CHAPTER III

MATERIALS AND METHODS

Chemical Substances

Chemical substances using in present study were listed in following details; from Sigma Chemical Co., USA

1. Vitamin C (L-ascorbic acid, 99%) (1g/L/day)
2. Streptozotocin (55mg/kgBW/i.v.)
3. Rhodamine 6G (0.3mg/ml, 0.9% normal saline)
4. Fluorescein isothiocyanate-labeled dextran, MW.150,000 (15 mg/kg BW)
5. Chemical for determination of of MDA
   -8.1% Sodium dodecyl sulfate (SDS)
   -20% of acetic acid solution (pH 3.5)
   -0.8% Thiobarbituric acid (TBA)
   -1,1,3,3- Tetramethoxypropane (TMP)
   -1.15% KCl in 0.1 M KH_2PO_4
6. Sodium pentobarbital (60mg/kg/BW/i.p.) from Sanofi, Thailand
7. Heparin

Experimental Animal

Male Wistar-Furth rats (National Laboratory Animal Center of Salaya Campus, Mahidol University) weighing 200-250 g were divided randomly into diabetic and non diabetic group. The animals were housed in a group four per 1 cage. The animals were kept in well-ventilated room in which the temperature was 28-32 °C with an automatic lighting schedule, which provided darkness from 7.00 PM to 6.00 AM. All animals were allowed freely access of food (purina laboratry Chow, Premium Quality feed, Zuelig Gold Coin Mills Pte., Singapore) and tap water.

The rats were divided into three groups.

1. **CON** group was represented the citrate buffer pH 4.5-injected non diabetic rats drinking ordinary water (n=30).

2. **STZ** group was represented the streptozotocin-injected diabetic rats drinking ordinary water (n=30).

3. **STZ-Vit C** was represented the streptozotocin-injected diabetic rats drinking 1 g/L/day of ascorbic acid (n=30).

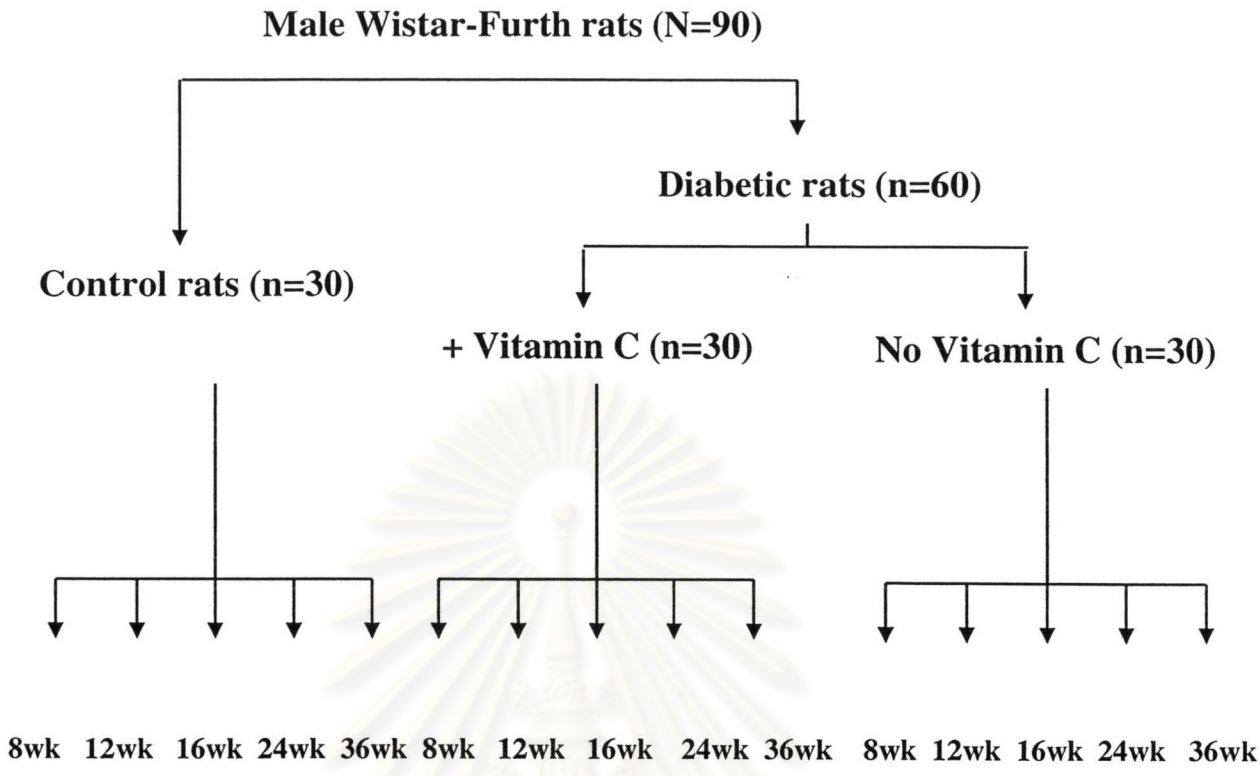

Figure 9. The diagram demonstrates the dividing of experimental animal groups

Diabetic Induction

Diabetes was induced by a single intravenous injection of streptozotocin (STZ; Sigma chemical Co. USA, 55 mg/kg BW). Streptozotocin was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma chemical Co. USA) and immediately single inject into the tail vein and same volume of citrate buffer pH 4.5 was injected by the same route to nondiabetic control animal. A diabetic condition defined as a plasma glucose concentration equal or greater than 250 mg/dl, and it usually was verified 48 hours after streptozotocin injection. A glucometer (Advance Glucometer, Bochringer Mannheim, Germany) was used for evaluation of plasma glucose from tail vein blood sample. Sample were analyzed by applying a drop of blood to a control strip inserted into the

monitor. Rats treated with streptozotocin that did not exhibit an elevation of blood glucose level at 48 hours (≥250 mg/dl) were excluded from the study (Jariyapongskul A et al., 1996).

Vitamin C supplementation

Supplementation of the rats with vitamin C (L-ascorbic acid, 99%, Sigma chemical Co., USA) started 48 hours after the administration of streptozotocin. Vitamin C was prepared daily by dissolving in drinking tap water at a concentration of 1g/L. And the experimental rats were freely access to this vitamin C drinking water (Jariyapongskul A et al., 2002).

Experiments

The experiments were performed at 8, 12, 16, 24, and 36 weeks after the injection of streptozotocin or citrate buffer pH 4.5.

On the day of experiments, rat was anesthetized with sodium pentobarbital (60 mg/kg BW, i.p.) and a thecheotomy was performed. They were ventilated mechanically with room air and supplemental oxygen.

A catheter was inserted into a femoral vein for injection of fluorescence tracer, and a femoral artery was cannulated for measurement of systolic and diastolic blood pressure (SBP and DBP) using a pressure transducer connected to a polygraph system (Nihon Kohden, Japan).

1. Iris blood flow-perfusion measurement

The iris blood flow-perfusion was measured using a Laser Doppler Flowmetry with the fiber optic needle probe (wavelength 780 nm) (model ALF 21, Advance Co. Ltd., Japan). The needle probe was fixed perpendicularly to and above the iris about 1 mm. Eight different measurement were performed at each time and the mean was then determined for each animal (Figure 10).

Principles of Laser Doppler Flowmetry

Laser Doppler Flowmetry (LDF) is an established technique for the real-time measurement of microvascular red blood cell (or erythrocyte) perfusion in tissue.

LDF works by illuminating the tissue under observation with low power laser light from a probe containing optic fiber light guides. Laser light from one fiber is scattered within the tissue and some is scattered back to the probe. Another optical fiber collects the back scattered light from the tissue and returns it to the adapter as which the result is demonstrated on the screen.

Intravital fluorescence microscopy

The iris microcirculation was observed by the technique of intravital fluorescence microscope. Briefly, after preparing the eye, the animal was then moved to the stage under fluorescence microscope. The FITC- dextran (MW 150,000 ; 15 mg/kg BW) in conjunction with blue light excitation was used to label plasma. The other florescent dye is rhodamine 6G (0.15 mg/kg BW) in conjunction with green light was used to stain mitochondria especially in leukocyte. Both fluorescent were use

and i.v. injected through the cannulated femoral vein. Both fluorescent substances can be used in pararellel by switching between two different filter blocks. The epifluorescent image was observed through the x 20 objective len and also by video camera, SIT (DAGE Co. USA), a low-light and real time throughout the experimental period. Simultanously, the image was also monitor (Sony) as showed in the figure 10. This videotape of each experiment was then play back flame by flame for further image processing analysis using the software called Global lab image II.

2. Leukocyte imaging

To visualize the leukocytes adhesion to vascular endothelium, fluorescence marker rhodamine 6G (R6G; Sigma chemical Co., USA; 0.3 mg/ml of normal saline) was administered intravenously.

Adherent leukocytes in pial postcapillary venules were recorded real time by SIT video camera mounted on an fluorescence microscope through out the experimental period. The emission wavelength of R6G lies between 530 and 540 nm.

During playback of the video tape, recording, the number of leukocyte adherence to endothelium of the postcapillary venule (20 to 50 μm diameter) were using the software called Global lab image. To measure diameter of postcapillary venule, of FITC-labeled dextran, MW, 150,000 ; 0.2 ml of 15mg/kg BW physiological saline solution) was injected into femoral vein to provide immediate contrast between plasma and interstitial.

The leukocyte that was counted as adherent one has to remain stationary for equal or longer than 30 second, the number of adherent cells were manually counted and reported by the number of cell per field of view (Kalia N et al., 2000).

3. Metabolic Changes

The parameters for metabolic changes were blood glucose, HbA_{1C}, and vitamin C. All these parameters were determined at the end of each experiment by collecting blood sample from femoral artery. Blood glucose was determined by using glucometer (Advance Glucometer, Bochringer Mannheim, Germany). Blood sample was divided into 2 sets. One set (1ml) was collected for the determine of HbA_{1C} analyzed by RIA lab CO,LTD. Where the daily internal control are performed by external quality control with Faculty of Mahidol Technology, Mahidol University. And the other set was centifused immediately for the collection of plasma. The plasma was kept at -80 °C for determination of plasma vitamin C level. Plasma vitamin C level was measured using enzyme-assisted spectrophotometric method (Liu TZ et al., 1982).

4. Free radical by products : Malondialdehyde (MDA)

Oxidation of polyunsaturated fatty acids leads to numerous peroxidic and aldehydic compounds, in particular the volatile low molecular weight aldehyde, malondialdehyde (MDA). The chemical composition of the end products of peroxidation will depend on the fatty acid composition of the lipid substrate used and upon what metal ions are present. Thus copper and iron ions give different end- product distributions as measured by the thiobarbituric acid (TBA) test. This is

one of the most commonly used method for detecting and measuring lipid peroxidation. The lipid material is simply heated with TBA at low pH, and the formation of a pink chromogen is measured at or close to 532nm. The chromogen is formed by reaction of one molecule of malondialdehyde (MDA) with two molecules of TBA (Ohgawa H et al., 1979).

5. Histological Examination

At the end of each experiment, the eye specimen was collected for further analysis of histology. Hematoxylin & Eosin technique was used. The observation of ultrastructure was performed by light microscope with 20x objective lens.

6. Statistical analysis

Results were expressed as mean ± standard deviation of mean (SD). Statistical analysis of the results was done using two-way analysis of variance followed by Post-Hoc test. A probability (P) of 0.05 or less was considered significant.

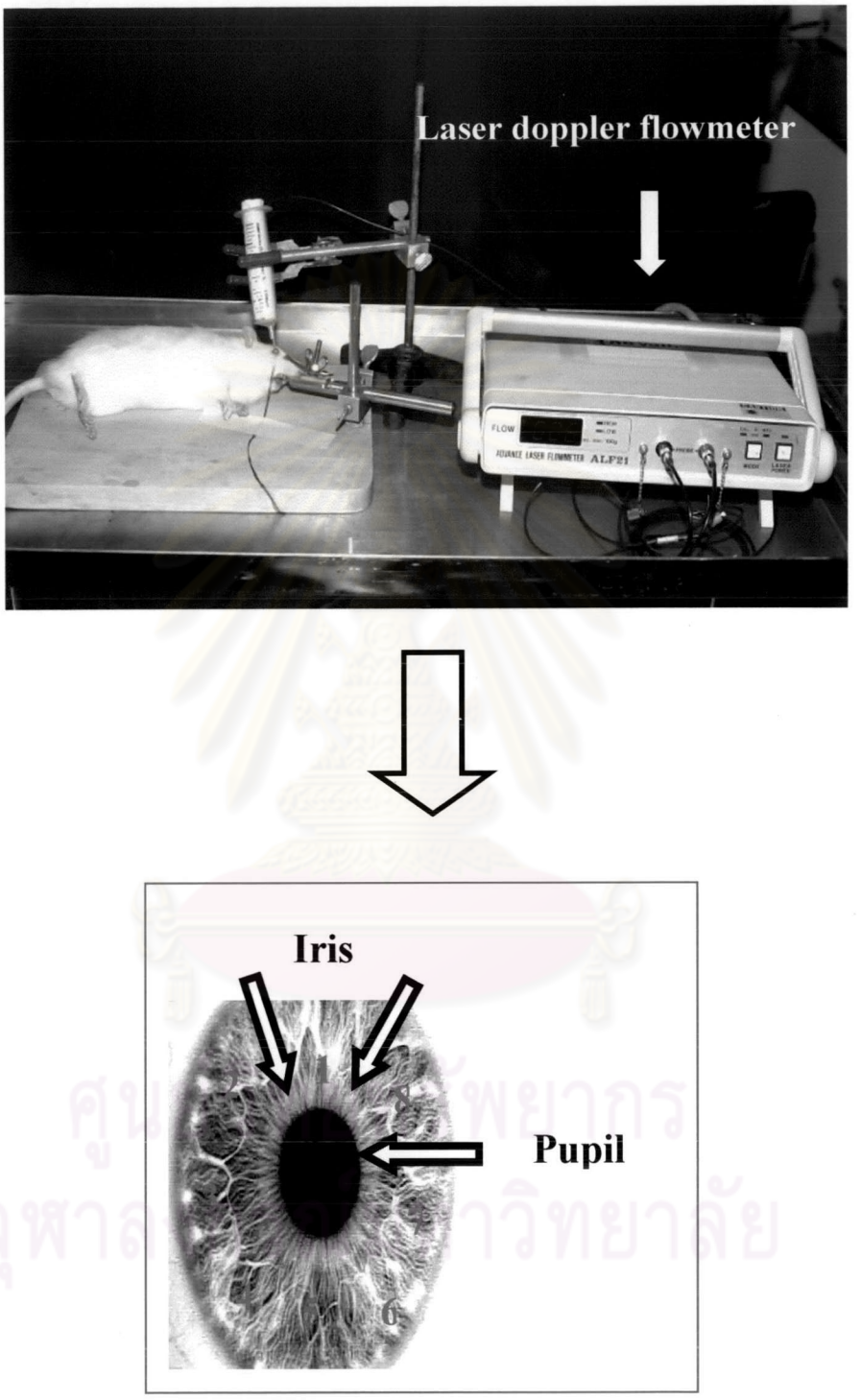

Figure 10. The method of measuring the regional iris blood-flow perfusion

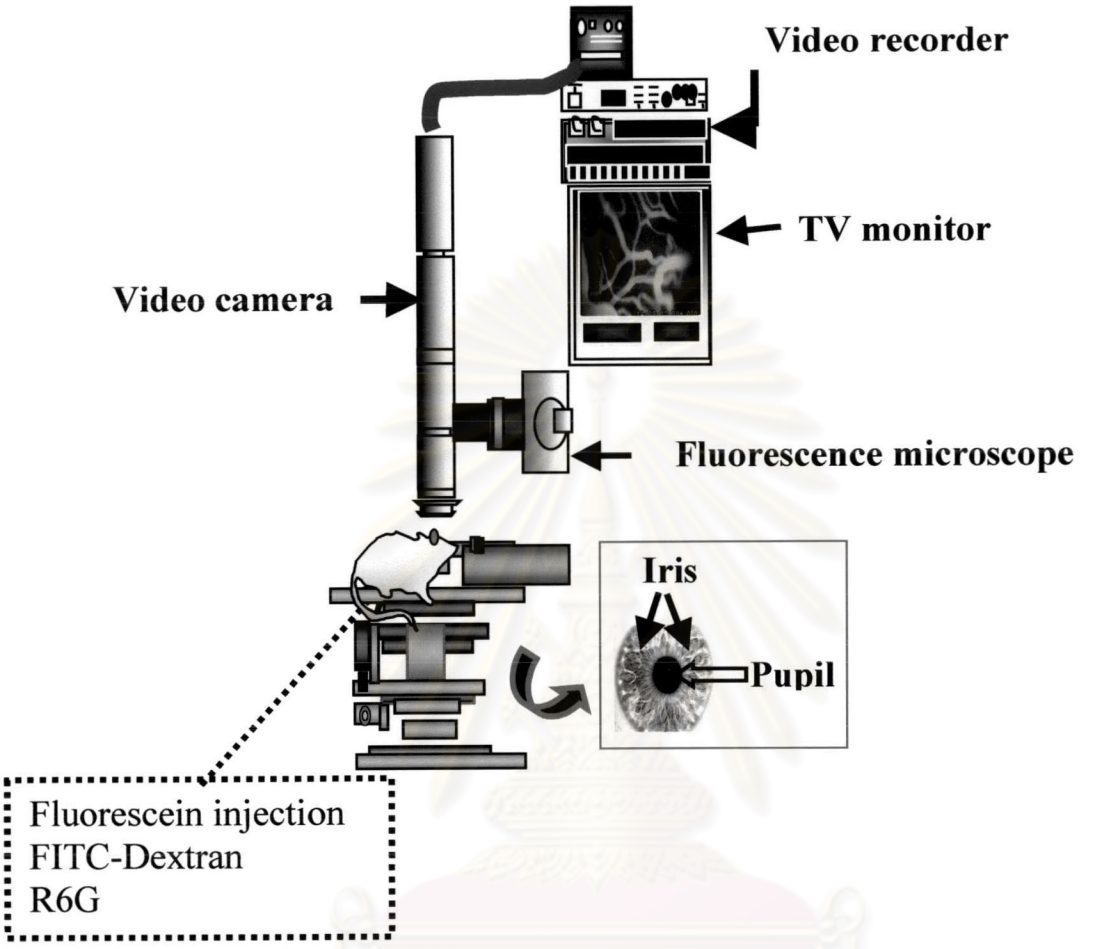

Figure 11. Schematic of setup for intravital microscopy of the iris microvasculature in the rat.