CHAPTER III

Materials and Methods

In present study, the ability of longterm supplement of vitamin C to prevent the impairment of cerebral endothelium function in streptozotocin-induced diabetic rat was studied. By using a closed cranial window technique, the effects of vitamin C on diabetic induced endothelial dysfunction were accessed through experiment protocols. Closed cranial window technique was performed to study by setting the experiment into 4 parts. First hemodynamics and metabolic changes in all animals groups were examined. Second, leukocyte-endothelium interaction was investigated. Third vasodilation responses of cerebral arterioles to endothelium dependent and independent vasodilation were examined. Moreover, the ultrastructural changes of cerebral microvessels were also investigated by using transmission electron microscopic technique.

Chemicals

Chemical substances using in present study were listed in following details.

1.	Vitamin C (L-ascorbic acid, 99%) (1 g/L/day)	Sigma, USA
2.	Streptozotocin (55 mg/kg /BW/i.v.)	Sigma, USA
3.	Fluorescence Isothiocyanate-Isomer I	Sigma, USA
4.	Rhodamine B Isothiocyanate-Dextran	Sigma, USA
5.	Rhodamine 6G (0.3mg/ml, 0.9% normal saline)	Sigma, USA
6.	Chemicals for preparation of artificial cerebrospinal fluid mM/l	

- NaCl (118.0) Riedel, Germany
- KCl (4.0) Riedel, Germany
- NaH_2PO_4 $2H_2O$ (1.2) Merck
- $NaHCO_3$ (25.0) Riedel, Germany
- Dextrose (5.0) Riedel, Germany
- $CaCl_2$ $2H_2O$ (1.5) Riedel, Germany
- $MgSO_4$ $7H_2O$ (1.2) Merck

7. Acetylcholine cholide (10^{-7} M) Sigma, USA
8. Adenosine-5'-diphosphate (10^{-6} M) Sigma, USA
9. Nitroglycerine (10^{-6} M)
10. Pentobarbital sodium (60mg/kg/BW/i.p.) Sanofi, Thailand
11. Ascorbate oxidase (0.05 ml) Sigma, USA

Animal Preparation

Male Wistar-Furth rats (National Laboratory Animal Center of Salaya Campus, Mahidol University) weighing 200-250 g were divided randomly into diabetic and nondiabetic group.

Diabetes was induced by a single intravenous injection of streptozotocin (STZ; Sigma, St. Louis, MO, USA, 55 mg/kg BW). Streptozotocin was freshly dissolved in 0.9% sterile saline (NSS) immediately before use and same volume of saline was injected by the same route to non diabetic control animals. A diabetic condition, defined as a glucose concentration 300 mg/dl or greater in tail vein blood samples, and was verified 48 hours after streptozotocin was injected and prior to each experiment with a glucometer

(Advance Glucometer, Bochringer Mannheim, Germany). In addition, diabetic condition was also confirmed by rat's manifestation of polyuria, polyphagia, and polydipsia.

Experiments

The experiments were performed at 12, 24 and 36 weeks (wks) after the injection of streptozotocin or saline solution.

On the day of experiment, rat was anesthetized with sodium pentobarbital (60 mg/kg BW, i.p.), and a tracheotomy 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 arterial blood pressure (ABP) and arterial blood gas (ABG). Arterial blood pressure was measured with a pressure transducer connected to a polygraph system (Nihon Kohden, Japan). Arterial blood gas tensions and pH were measured with CIBA Corning electrode and maintained within normal limit throughout the experiment (pCO_2 35-45 mmHg, pO_2 90-100 mmHg, pH 7.35-7.45).

For visualization of cerebral microcirculation, a craniotomy was prepared to expose the anterior pial cerebral vessels. The dura was opened using a microneedle. A stainless metallic frame with a circular glass window (7 mm diameter) was fixed to the cranial opening. An artificial cerebrospinal fluid (CSF) was infused into the intracranial space. The cranial window was connected to an infusion pump with rate 1.5 ml/min which allowed infusion of substances and artificial CSF.

Vitamin C supplementation

The rats were devided into four groups.

2.1) CON group was represented the saline-injected non-diabetic rats drinking ordinary water (n=18).

2.2) CON-vitC group was represented the saline-injected non-diabetic rats drinking 1g/L/day of ascorbic acid (n=18).

2.3) STZ group was represented the streptozotocin-injected diabetic rats drinking ordinary water (n=18).

2.4) STZ-vitC group was represented the streptozotocin-injected diabetic rats drinking 1 g/L/day of ascorbic acid (n=18).

Supplementation of the rats with vitamin C (L-ascorbic acid, 99%, Sigma, USA) started 12 hours after administration of streptozotocin or saline solution. 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.

Intravital fluorescence microscopy

Then, the cerebral microcirculation was observed by the technique of intravital fluorescence microscope. Briefly, after wareing the cranial window, the animal was then moved to the stage under fluorescence microscope. The RITC- dextran with the concentration of 25 mg/ml, MW 40,000 were used and i.v. injected through the cannulated femoral vein. The epifluorescent image of cerebral microcirculation was observed through the x 20 objective len and also be recorded by video camera, SIT (Hamamutsu Photonics), a low-light and

real time throughout the experimental period. Simultanously, the image was also monitored through the video monitor (Sony) as showed in the figure 10. This videotape of each experiment was then play back frame by frame for further image processing analysis using the software called Global lab image.

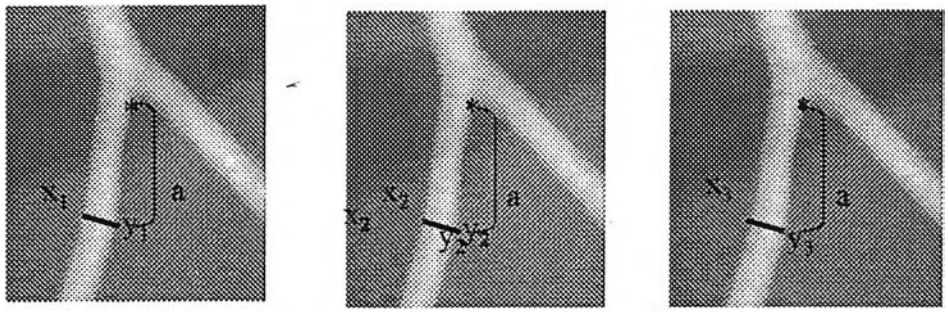
1. Leukocyte imaging

To visualize the leukocytes adhesion to vascular endothelium, fluorescence marker rhodamine 6G (R6G; Sigma Chemical) was administered intravenously .

Adherent leukocytes in pial postcapillary venules were recorded to videotape real time with use of SIT videocamera mounted on an fluorescence microscope. The emission wavelength of R6G lies between 530 and 540 nm.

During playback of the video recording, the number leukocyte adherence to endothelium of the postcapillary venule (10 to 50 μm diameter) were manually counted .

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 totally expressed as the number per 100 μm length the postcapillary venule (diameter 10-50μm) . If there are many cells of leukocytes that adhere to endothelial wall as a group, will be ignore and not be counted.

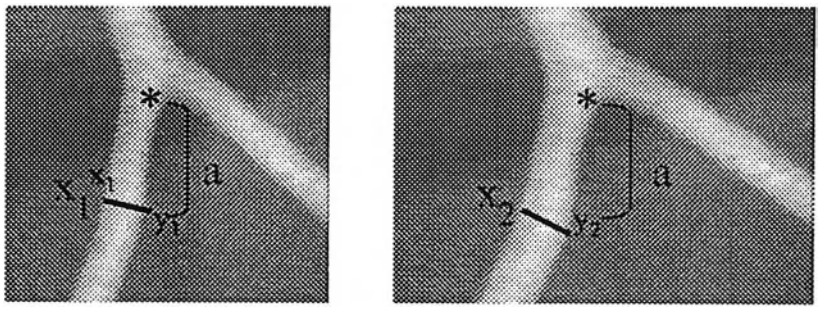


Frame 1: t=00:00:01 Frame 2: t=00:00:02 Frame 3: t=00:00:03

$$\text{mean arteriolar diameter} = \frac{x_1y_1+x_2y_2+x_3y_3}{3}$$

Figure 10. Method for measurement of arteriolar diameter.

First is defining the (x,y) position of the reference point (*) and then plus the "y" value with " a distance" in order to get x_1y_1 straight line that perpendicular to the vessel that will be measured for its diameter.



T_0=00:00:00 T_5=00:00:00

before application of substances 5 min after application of substances

$$\% \text{ change of arteriolar diameter from baseline} = \frac{x_2y_2 - x_1y_1}{x_1y_1} \times 100$$

x,y = reference point (*) of the branching

Figure 11. Method for calculation the % change of arteriolar diameter

2. Measurement of pial arteriolar diameter and red blood cell velocity

Rhodamine B isothiocyanate-labeled dextran (RITC, MW 40,000) was dissolved in a phosphate buffer saline solution (PBS) pH 7.4 to a concentration of 25 mg/ml. Based on RITC images , pial artertiolar diameter in third order arterioles was measured with a Global Lab Image device. The arteriolar diameter in micrometer(μm) was calculated as the mean of triple measurements from three video frames by using the same reference point (*) as a marker for measured each vessel in each frame as shown in Figure 10.

Using fluorescence isothiocyanate (FITC) labeled red blood cell(RBC) , RBC flow velocity in third order arterioles (diameter 20-30μm) was measured by using dual windows technique. Two windows were set on a nearly straight vessel which had no branching, and set as far apart as possible to ensure that the normalized cross-correlation. Videodensitometric signals from the windows were obtained with a videophotometric analyzer (IPM, model 204). A normalized cross-correlation on the signals was directly computed with a microcomputer (NEC,PC 9801).

For labeling of red blood cell with FITC :

Red blood cells(RBCs) were removed from plasma and buffy coat, and were washed three times with saline solution. They were mixed in FITC which was dissolved in a phosphate buffer saline pH 7.8(1mg/ml). The FITC-RBCs were incubated at room temperature for 60 minutes. The FITC-RBCs were washed again (three times) with saline solution and suspended in saline solution at a concentration of 15%(vol/vol).

0.3 ml of RITC-labeled dextran solution was intravenously injected through femoral vein prior to the injection of FITC-labeled RBCs to record the microvascular morphology and red blood cell velocity, respectively.

Experimental protocol

For studying the effect of vitamin C on endothelial function of cerebral microvessels. After the surgical procedure was completed. Rat's cerebral microvasculature was kept for 30 minutes equilibrium period under infusion of artificial CSF prior to start the experimental protocol.

Protocol 1: To examine the antioxidant effect of vitamin C supplementation on hemodynamics and metabolic changes

Hemodynamics changes

The parameters for determination of physiological changes in the present study were:-

1.1 arterial pressure (AP) which was measured via a cannular inserted into the femoral artery and monitored through out the experiment. Arterial pressure was reported in term of mean arterial pressure (MAP).

1.2 arteriolar flow rate (Q;ml/min) which was calculated by $Q=V_m r^2 \pi$, where V_m (mean RBC velocity) was obtained by dual window technique, r represented radius of blood vessels.

Metabolic changes

The parameters for determination of metabolic changes were blood glucose, plasma triglyceride, cholesterol and vitamin C. All these parameters were determined at the end of experiment by withdrawn from femoral artery via catheter. Blood glucose was determined by using glucometer (Advance Glucometer, Bochringer Mannheim, Germany). Blood samples were centifused immediately for the collection of plasma. Plasma was divided into 2 sets. One set (0.5 ml) was kept at -80°C for determination of plasma vitamin C level, and the other set was collected for the determination of plasma cholesterol and triglyceride. Plasma cholesterol and triglyceride were analyzed by RIA lab CO, LTD. that have the dialy internal control and external quality control with Faculty of Mahidol Technology, Mahidol University. Plasma vitamin C level was measured using enzyme-assisted spectrophotometric method.

Protocol 2: To examine the antioxidant effect of vitamin C on leukocyte-endothelium interaction

To quantify in vivo leukocyte-endothelial interaction, leukocytes were labeled by intravenous injection of rhodamine 6G in all animal groups. The rhodamine 6G was injected after 30 minutes equilibrium period. Immediately thereafter, leukocyte dynamics in the pial microciculation were continuously recorded to videotape(super VHS) throughout the experiment.

The number of leukocytes adherent to the postcapillary venule endothelium was determined 3 minutes after the injection of rhodamine 6G by manually counting during repeated videotape playback.

Protocol 3: To examine the effect of vitamin C on responses of cerebral arteriole to endothelium dependent and independent vasodilation

After 20 minutes recording of leukocyte images, FITC-labeled RBCs was intravenously injected following by RITC-labeled dextran for measurement red blood cell velocity at baseline period and examining response of cerebral arteriole to the endothelium-dependent and independent vasodilators, respectively. Red blood cell velocity was measured at baseline period from the recorded fluorescence images of FITC-labeled RBCs using a dual window technique . .

Vasodilation responses to topical application of endothelium dependent vasodilator (acetylcholine ; Ach 10^{-7} M , adenosine-5 diphosphate ;ADP 10^{-6} M) of pial arterioles were examined in all animal groups. Responses of cerebral arterioles to endothelium-independent vasodilator (nitroglycerine 10^{-6} were also examined. In each experiment the vasoactive agent was first mixed in artificial CSF and then superfused over the cranial space. Then the responses were monitored from the third order arterioles.

Each substance was superfused over the cranial space for 5 minutes using infusion pump with rate 1.5 ml/min. After application of each substances, artificial CSF was superfused within 5 minutes for washing, and the diameter of cerebral arterioles returned to baseline within 2-3 minutes after

application of substance was stopped. Diameter was measured before and after 5 minutes after application of cerebral arterioles

In the present study, increased arteriolar diameter was started at 2-3 minutes and reached to steady state at 5 minutes after application of substance. Vasodilation responses were expressed as percentage changes (%) of vasodilation from baseline.

Protocol 4: To examine the effect of vitamin C on ultrastructural changes of cerebral microcirculation

In present study the thickness of small cerebral arterioles (10-20 μm) and capillary (4-7 μm) was measured by using analysing program (Vascular wall structure mearuring tool program) which was developed by Department of Computer Engineering Chulalongkorn University . In addition, general morphological observation of endothelial cell, endothelial junction, and mitochondria were included in the present study.

Preparation for Transmission electron microscopy (TEM)

Cerebral tissues were immediately excised under anesthesia and during blood was withdrawn. These tissues were taken from the same area which was previously investigated by the fluorescence technique. The tissues were cut into 1 x 1 mm blocks and immersed in 0.1 M phosphate buffer saline solution at 4° C overnight .

The cerebral sections were post fixed in 1% O_s0_4 and dehydrated through a graded series of ethanol, passed through two changes of propylene oxide, and embedded in Epon 812. The thick sections were cut and stained with toludine blue. The thin sections on grids were stained with uranyl acetate and lead citrate for examination by a JEOL, JEM 1210. For qualitative analysis, electron photomicrographs of 34,000 x magnification were taken. The thickness of capillary and arteriolar basement membrane was measured .

Data analysis

Results were expressed as mean ± standard error of mean (SEM) . Statistical analysis of the results was done using two-way analysis of variance followed by student's t –test. A probability(P) of 0.05 or less was considered significant.

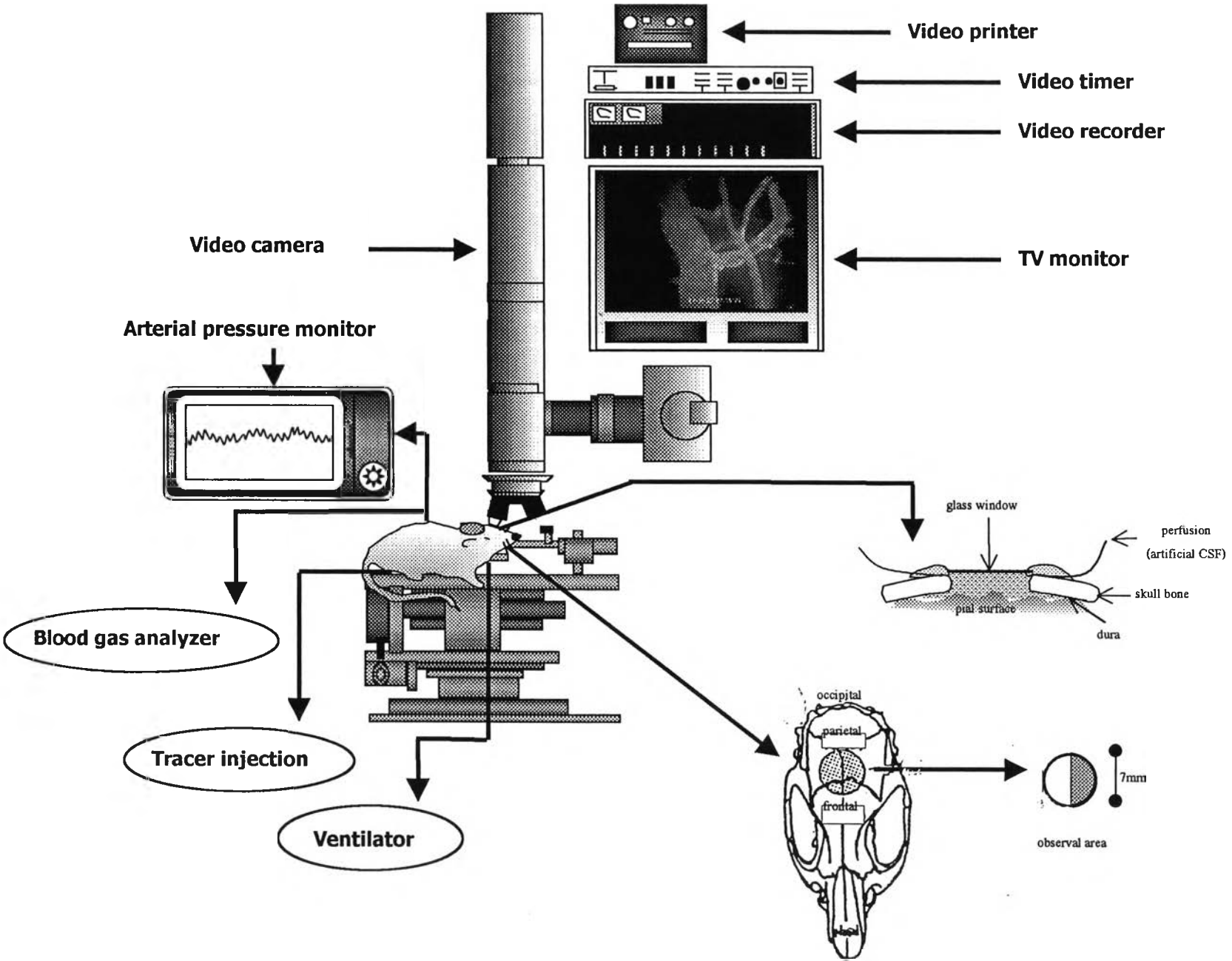


Figure12 Schematic of a setup for intravital microscopy of the cerebro microvasculature in the rat. Epifluorescence imaging is done through a closed cranial window implanted unilaterally over the frontoparietal cortex