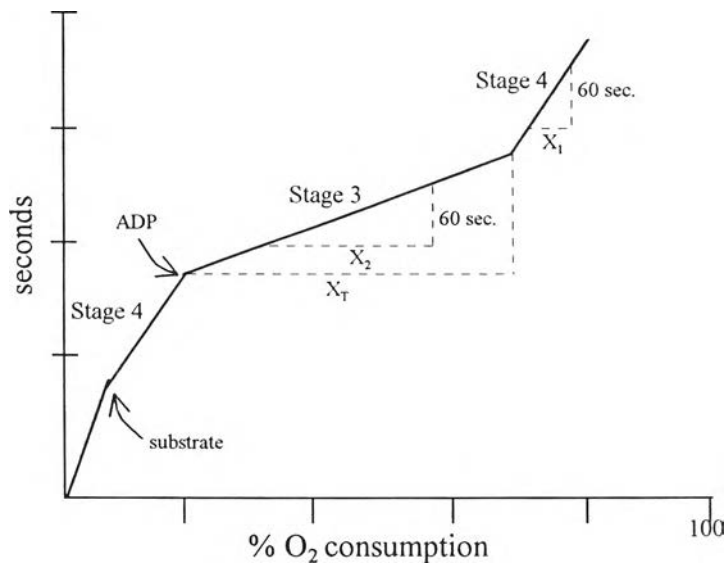
Procedure

The mitochondrial activity was carried out in a close chamber with Clark-type electrode and continuously stirred by magnetic stirring bar at 25°C. The mitochondrial oxygen consumption (Vo_2) was measured using an oxygen consumption monitor (YSI Model 5300, Biological oxygen monitor, Scientific Division, Yellow Springs Instrument Co., Inc.). The oxygen consumption tracing was printed out by Flatbed Recorder (Dual Channel Model BD112, Kipp & ZONEN DELFT Holland).

Incubation of 100 µl mitochondrial homogenate and substrates (the mixture of 5 mM glutamate and 5 mM malate for site I) in the incubation medium was performed. The oxygen consumption change (respiratory stage 3) was detected when the phosphorylation was stimulated with 600 nM of ADP. The phosphorylation reaction was going on until a change of oxygen consumption appeared (respiratory stage 4). That indicated the end of the respiratory stimulation of ADP.

After completing the experiment for mitochondrial activity within site I, the experiment of mitochondrial activity within site II was achieved in the same procedure of mitochondrial activity within site I but using 10 mM succinate instead of glutamate and malate. In addition the concentration of ADP was changed to 300 nM.

The ratio of oxygen consumption (Vo_2; ng atom of O_2) in state 3 to Vo_2 in state 4 termed the respiration control index (RCI) was determined. Moreover, the ratio of ADP concentration added respected to the total oxygen consumption in stage 3 (P/O ratio) was calculated (Estrabrook, 1967). A tracing illustration of mitochondrial respiration is shown as follows



Calculation for the measurements of renal mitochondrial activities

Rate of mitochondrial respiration in stage 4 (X_1) (ng atom O_2/min/mg protein) = $\frac{Vo_2 \text{ in stage 4}}{\text{mg of mitochondrial protein}}$

Rate of mitochondrial respiration in stage 3 (X_2) (ng atom O_2/min/mg protein) = $\frac{Vo_2 \text{ in stage 3}}{\text{mg of mitochondrial protein}}$

Respiratory control index (RCI) = X_2/X_1

P/O ratio = $\frac{\text{nmole of ADP}}{\text{Total } Vo_2 \text{ in stage 3 respiration } (X_T)}$

3.3 Determination of the effect of L-ascorbic acid supplementation on lipid peroxidation in renal cortex in STZ-induced diabetic rats

Preparation of renal cortex homogenate

At the end of the renal function study experiment, the kidney was removed and the renal cortex was isolated under a binocular scope in ice-cold condition. After weighing, the renal cortex was cut into small pieces and homogenized in 0.5 ml of ice-cold 0.1 M phosphate buffer (PBS), pH 7.4 with a homogenizer (Ika T25 Basic, Ultra turrax; Ika works) at 13,000 rpm for 5 minutes. Then, the homogenate was centrifuged at 3,500 rpm, 4 °C for 20 minutes. The supernatant was decanted and divided in aliquots and kept at −70 °C until the analyses were performed. The homogenate was determined total protein to correct the concentrations of MDA, TGF-β1 and Glut1 that were examined in this study.

Determination of total protein

To correct the concentrations of MDA, TGF-β1, and Glut 1, total protein in the renal cortex homogenate was determined by Lowry's method (Lowry et al., 1951) as following:

Chemicals for protein content

Fresh reagent was composed of 100 ml of 2%Na_2CO_3 in 0.1 M NaOH, 1 ml of 2% $Na_2C_4H_4O_6.2H_2O$ and 1 ml of 1% $CuSO_4.5H_2O$.

1N Folin and Ciocalteu's phenol reagent

20, 40, 80, 100 mg% bovine serum albumin (BSA)

Procedure

The renal cortex homogenate was diluted with 0.1 M phosphate buffer in the ratio of 1:50. The diluted homogenate of 100 µl was mixed in 3 ml of fresh reagent. After mixing and incubating at room temperature for 10 minutes, 300 µl of 1N Folin and Ciocalteu's phenol reagent was added and mixed. The homogenate mixture was incubated at room temperature for 30 minutes. The absorbance was read at 650 nm against the reagent blank. The total protein concentration was calculated in milligram per deciliter respected to the BSA standard curve.

Determination of lipid peroxide

MDA is usually assayed by their reaction with 2-thiobarbituric acid (TBA), which forms chromogen adduct at acid pH, under heating (100°C). The chromogen are then measured spectrophotometrically at 532 nm by using 1,1,3,3-tetraethoxy-propane for MDA standard.

Chemicals for lipid peroxide

8.1 % sodium dodecyl sulfate (SDS)

0.8 % thiobarbituric acid (TBA)

mixture of N-butanol and Pyridine (15:1 v/v)

Malondialdehyde bis-dimethylacetal (1,1,3,3 tetramethoxypropane) (TMP)

Procedure

Renal cortex homogenate was determined for malondialdehyde (MDA) by thiobarbituric acid reaction (Ohkawa et al., 1979) as following

Fifty microliter of the renal cortex homogenate was diluted with 350 µl of distilled water. The reaction was generated by adding 100 µl of 8.1% sodium dodecylsulfate (SDS), 750 µl of 20% acetic acid (pH 3.5) and 750 µl of 0.8 % thiobarbituric acid into the diluted homogenate. The mixture was mixed and heated at 95 °C for 60 minutes. After cooling down at room temperature, the mixture of sample was added with 0.5 ml of distilled water and extracted the chromogen with adding 2.5 ml of the mixture of N-butanol and pyridine with the ratio of 15:1 (v/v) into the tubes and thoroughly mixed. The mixture was centrifuge at 4,000 rpm for 15 minutes. The organic supernatant (pink color) was read the absorbance at 532 nm. The amount of

MDA was calculated as nanomole per milligram protein of renal cortex homogenate respected to the MDA standard curve.

3.4 Determination of the effect of L-ascorbic acid supplementation on the transforming growth facter-beta1 (TGF-β1) concentration in renal cortex of STZ-induced diabetic rats

The renal cortex homogenate aliquots from the rats in four groups of four periods were analyzed for the concentration of transforming growth facter-β 1 (TGF-β1) protein by enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH, Germany). The immuno enzyme sandwich complex was formed and the absorbance at 450 nm was measured with a microtiterplate reader. The concentration of TGF-β1 was calculated as picomole per milligram protein of renal cortex homogenate respected to the TGF-β1 standard curve.

TGF-β1 standard and renal cortex homogenate was diluted in assay buffer to obtain the serial concentration of 600, 300, 150, 75, 38 and 0 picomole/ml of TGF-β1 standard and the dilution of 1:100 for the homogenate The desired number of anti-TGF-β1 antibody coated microtiter wells were secured in a holder. To assay the concentration of TGF-β1 protein, 100 µl of each diluted homogenate was pipetted into the microtiter wells. The plate was covered and incubated over night (8-16 hours) at 4 °C. After incubation, the plate was briskly shaken out the contents of the wells. The wells were rinsed 3 times with 350 µl of diluted wash solution per well and stroke sharply on absorbance paper to remove the residual droplets. One hundred microliters of specific antibody (monoclonal mouse anti-TGF-β1 antibody) were added into each well. After incubation for two hours at room temperature, the contents of the well were briskly shaken out and rinsed 3 times with 350 µl of diluted wash solution per well and stroke sharply on absorbance paper to remove the residual droplets. One hundred microliters of Conjugate (anti mouse IgG biotin) were added into each well. After incubation for 45 minutes at room temperature, the contents of the well were briskly shaken out and rinsed 3 times with 350 µl of diluted wash solution per well and stroke sharply on absorbance paper to remove the residual droplets. One hundred microliters of Enzyme Complex (streptavidin peroxidase)

were added into each well. After incubation for 45 minutes at room temperature, the contents of the well were briskly shaken out and rinsed 3 times with 350 µl of diluted wash solution per well and stroke sharply on absorbance paper to remove the residual droplets. One hundred microliters of substrate solution (TMB) were added into each well. After incubation for 15 minutes at room temperature, 50 µl of Stop solution (0.5 M H_2SO_4) was added into each well in the same timed intervals as substrate solution addition. The TGF-β1 concentration was determined by reading the absorbance at 450 ± 10 nm within 10 minutes following the addition of the Stop solution.

3.5 Determination of the effect of L-ascorbic acid supplementation on glucose transporter 1(Glut1) protein in renal cortex of STZ-induced diabetic rats

The renal cortex homogenate aliquots from rats of four groups at week 16 were indirectly determined for the concentrations of glucose transporter 1 (Glut 1) protein by using Indirect ELISA principle. To confirm the change in Glut1 of the control and experimental groups, two amounts of total protein concentrations of the samples were fixed at 25 and 40 µg/well. The absorbance was the reversed proportion to the Glut 1 concentrations in the samples. The Glut 1 content was examined by comparing OD of control and experimental groups in each assay.

Indirect ELISA for Glut1

The microtiterplate was coated with primary antibody for Glut1 (USBiological, USA.) 0.15 µg/well in carbonate coating buffer pH 9.6 and incubated at 4° C overnight (16-18 hours). After incubation, the plate was briskly shaken out the contents of the wells. The wells were rinsed 4 times with 300 µl of washing buffer of 0.01 M PBS + 0.5 % Tween 20 (PBST) per well and stroked sharply on absorbance paper to remove the residual droplets. The coated plate was blocked with 1% gelatin 300 µl/well , incubated at room temperature for 2 hrs. After washing with PBST 4 times, 25 or 40 µg /well of total protein of the renal homogenate samples were added and incubated at room temperature for 2 hrs.

After washing with PBST 4 times, 50 µl/well of anti-IgG-Hrp (1:10000) was added and incubated at room temperature 2 hrs. Then, 50 µl/well of

o-phenylenediamine dihydrochloride (OPD) solution was added after washing the wells with PBST 4 times. The plate was incubated at room temperature for 10 min. before stop reaction with 4 N H_2SO_4 25 µl/well. Glut1 was determined by reading the absorbance at 492 nm within 10 minutes following the addition of the stop solution.. The values of OD were the reversed proportions to the GLUT1 concentrations in the sample. The relation histogram of OD and µg of total protein was examined.

3.6 Pathological study of glomerular part in STZ-induced diabetic rats supplemented with L-ascorbic acid

The percentage of kidney weight per body weight as a factor to determine the renal pathology was considered. The kidneys from the experiment in 3.1 were performed cross-section at the middle part into a 2 mm-thickness piece and preserved in 10 % formalin. The renal tissues were processed by paraffin embedding and periodic acid-Schiff staining. The sections were examined under light microscope for the glomerulosclerosis, which was characterized by vascular injury (loop collapse), regional adhesion of the glomerular tuft to Bowman's capsule and expansion of the mesangial matrix. Of one hundred glomeruli, numbers of glomeruli which express glomerulosclerosis were counted (Reyes et al., 1992; Fornoni et al., 2003).

Periodic acid-Schiff staining technique

The 0.5% periodic acid solution was prepared by dissolving 0.5 g of periodic acid in 100 ml of distilled water.

Schiff reagent was tested by pouring 10 ml of 37% formalin into a watch glass. A few drops of the Schiff reagent were added for testing. A good Schiff reagent would rapidly turn a red-purple color. A deteriorating Schiff reagent would give a delayed reaction and the color produced will be a deep blue-purple.

Procedure:

The staining technique was prepared as following:

The paraffin embedded samples were deparaffinized and hydrated to water. Then, the deparaffinized sample was allowed to oxidize in 0.5% periodic acid solution for 5 minutes and rinsed in distilled water. The sample was placed in Schiff reagent

for 15 minutes (Sections become light pink color during this step) and washed in lukewarm tap water for 5 minutes (Immediately sections turn dark pink color). Then , the sample was counterstained in Mayer's hematoxylin solution for 1 minute, washed in tap water for 5 minutes. The sample was coversliped using a synthetic mounting medium after the dehydration.

Glycogen, mucin and some basement membranes showed red/purple and background showed blue.

4. Statistical analyses

All data were expressed as means with standard deviations. The statistically significant differences were analyzed by ANOVA using Least significant difference (LSD) and Thamhane's as the post hoct tests. Significant comparisons were indicated at p-value < 0.05.