

รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของวิตามินซีต่อการเปลี่ยนแปลงโครงสร้างของหลอดเลือด
และการทำงานของเอนโดทีเลียมในหลอดเลือดขนาดเล็กบริเวณม่านตา
ในหนูเบาหวาน : โดยวิธี Intravital Fluorescence Microscopy

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ให้ทุนส่งเสริมนักวิจัยรุ่นใหม่ ประจำปี 2545 เพื่อสนับสนุนการวิจัย และเปิดโอกาสให้ผู้วิจัยได้ใช้ความรู้ความสามารถในการคิดและสร้างงานวิจัยในสาขา Physiology (Microcirculation) เพื่อเป็นการเพิ่มประสบการณ์การทำวิจัย การพัฒนาความคิดและความรู้ทางวิชาการ ผู้วิจัยขอขอบคุณ รศ.ดร.สุทธิลักษณ์ ปทุมราช จากภาควิชาสรีรวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย นักวิจัยพี่เลี้ยงที่ให้คำปรึกษาแก่ผู้วิจัยมาโดยตลอด รวมทั้ง Dr. Niimi Hideyuki จาก National Cardiovascular Research Institute, โอซากา ประเทศญี่ปุ่นที่ให้คำปรึกษาและสนับสนุนเครื่องมือในการทำวิจัย อีกทั้งการเตรียมผลงานวิจัยเพื่อตีพิมพ์ในวารสารวิชาการระดับนานาชาติ และขอขอบคุณ ภาควิชาสรีรวิทยา คณะแพทยศาสตร์มหาวิทยาลัยศรีนครินทรวิโรฒ ที่ให้การสนับสนุนการทำวิจัยมาโดยตลอด

บทคัดย่อ

รหัสโครงการ : TRG4580093

ชื่อโครงการ : ผลของวิตามินซีต่อการเปลี่ยนแปลงโครงสร้างของหลอดเลือดและการทำงานของเอนโดทีเลียมในหลอดเลือดขนาดเล็กบริเวณม่านตาในหนูเบาหวาน : โดยวิธี Intravital Fluorescence Microscopy

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โครงการวิจัยนี้ทดสอบผลต้านอนุมูลอิสระของวิตามินซีต่อการเปลี่ยนแปลงโครงสร้างของหลอดเลือดและการทำงานของเอนโดทีเลียมในหลอดเลือดขนาดเล็กบริเวณม่านตาในหนูเบาหวาน ศึกษาในหนูวีสตาร์เพศผู้ น้ำหนัก 200-250 กรัม ทำหนูให้เป็นเบาหวานโดยวิธีฉีดสารสเตรปโตโซโตซินเข้าทางหลอดเลือดดำขนาดความเข้มข้น 55 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว แบ่งสัตว์ทดลองแบบสุ่มเป็น 3 กลุ่มคือ กลุ่มควบคุม (CON) กลุ่มเบาหวาน (STZ) และกลุ่มเบาหวานที่ได้รับวิตามินซี (STZ-Vit C) ให้วิตามินซีเสริมโดยให้สัตว์ทดลองดื่มน้ำ ซึ่งผสมวิตามินซีขนาดความเข้มข้น 1 กรัมต่อน้ำ 1 ลิตรให้กินอย่างอิสระ ทำการทดลองหลังจากสัตว์ทดลองได้รับการฉีดสารละลายซิเตรทบัฟเฟอร์หรือสเตรปโตโซโตซินไปแล้ว 8, 12, 16, 24 และ 36 สัปดาห์ พารามิเตอร์ที่ใช้ศึกษาได้แก่ ค่าน้ำหนักตัว ระดับน้ำตาลในเลือด ไกลโคซิเลทเท็ดฮีโมโกลบิน วิตามินซีในพลาสมา ความดันเลือดแดง การไหลเวียนเลือดที่ไปเลี้ยงบริเวณม่านตา และการเกาะติดของเม็ดเลือดขาวที่เอนโดทีเลียมภายในหลอดเลือดดำขนาดเล็ก หลังจากสิ้นสุดการทดลองตัดเก็บลูกตาทันทีเพื่อนำมาใช้ในการวิเคราะห์หาระดับมาลอนไดอัลดีไฮด์

จากผลการทดลองพบว่าในหนูกลุ่มเบาหวานทุกช่วงอายุมี ระดับน้ำตาลในเลือด ไกลโคซิเลทเท็ดฮีโมโกลบิน และมาลอนไดอัลดีไฮด์ในเนื้อเยื่อ สูงขึ้นอย่างมีนัยสำคัญทางสถิติ ขณะที่น้ำหนักตัวและระดับวิตามินซีในพลาสมามีค่าต่ำกว่ากลุ่มควบคุมที่ช่วงอายุเดียวกัน ในหนูเบาหวานที่ได้รับวิตามินซีเสริม มีระดับไกลโคซิเลทเท็ดฮีโมโกลบิน และระดับมาลอนไดอัลดีไฮด์ ต่ำกว่ากลุ่มเบาหวานอย่างมีนัยสำคัญทางสถิติ โดยที่ระดับวิตามินซีในพลาสมามีค่าสูงขึ้นเมื่อเปรียบเทียบกับหนูเบาหวาน อย่างไรก็ตามเฉพาะในกลุ่ม 36 สัปดาห์เท่านั้นที่ระดับน้ำตาลในพลาสมามีค่าต่ำกว่ากลุ่มเบาหวานอย่างมีนัยสำคัญทางสถิติ สำหรับอัตราการไหลเวียนเลือดที่ไปเลี้ยงบริเวณม่านตามีค่าต่ำ ขณะที่การเกาะติดของเม็ดเลือดขาวมีจำนวนสูงในหนูกลุ่มเบาหวานทุกช่วงอายุ ในหนูเบาหวานที่ได้รับวิตามินซีเสริมการไหลเวียนเลือดที่ไปเลี้ยงบริเวณม่านตามีค่าสูงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มเบาหวาน ขณะที่การเกาะติดของเม็ดเลือดขาวมีจำนวนต่ำอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มเบาหวาน เฉพาะในกลุ่ม 24 และ 36 สัปดาห์

โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นถึงการบาดเจ็บของเอนโดทีเลียมในภาวะเบาหวาน ความผิดปกติที่เกิดขึ้นดังกล่าวนี้เป็นผลจากอนุมูลอิสระที่เกิดขึ้นจากระดับน้ำตาลในเลือดที่สูงกว่าปกติเป็นระยะเวลานานก่อให้เกิดการความบกพร่องในการทำงานของเซลล์เอนโดทีเลียมมีผลทำให้ปริมาณการเกาะติดของเม็ดเลือดขาวมีค่าเพิ่มขึ้น การไหลเวียนเลือดที่ไปเลี้ยงบริเวณม่านตาลดลง เป็นที่น่าสนใจว่าจากการทดลองครั้งนี้อาจกล่าวได้ว่าไม่เพียงแต่การให้วิตามินซีเสริม สามารถป้องกันการสูญเสียหน้าที่ของเอนโดทีเลียมที่เกิดจากภาวะเบาหวาน แต่ยังอาจเป็นสารที่นำมาใช้ป้องกันการเกิดโรคหลอดเลือดเรื้อรังในผู้ป่วยภาวะเบาหวานที่นำไปสู่การขาดเลือดได้อย่างดีเยี่ยม

คำหลัก วิตามินซี อนุมูลอิสระ หนูเบาหวาน น้ำหนักของเซลล์เอนโดทีเลียม การเกาะติดของเซลล์เม็ดเลือดขาว การไหลเวียนเลือดที่บริเวณม่านตา

Abstract

Project Code : TRG4580093

Project Title : Effect of Vitamin C on Changes of Vascular Structure and Endothelial

Function of Iris Microcirculation in Diabetic Rats : Intravital Fluorescence Microscopy

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The effects of long-term supplementation of antioxidant; vitamin C on vascular structure and endothelial function of Iris microcirculation in diabetic rats. Diabetes was induced in male Wistar Furth by intravenous injection of streptozotocin, 55 mg/kg body weight. The rats were divided randomly into three groups of control (CON), streptozotocin (STZ) and streptozotocin supplementation with vitamin C (STZ-Vit C). The supplementation of vitamin C was performed by allowing the animals free access to drinking water added 1 g/L of ascorbic acid (Sigma, Chemical Co., USA). The experiments were performed at 8, 12, 16, 24 and 36 weeks after injection of STZ and citrate buffer. On the day of experiment, body weight (BW), blood glucose (BG), glycosylated hemoglobin (HbA_{1c}), plasma vitamin C, arterial blood pressure, iris blood-flow perfusion were measured. Endothelial function was examined by manually counted of leukocyte that adhered to endothelial cells of iris postcapillary venule in all experimental groups. At the end of each experiment, plasma vitamin C from venous blood was analyzed and the eye was immediately isolated for evaluate oxidative stress status; malondialdehyde (MDA) analysis.

The results showed that all groups of 8, 12, 16, 24 and 36 weeks STZ-rat had the significantly increased in BG, HbA_{1c} and tissue MDA, but decreased in BW and plasma vitamin C levels as compared to their age-match control groups. However, STZ-Vit C-rat was significantly decreased in BG (only at 36 weeks of STZ-Vit C rats), HbA_{1c} and MDA but increased in BW and plasma vitamin C levels as compared to STZ group. Moreover, all STZ groups have the significantly decreased in iris blood-flow perfusion but increased in number of leukocyte adhesion. STZ-Vit C was significantly increased iris blood-flow perfusion but decreased leukocyte adhesion as compared to STZ group only in 24 and 36 week groups. In the present STZ-diabetic rats, hyperglycemia and increased tissue lipid peroxidation developed. These abnormalities were prevented by long-term supplementation of vitamin C in STZ-diabetic rats. Moreover, the present study has demonstrated that the endothelial dysfunction induced by diabetes has been resulted to the increased in leukocyte adhesion and to the decreased in iris blood flow.

In conclusion the preventive effect on endothelial dysfunction and impaired iris blood flow is due to the antioxidant capacity of vitamin C. Therefore, vitamin C might be a great therapeutic agent for preventing diabetic retinopathy.

Keywords: vitamin c, diabetic rats, endothelial function, leukocyte adhesion, iris blood flow

Introduction

The major cause of morbidity and mortality in both type I and type II diabetic patients is vascular disease which include microangiopathy and macroangiopathy. Microangiopathy is the hallmark of retinopathy, neuropathy and nephropathy (Standl E. et al, 1996). Diabetes is the leading cause of blindness . In diabetic patients the whole vascular system of the eye can be affected by microangiopathy. Most of severe manifestation of vascular changes in diabetic eye are iridopathy and retinopathy, therefore,diabetic eye diseases are not limited only to the retina (Bandello F. et al, 1994). Diabetic retinopathy is one of major complications that cause most suffering in diabetic patients. In diabetes, interaction between leukocyte and endothelium may be enhanced markedly. In fact, Schroder et al,1991. showed the appearance of capillary occlusion with leukocyte in the retinopathy of diabetic rats. The leukocytes that adhered to endothelial cells particularly in the small vessels are caused of capillary occlusion. The harmful of these phenomenon is that contact of circulating leukocytes with the vascular endothelium promotes a cascade of events leads to further leukocyte activation. Once activated, PMNs release reactive oxygen species (ROS) and mediator of proteolytic tissue degradation, contributing to oxidative stress, subsequent inflammation, and causing surrounded endothelial cells even more damaged (Semdly LA et al, 1986). Among the mechanism proposed as mediators of the endothelial dysfunction and increased leukocyte adhesion observed in diabetes, hyperglycemia plays a key pathogenic role in the development of diabetic vascular diseases. High blood glucose concentrations result in endothelial dysfunction that is associated with loss of endothelium derived NO, increased vascular permeability, increased endothelial adhesives, and thickening of the basement membrane of blood vessels, and increased generation of oxygen free radical (Jones et al, 1987). Diabetes increases oxidative stress in tissue and plasma both human and experimental animals, increassing oxidative stress might play a role in the development of diabetic complication. Oxidative stress develops in the retina of diabetic animals and galactos-fed animals (Halliwell B, 1999; Gutteridge JM et al, 2000), indicating that oxidative stress is associated with the development of retinopathy. Currently, the potential contribution of increased oxidative stress to the development of endothelial dysfunction in diabetes has received much of interest. Enhanced oxidative stress in the blood and tissue is thought to play an important role in the onset and progression of microvascular complications in diabetic patients. Particularly, the molecular mechanisms of the enhancement of oxidative stress in diabetes have been characterized by two dependable domains which are 1) increased production of reactive oxygen species (ROS) and 2) impaired endogenous antioxidant defenses. Several suggestions regarding the origins of oxidative stress in diabetes,

are free radical generated by glycation of proteins, consumption of NADPH through the polyol pathway, glucose autooxidation, hyperglycemia-induced pseudohypoxia, and activation of protein kinase C (VanderJagt DJ, 2000). Normally oxidative level controlled by a variety of cellular antioxidant defense mechanisms consisting of enzymatic and nonenzymatic scavengers (Aydin A, 2001). Such enzymes include: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Such those play an important role in protecting cell and tissue from oxidative stress. And the endogenous nonenzymatic antioxidants including vitamin C, vitamin E, reduced glutathione, β -carotene, various amino acids, proteins, uric acid and bilirubin, etc. They all can directly scavenge reactive oxygen species (Kashiwagi A, 2001). Under hyperglycemic conditions, the intracellular concentrations of reduced vitamin C, reduced glutathione and vitamin E are reported to be decreased (Giugliano D et al, 1996). Accordingly, it can be said that antioxidant supplementation is desirable in patients with diabetes mellitus.

Vitamin C, or namely ascorbic acid, is an antioxidant agent, two major properties of vitamin C make it an ideal antioxidant. The first is low one-electron reduction potentials of both ascorbate and its one-electron oxidation product, the ascorbyl radical, which is derived from the one-diol functional group in the molecule (Halliwell B, 1996). These low reduction potentials enable ascorbate and the ascorbyl radical to react with and reduce basically all physiologically relevant radical and oxidants. The second major property that makes vitamin C such an effective antioxidant is the stability and low reactivity of the ascorbyl radical formed when ascorbate scavenges a reactive oxygen or nitrogen species (Carr AC and Frei B, 1999). Levels of vitamin C in plasma and in various tissues are decreased in diabetic patients and in animals with experimentally induced diabetes. Cellular deficiency of vitamin C has been implicated in some of the cellular pathology and complications of diabetes mellitus such as angiopathy. It has been suggested that vitamin C supplementation may help to prevent the development of some diabetic complications as well (Dai S and McNeill JH, 1995). Our previous study (Jariyapongskul et al, 2002) demonstrated that daily supplementation of vitamin C could improve the diabetic-induced endothelial dysfunction in STZ-rats. In particular, we have showed that long term supplementation of vitamin C could decrease leukocyte adhesion to the cerebral postcapillary venules in diabetic-rats. In regard to the literature view from the above, it might be said that vitamin C, as an antioxidant, is likely to ameliorate diabetic-induced endothelial dysfunction. As which it might help to prevent the leukocyte-endothelial cell interaction that mostly enhanced the vaso-occlusion and consequently brought about hypoxic condition in diabetic eye. Together, with the idea of vitamin C could reduce the abnormality of diabetic-induced endothelial-dependent vasomotion, Therefore, this present study is designed to evaluate the possible effects of long-term vitamin C supplementation on diabetic iridopathy.

characterization by decreased iris blood-flow perfusion and increased number of leukocyte-endothelial cell interaction.

Materials and methods

1. 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 rats 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.

1.1 Diabetic induction

The rats were divided into three groups.

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

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

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

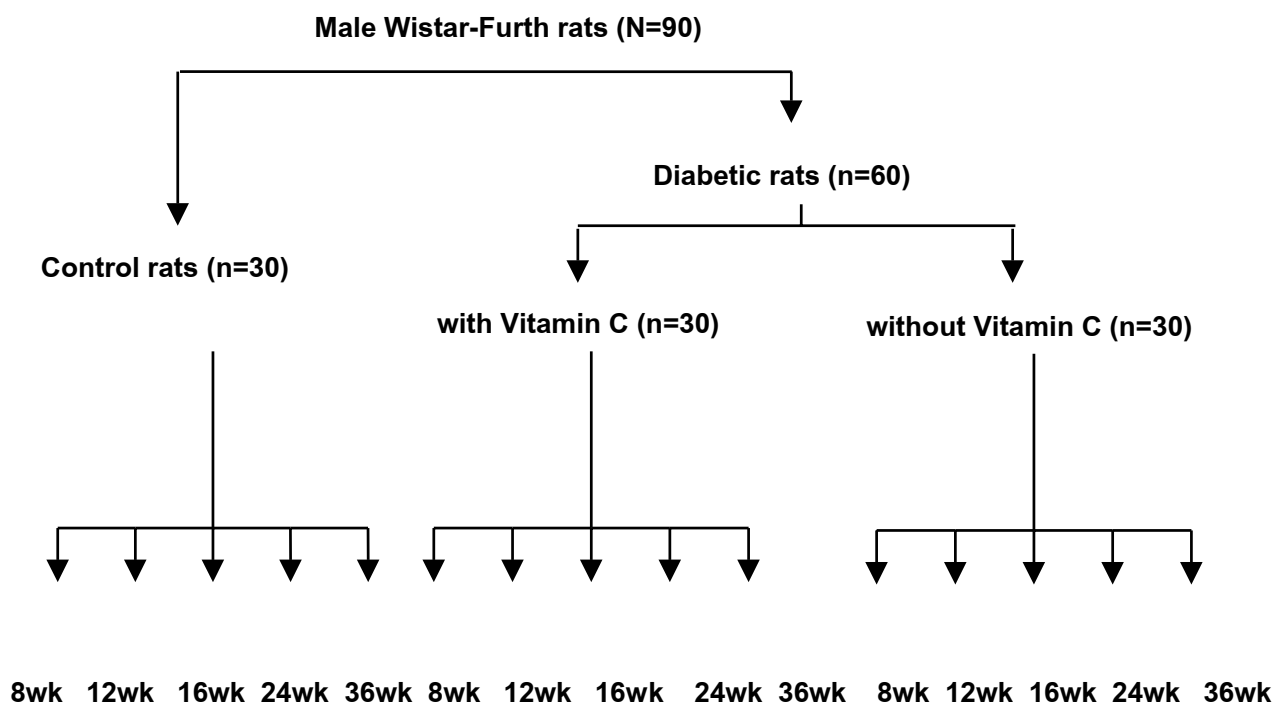


Figure 1. The diagram demonstrates the dividing of experimental animal groups.

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 injected 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, Boehringer Mannheim, Germany) was used for evaluation of plasma glucose from tail vein blood sample. 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).

1.2 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).

2. 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 experiment, rats were 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 systolic and diastolic blood pressure (SBP and DBP) using a pressure transducer connected to a polygraph system (Nihon Kohden, Japan).

2.1. 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 measurements were performed at each time and the mean was then determined for each animal (Figure 2).

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.

2.2 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 fluorescent 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 used and intravenously injected through the cannulated femoral vein. The epifluorescent image was observed through the x 20 objective lens and also by video camera, SIT (DAGE Co. USA), a low-light and real time throughout the experimental period. Simultaneously, the image was also monitored (Sony) as showed in the figure 2. This videotape

of each experiment was then play back frame by frame for further image processing analysis using the software called Global lab image II. 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 interstitium.

2.3. 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 iris postcapillary venules (20 to 50 μ m diameter) 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. 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).

2.4. 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, Boehringer Mannheim, Germany). Blood sample was divided into 2 sets. One set (1ml) was collected for the determination 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 centrifused 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).

2.5. 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 presented. 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).

3. Statistical analysis

Results were expressed as mean $\pm$ standard deviation of mean (SD). 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.

Results

Part 1. The antioxidant effects of vitamin C supplementation on metabolic and hemodynamics changes

1.1 Metabolic changes

The tail vein injection of streptozotocin 55 mg/kg/BW into 200-250g Wistar Furth rat resulted in polydipsia, polyuria, polyphagia and hyperglycemia within 48 hours and showed persistent hyperglycemia through out the experiment. In the present study, the criteria used for diabetic rats was the blood glucose level that had to be higher than 250 mg/dl.

Results of blood glucose, body weight and glycosylated hemoglobin of CON, STZ and STZ-Vit C rats were shown in Table 1. Eight weeks after streptozotocin injection body weight were 39% lower in STZ-rats compared with non diabetic control rats ($p < 0.001$) and up to 51% in 36 weeks (Table 1.). Blood glucose levels were significantly elevated in STZ rats (397.12 ± 65.48 mg/dl to 463.37 ± 106.52) compared with non diabetic control rats (83.5 ± 11.86 to 100.9 ± 11.51 ; $p < 0.001$) (Table 1. and Figure 3.). Plasma glycosylated hemoglobin level was significantly elevated in STZ rats (9.52 ± 1.66 to $10.13 \pm 1.88\%$) compared with non diabetic control rats (3.91 ± 0.15 to $4.51 \pm 0.58\%$; $p < 0.001$) in all five monitored time points. Interestingly, at 36week vitamin C supplemented of diabetic rats significantly improved in body weight, decreased in blood glucose and decreased in HbA_{1c}.

1.2 Plasma vitamin C was significantly reduced in 12, 24, and 36 weeks STZ-rats (23.01 ± 0.92 , 21.47 ± 1.87 and 15.95 ± 2.02 μ mol/L respectively compared with 12, 24 and 36 week of control rats (44.59 ± 2.12 , 43.58 ± 1.19 and 44.89 ± 2.93 ; respectively). Vitamin C supplementation can improved the plasma vitamin C concentration in all three monitored time points of STZ-Vit C rats (43.66 ± 3.92 , 39.44 ± 2.04 and 38.65 ± 2.02 ; $p < 0.01$).

1.3 Tissue lipid peroxidation

In this present study malondialdehyde (MDA), product of lipid peroxidation was used as indicator of oxygen free radical. The MDA values were significant higher in 8, 12, 16, 24, and 36 weeks diabetic rats eyes (103.32 ± 25.28 , 107.11 ± 35.75 , 146.26 ± 25.73 , 137.59 ± 37.88 and 145.63 ± 28.80) than in 8, 12, 16, 24, and 36 weeks control rats (58.36 ± 27.76 , 72.95 ± 21.41 , 69.85 ± 23.64 , 70.49 ± 31.39 and 73.65 ± 27.91 ; respectively). Interestingly, vitamin C supplemented can reduced MDA values in 16, 24, and 36 weeks of STZ-Vit C rats (84.93 ± 13.93 , 81.70 ± 21.39 and 80.88 ± 20.84 ; respectively) compared with STZ rats ($p < 0.01$).

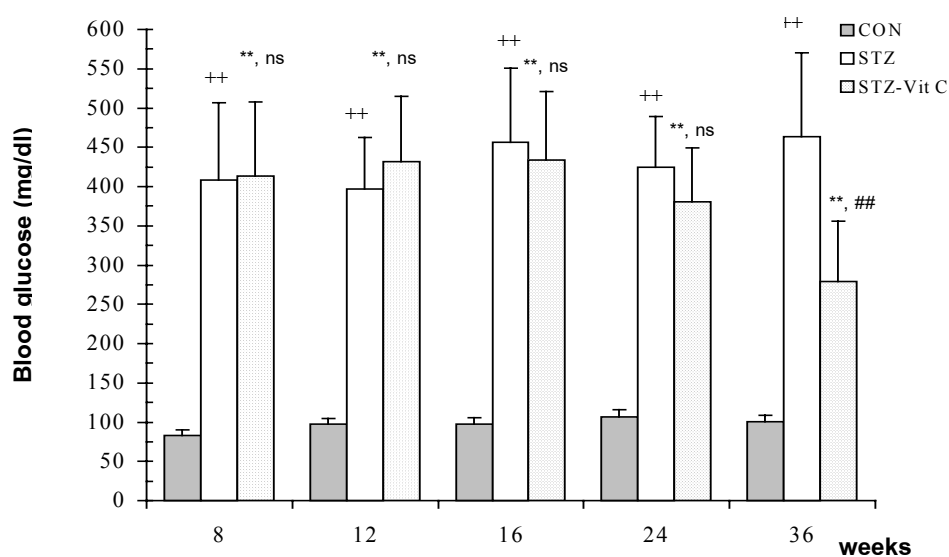
Table 1. Body weight, blood glucose and plasma glycosylated hemoglobin (HbA_{1c}) levels of control rats(CON), streptozotocin rats (STZ) and streptozotocin rats supplementation with vitamin C (STZ-Vit C)

	Body weight (g)	Blood glucose (mg/dl)	Glycosylated hemoglobin A_{1c} (HbA_{1c} ;%)
CON 8 WK	393±31.38 (n=10)	83.5±11.86 (n=10)	3.96±0.23 (n=6)
12 WK	443.07±22.41 (n=13)	97±13.81 (n=12)	4±0.61 (n=6)
16 WK	471±37.10 (n=10)	97.9±11.20 (n=10)	4.13±0.71 (n=8)
24 WK	507±35.55 (n=13)	106.84±10.49 (n=13)	4.51±0.58 (n=6)
36 WK	566.45±44.13 (n=11)	100.9±11.51 (n=11)	3.91±0.15 (n=7)
STZ 8 WK	238.8±28.75 (n=10) ⁺⁺	408.62±97.82(n=9) ⁺⁺	10.13±1.88 (n=9) ⁺⁺
12 WK	223.25±36.80 (n=8) ⁺⁺	397.12±65.48(n=8) ⁺⁺	10.57±1.55 (n=7) ⁺⁺
16 WK	261±35.71 (n=9) ⁺⁺	456.37±94.05 (n=8) ⁺⁺	10.18±1.43 (n=6) ⁺⁺
24 WK	246.00±19.77 (n=8) ⁺⁺	425.00±64.43 (n=7) ⁺⁺	9.72±0.52 (n=5) ⁺⁺
36 WK	278.33±55.17 (n=9) ⁺⁺	463.37±106.52 (n=8) ⁺⁺	9.52±1.66 (n=4) ⁺⁺
STZ-vit C 8 WK	236.5±35.11 (n=10) ^{**, ns}	413.62±94.09 (n=8) ^{**, ns}	9.5±1.00 (n=8) ^{**, ns}
12 WK	229.6±47.71 (n=10) ^{**, ns}	431.4±83.22 (n=10) ^{**, ns}	9.13±1.76 (n=6) ^{**, ns}
16 WK	245.12±38.33 (n=8) ^{**, ns}	433.42±87.98 (n=7) ^{**, ns}	8.71±0.90 (n=7) ^{**, ns}
24 WK	276.88±39.25 (n=9) ^{**, ns}	380.27±68.96 (n=11) ^{**, ns}	9.75±0.70 (n=6) ^{**, ns}
36 WK	362.33±38.91 (n=9) ^{**, ##}	279.37±77.04 (n=8) ^{**, #}	6.60±2.03 (n=5) ^{*, #}

Values are expressed as mean ± SD

⁺⁺ p<0.001 as compared to CON, ^{*} p<0.05 as compared to CON, ^{**} p < 0.01 as compared to CON ,
[#] p<0.05 as compared to STZ, ^{##} p<0.01 as compared to STZ , ^{ns} not significant different as compared to STZ

Figure 3. The antioxidant effects of vitamin C supplementation on blood glucose

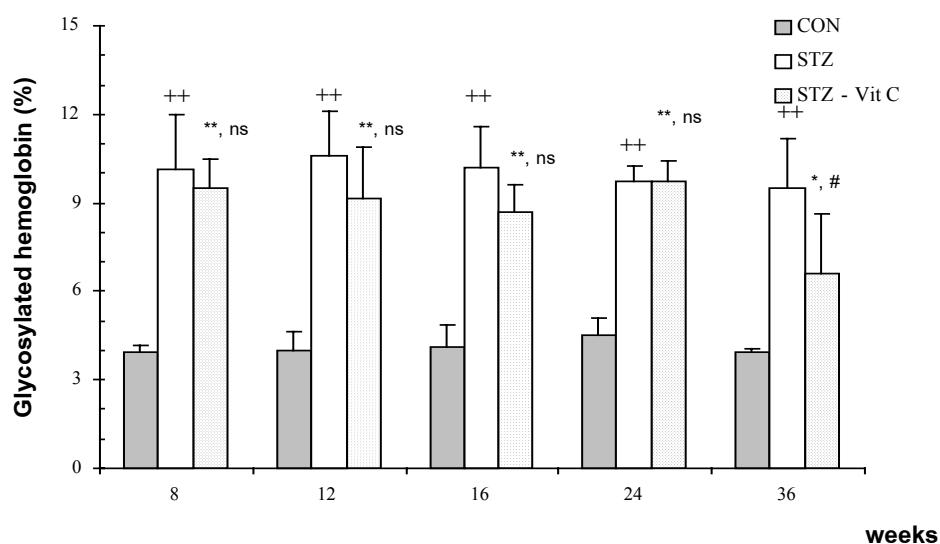


Values are expressed as mean $\pm$ SD

CON ; control rats, STZ ; streptozotocin rats, STZ-Vit C ; streptozotocin rats supplementation with vitamin C

** Significantly different as compared to CON ($p < 0.001$); ⁺⁺ Significantly different as compared to CON ($p < 0.001$), ## Significantly different as compared to STZ ($p < 0.01$), ^{ns} no significantly different as compared to STZ

Figure 4. The antioxidant effects of vitamin C supplementation on Glycosylated hemoglobin



Values are expressed as mean $\pm$ SD

*Significantly different as compared to CON ($p < 0.05$), **Significantly different as compared to CON ($p < 0.001$), ⁺⁺ Significantly different as compared to CON ($p < 0.001$), # Significantly different as compared to STZ ($p < 0.05$), ^{ns} no significantly different as compared to STZ

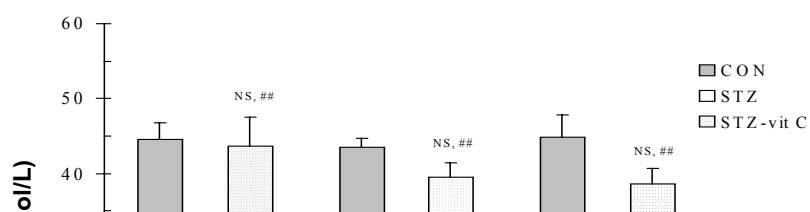
Table 2 Plasma vitamin C (Vit C) and tissue malondialdehyde (MDA) of control rats(CON), streptozotocin rats (STZ) and streptozotocin rats supplementation with vitamin C (STZ-Vit C)

	VIT C (μmol/L)	MDA (nmol/100mg wet wt.)
CON 8 WK	-	58.3 $\pm$ 27.7 (n =7)
12 WK	44.5 $\pm$ 2.1 (n = 5)	72.9 $\pm$ 21.4 (n = 10)
16 WK	-	69.85 $\pm$ 23.64 (n=9)
24 WK	43.5 $\pm$ 8 1.1 (n = 8)	70.4 $\pm$ 31.3 (n = 11)
36 WK	44.89 $\pm$ 2.93 (n = 5)	73.6 $\pm$ 22.9 (n = 10)
STZ 8 WK	-	103.3 $\pm$ 25.9 (n = 9) *
12 WK	23.0 $\pm$ 0.9 (n = 8) ***	107.1 $\pm$ 35.7 (n = 6) *
16 WK	-	146.26 $\pm$ 25.73 (n=8) **
24 WK	21.4 $\pm$ 1.8 (n = 8) ***	137.5 $\pm$ 37.8 (n = 6) ***
36 WK	15.9 $\pm$ 2.0 (n = 5) ***	145.6 $\pm$ 28.8 (n = 11) ***
STZ-vit C 8 WK	-	67.7 $\pm$ 29.0(n = 6) ^{NS}
12 WK	43.6 $\pm$ 3.92 (n = 5) ^{NS}	84.1 $\pm$ 13.4 (n = 6) ^{NS}
16 WK	-	84.93 $\pm$ 13.93 (n=7) ^{NS, ##}
24 WK	39.4 $\pm$ 2.04 (n = 5) ^{##}	84.9 $\pm$ 13.9 (n = 10) ^{##}
36 WK	38.5 $\pm$ 2.0 (n = 5) ^{##}	80.8 $\pm$ 20.8 (n = 8) ^{##}

Values are expressed as mean $\pm$ SD

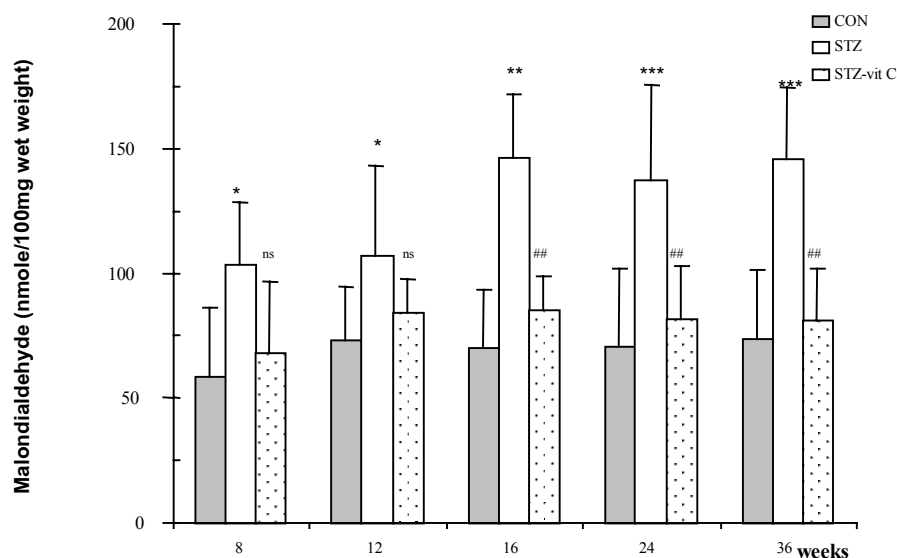
* p< 0.05 as compared to CON, ** p<0.01 as compared to CON, *** p < 0.001 as compared to CON, ^{##} p< 0.01 as compared to STZ, ^{NS} no significant different as compared to CON

Figure 5. The antioxidant effects of vitamin C supplementation on Plasma Vitamin C



Values are expressed as mean $\pm$ SD. ⁺ Significantly different as compared to CON (p<0.05), ⁺⁺ Significantly different as compared to CON (p<0.001), ^{##} Significantly different as compared to STZ (p<0.01), ^{ns} no significantly different as compared to STZ, ^{NS} no significantly different as compared to CON

Figure 6. The antioxidant effects of vitamin C supplementation on Lipid peroxidation



Values are expressed as mean $\pm$ SD. ^{*} Significantly different as compared to CON (p<0.05), ^{**} Significantly different as compared to CON (p<0.01), ^{***} Significantly different as compared to CON (p<0.001), ^{##} Significantly different as compared to STZ (p<0.01).

Part 2. The antioxidant effects of vitamin C supplementation on leukocyte-endothelial cell interaction

The leukocyte that was counted as adherent one has to remain stationary for equal or longer than 30 second (Joussen AM et al, 2002). The leukocyte adhesion was totally count per field of view of postcapillary venule (diameter 20-50 μm) as described previously.

In the present video microscopic visualization showed clear image of leukocytes adhering to the endothelium of postcapillary venules in control non diabetes and diabetes rats all five monitored time points. The average numbers of leukocyte that adhered on endothelium in a field of view in rats 8, 12, 16, 24, 36 weeks of STZ rats (6.14 ± 1.60 , 6.05 ± 0.57 , 6.93 ± 1.79 , 12.90 ± 3.20 , and 22.28 ± 4.35 ; respectively) were significantly high compared with control rats at the same monitored time points (2.20 ± 0.65 , 2.48 ± 0.79 , 2.45 ± 0.69 , 2.27 ± 0.48 and; 2.56 ± 1.03 respectively). Interestingly, vitamin C supplementation had effects to reduced the number of leukocyte adhesion to endothelial of postcapillary venule in STZ-Vit C rats.

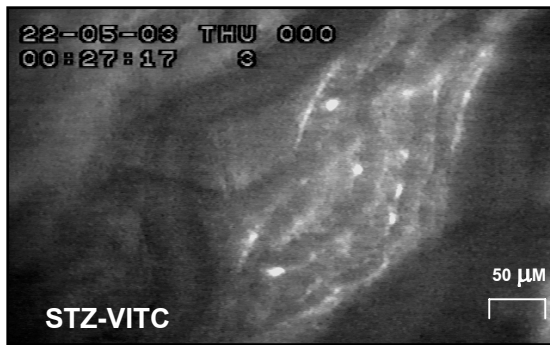
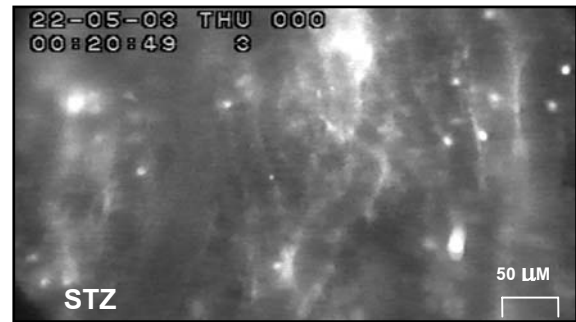


Figure 7. Fluorescence images of leukocyte -endothelial interaction in iris post-capillary venules of 8 weeks control, STZ with and without vitamin C supplementation. White dot represents leukocyte that adhered to endothelial cell



Figure 8. Fluorescence images of leukocyte-endothelial interaction in iris post-capillary venules of 36 weeks control, STZ with and without vitamin C supplementation. White dot represents leukocyte that adhered to endothelial cell.

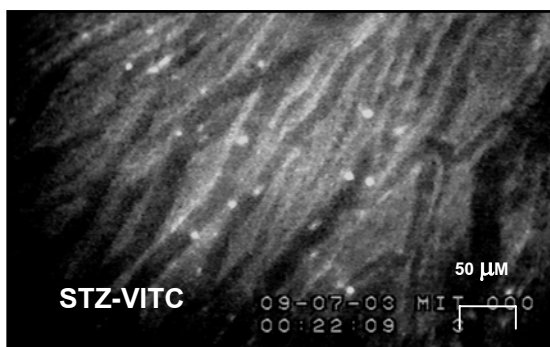
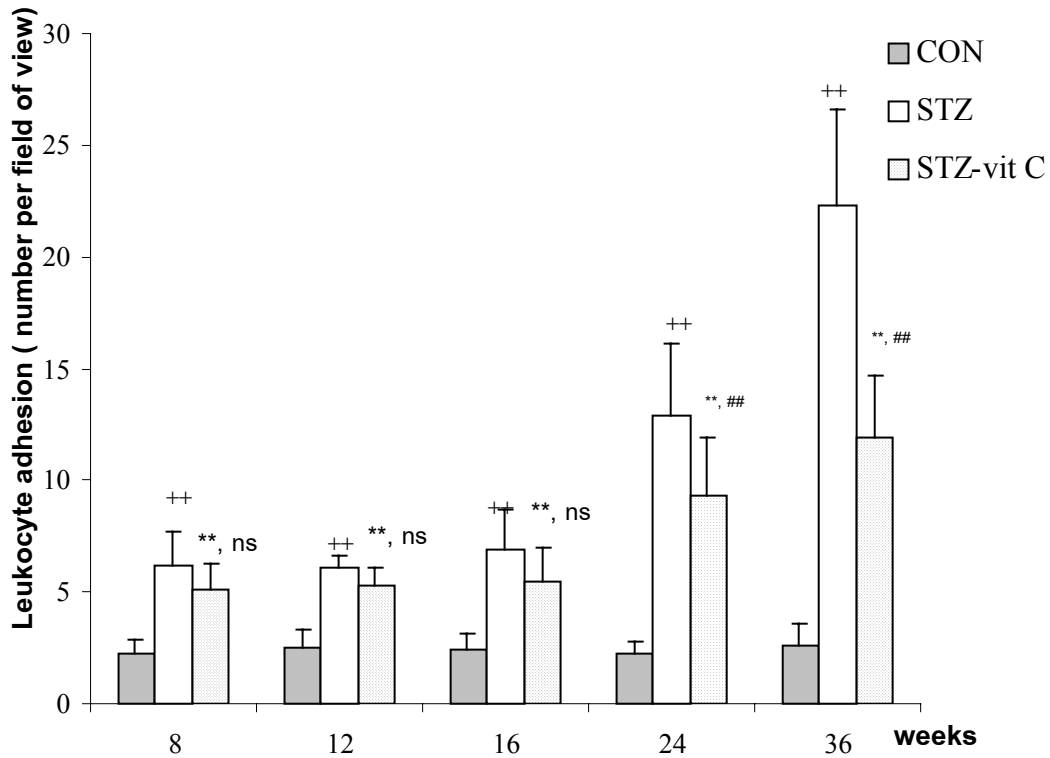


Figure 9. The antioxidant effects of vitamin C supplementation on leukocyte adhesion



Values are expressed as mean $\pm$ SD.

⁺⁺ Significantly different as compared to CON ($p < 0.001$), ^{**} Significantly different as compared to CON ($p < 0.001$), ^{##} Significantly different as compared to STZ ($p < 0.01$), ^{ns} no significantly different as compared to STZ.

Part 3. The antioxidant effects of vitamin C supplementation on hemodynamics changes

3.1 By using laser doppler flowmetry the iris blood-flow perfusion was evaluated from eight areas of the iris around the pupil as described previously. Means iris blood-flow perfusion were summarized in the Table 5. and Fig 10. Means regional iris blood-flow perfusion of STZ rats were significantly reduced to 33.64 %, 50.46 %, 57.95, 57.60 % and 56.12 %, respectively compared with control rats on 8,12, 16, 24, and 36 weeks. However, the significant difference between STZ and STZ-Vit C were observed on 24 and 36 weeks.

3.2 Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean regional iris blood-flow perfusion of control, STZ and STZ-Vit C rats.

The results of SBP value were shown in Table 3. And the results DBP value were shown in Table 4. In the present study, diabetic state had effect on change of SBP and DBP in 16 and 24 weeks of STZ and control rats. The SBP and DBP were significantly different between STZ and STZ-Vit C at 16-36 weeks.

Table3. Systolic blood pressure control rats(CON), streptozotocin rats

(STZ) and streptozotocin rats supplementation with vitamin C (STZ-Vit C)

Duration (weeks)	Systolic blood pressure (mmHg)		
	CON	STZ	STZ-Vit C
8	88.57 ± 22.49 (n=7)	109.28 ± 9.75 ^{NS} (n=7)	95.00 ± 22.20 ^{NS, ns} (n=8)
12	103.00 ± 20.18 (n=5)	115.00 ± 18.70 ^{NS} (n=5)	109.28 ± 12.39 ^{NS, ns} (n=7)
16	93.33 ± 23.16 (n=6)	125.83 ± 13.57 ⁺ (n=6)	112.50 ± 8.80 ^{NS, ns} (n=6)
24	105.55 ± 14.45 (n=9)	116.00 ± 12.94 ^{NS} (n=5)	103.00 ± 5.70 ^{NS, ns} (n=5)
36	104.00 ± 8.94 (n=5)	132.00 ± 7.52 ⁺ (n=10)	111.66 ± 7.52 ^{NS,##} (n=6)

Values are expressed as mean ± SD.

⁺ Significantly different as compared to CON (p<0.05), ^{##} Significantly different as compared to STZ (p

<0.01), ^{ns} no significantly different as compared to STZ,

^{NS} no significantly different as compared to CON

Table 4. Diastolic blood pressure control rats(CON), streptozotocin rats**(STZ) and streptozotocin rats supplementation with vitamin C (STZ-Vit C).**

Duration (weeks)	Diastolic blood pressure (mmHg)		
	CON	STZ	STZ-Vit C
8	79.28 ± 21.68 (n=7)	97.85 ± 8.09 ^{NS} (n=7)	85.62 ± 20.07 ^{NS, ns} (n=8)
12	90.00 ± 19.68 (n=5)	106.00 ± 21.62 ^{NS} (n=5)	92.85 ± 14.09 ^{NS, ns} (n=7)
16	79.16 ± 20.83 (n=6)	109.16 ± 12.41 ⁺ (n=6)	105.00 ± 7.07 ^{NS, ns} (n=6)
24	90.00 ± 13.91 (n=9)	90.00 ± 11.72 ^{NS} (n=5)	95.00 ± 8.66 ^{NS, ns} (n=5)
36	88.00 ± 10.36 (n=5)	118.50 ± 8.83 ⁺ (n=10)	103.33 ± 6.05 ^{*,##} (n=6)

Values are expressed as mean ± SD.

* Significantly different as compared to CON (p<0.05), ⁺ Significantly different as compared to CON (p<0.05), ^{##} Significantly different as compared to STZ (p<0.01), ^{ns} no significantly different as compared to STZ, ^{NS} no significantly different as compared to CON.

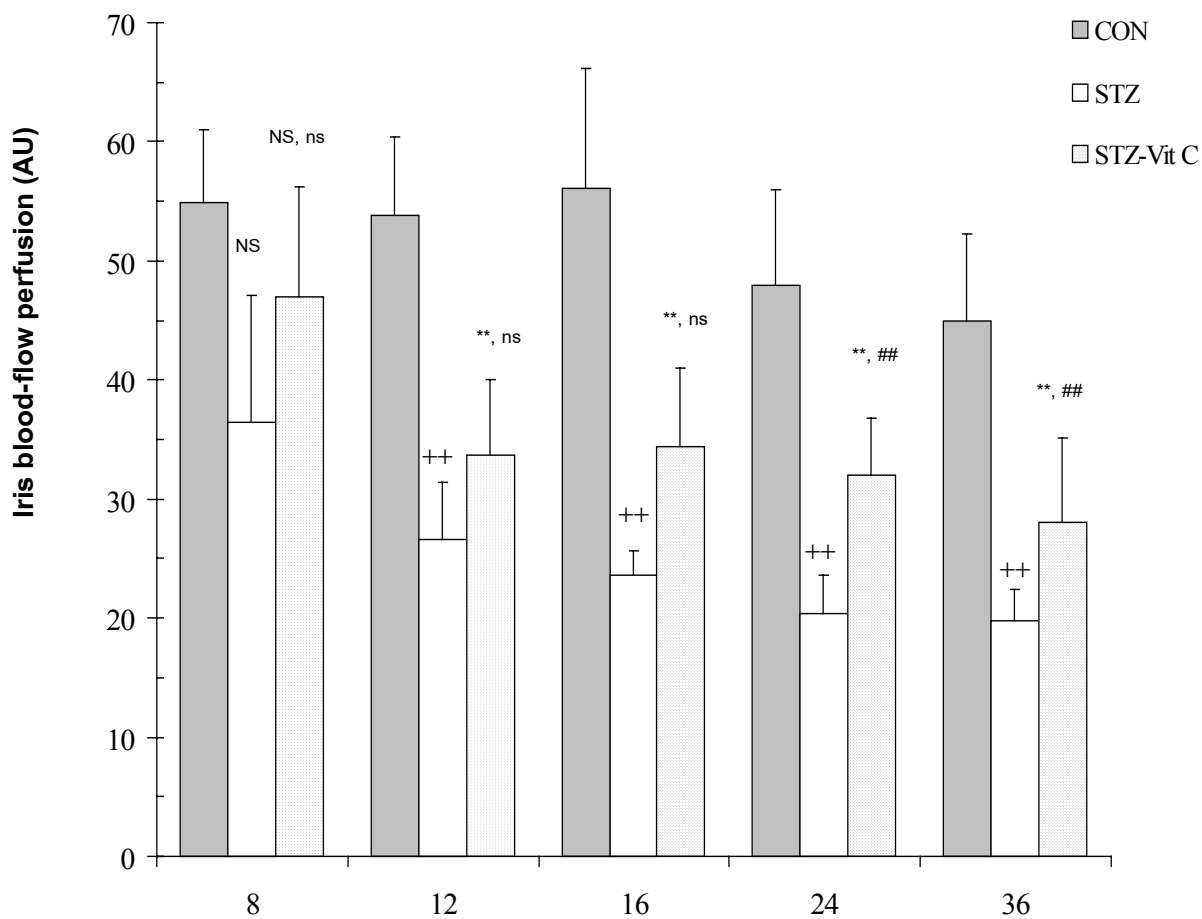
Table 5. iris blood-flow perfusion of control rats(CON), streptozotocin rats (STZ) and streptozotocin rats supplementation with vitamin C (STZ-Vit C).

Duration (weeks)	Iris blood-flow perfusion (AU)		
	CON	STZ	STZ-Vit C
8	54.95±6.05 (n=10)	43.05±8.18 ^{NS} (n=7)	46.97±9.23 ^{NS, ns} (n=9)
12	53.82±6.59 (n=13)	26.66±4.76 ⁺⁺ (n=7)	33.74±6.30 ^{**, ns} (n=8)
16	56.06±10.11 (n=10)	23.57±2.08 ⁺⁺ (n=6)	34.36±6.62 ^{**, ns} (n=5)
24	48±7.97 (n=13)	20.35±3.24 ⁺⁺ (n=6)	32.06±4.72 ^{**, ##} (n=7)
36	44.94±7.30 (n=10)	19.72±2.67 ⁺⁺ (n=12)	27.99±7.07 ^{**, ##} (n=8)

Values are expressed as mean ± SD.

⁺⁺ Significantly different as compared to CON (p<0.001), ^{**} Significantly different as compared to CON (p<0.001), ^{##} Significantly different as compared to STZ (p<0.01), ^{ns} no significantly different as compared to STZ, ^{ns} no significantly different as compared to CON

Figure 10. The antioxidant effects of vitamin C supplementation on iris blood-flow perfusion

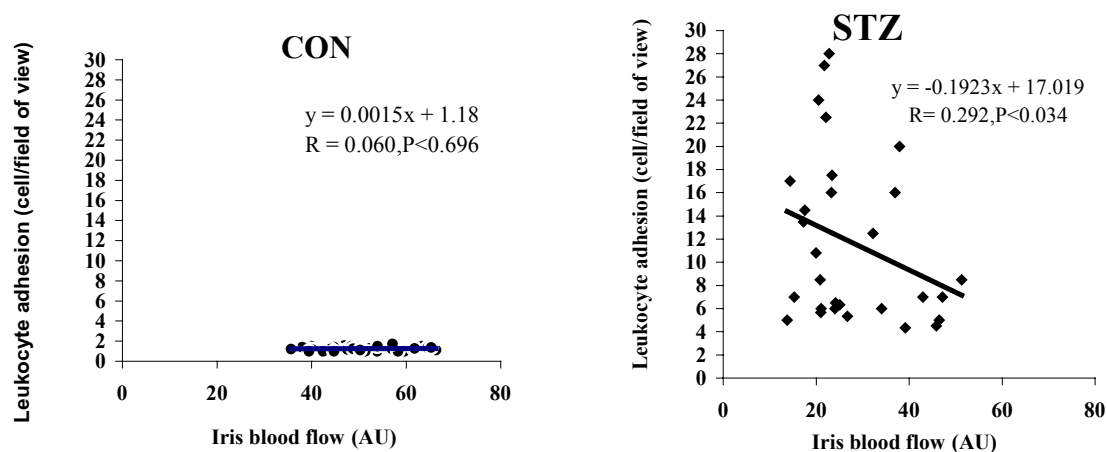


Values are expressed as mean $\pm$ SD.

⁺⁺ Significantly different as compared to CON ($p < 0.001$), ^{**} Significantly different as compared to CON ($p < 0.001$), ^{##} Significantly different as compared to STZ ($p < 0.01$), ^{ns} no significantly different as compared to STZ, ^{NS} no significantly different as compared to CON

Part 4. Study relationship on iris blood-flow perfusion and leukocyte endothelial cell interaction

Linear regression analysis was used to examine the relationship on iris blood-flow perfusion and leukocyte-endothelial cell interaction. The result of this relationship was shown in Figure 11. Iris blood-flow perfusion values and leukocyte adhesion (cell per field of view of postcapillary venule of all five monitored time points of diabetic rats were plotted and these two parameters were correlated equation $y = -0.447x + 32.80$, $r = -0.317$, $p < 0.034$. Interestingly, the correlation was more clarified and supported by the results of STZ-Vit C, $y = -1.862x + 47.103$, $r = -0.517$, $p < 0.001$. The data demonstrates that the presences of diabetes are necessary for measurable effect on iris blood-flow perfusion can occur in diabetes, not founded relationship in control rats. From the result, it can be explained that when leukocyte adhesion was raised as the iris blood-flow perfusion was reduced in diabetic rats.



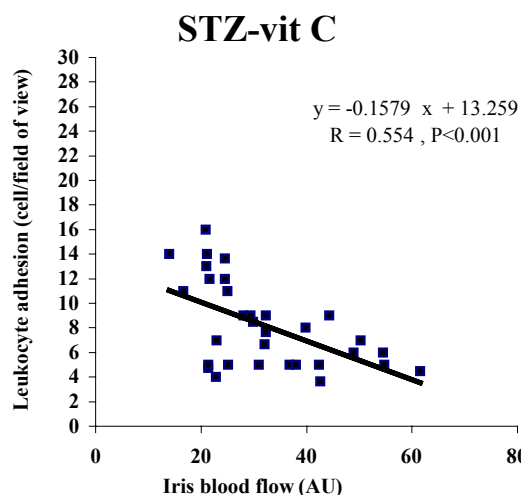


Figure 9. Correlation of leukocyte adhesion and iris blood flow perfusion in the postcapillary venule from 8-36 week of Control, STZ-rat, STZ with vitamin C supplementation.

Discussion

In the present study, the experiments were performed to investigate the effects of long-term vitamin C supplementation on vascular structure and endothelial function of Iris microvessels in diabetic rats.

Streptozocin (STZ) induce β -cells of Islets of Langerhans damage by initiating biochemical events which cause DNA strand breaks. This substance has been widely used to experimentally imitate Type I diabetes mellitus (IDDM) in rats. STZ action on β -cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. Finally, hyperglycemia develops and blood insulin level decreased (West E et al, 1996). In the present study, hyperglycemia occurs within 48 hours after the single dose 55 mg/kg BW. of STZ intravenous injection and persist throughout the experiment. Metabolic derangement after STZ administration include marked hyperglycemia, polyphagia, polydipsia, polyuria and weight loss.

I The metabolic changes in diabetic model : Role of vitamin C

In present study, STZ-diabetic rats lost of their body weight compared to the non-diabetic control rats (CON). Eight weeks after STZ injection body weight were 39% lower in STZ-rats compared with non diabetic control rats ($p < 0.001$) and up to 51% in 36 weeks. With a deficiency of insulin, the metabolism was then shifted from insulin-promoted anabolism to

catabolism of proteins and fats, therefore, it trends to induce a negative energy balance, resulting in weight loss.

1.1 Plasma Vitamin C

In this study, the plasma vitamin C in diabetic rats was chronologically monitored by using a specific enzymatic spectrophotometric method with the absorbance at 593 nm (Liu TZ et al 1982). The findings demonstrated that plasma vitamin C was significantly reduced in 12, 24, 36 weeks of STZ-rats (23.01 ± 0.92 , 21.47 ± 1.87 and 15.95 ± 2.02 $\mu\text{mol/L}$; respectively) as compared those of control rats (44.59 ± 2.12 , 43.58 ± 1.19 and 44.89 ± 2.93 ; respectively). The metabolism of vitamin C has become abnormal in diabetes. And this finding supports the previous study of Kashiba M et al (2002). They demonstrated that plasma vitamin C concentrations as well as in tissues were decreased in STZ-induced diabetic rats. The mechanism which is responsible for the matter might be due to the competitive inhibition between vitamin C and glucose molecules. Since, transport of vitamin C through biological membrane is facilitated by diffusion through the ubiquitous glucose transporter proteins (GLUTs) (Welch RW et al, 1995). GLUTs is normally facilitate dehydroascorbic acid (DHA) diffusion into the cell. Therefore, hyperglycemic condition will be downregulated the V_{max} of the uptake mechanism of DHA. Besides, plasma vitamin C was also depleted through its antioxidant property; scavenging with hyperglycemia induced free radical. By means of these reasons, both plasma and intracellular vitamin C levels were reduced in diabetic state.

1.2 Blood glucose, HbA_{1c}

From the present result, blood glucose, plasma glycosylated hemoglobin levels were significantly elevated in STZ rats compared with non diabetic control rats ($p < 0.001$). The data of plasma glycosylated HbA_{1c} reflected long-term exposure to high blood glucose levels, to which diabetologists use as an index for how well of glycemic control (Cerami A et al, 1979). In this study supplementation of vitamin C was able to reduce plasma glucose, however, significant only at 36 weeks of supplemented. These findings confirmed our previous study (Jariyapongskul A. et al 2002). This unpredicted result might be explained by the beneficial effect of a rise in plasma vitamin C on increasing insulin action (Paolisso G et al, 1994). In addition the increased plasma vitamin C with a simultaneous reduction in plasma GSSG/GSH ratio could enhanced the glucose transport. Since the intravenous injection of streptozotocin 55 mg/kg BW, normally does not damage the whole β cells, therefore, the small amount of plasma insulin can be detected in streptozotocin diabetic rats. In other word the STZ-rat model basically represents for hypoinsulinemic state. According to the finding of Paolisso (1994), our data on hypoglycemic effects of vitamin C may be explained through the possible mechanisms

of vitamin C on reducing blood glucose via its scavenging free radicals result, by which the simultaneous plasma GSSG/GSH ratio was increased, and then increase in insulin action which the consequence of decreased plasma glucose level.

In addition, the glycosylated hemoglobin in diabetic rats was found to be significantly decreased in 36 weeks of STZ-Vit C as well. Therefore, it further confirmed the benefit effect of vitamin C on reducing plasma blood glucose.

1.3 Malondialdehyde (MDA) level in the eye

Lipid peroxidation is initiated by the attack free radical on fatty acid or fatty acyl side chain. Any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. Malondialdehyde (MDA) is generated by both lipid oxidation and as a by-product of prostaglandin and thromboxane synthesis. Its plasma concentration is increased in diabetes mellitus and it is found in the atherosclerotic plaque deposits promoted by diabetes as well (Kume S et al, 1995). In our study, the MDA levels were significantly higher in 8, 12, 16, 24, and 36 weeks of STZ-rats' eyes than in control rats with aged match control. These concentrations are increased in diabetes mellitus where as it is that hyperglycemia can accelerate lipid oxidation (Niskanen LK et al, 1995). Santini SA (1997) indicated the increased lipid hydroperoxides (ROOH) and also its conjugated diene plasma levels in IDDM patients. Losada M (1997) reported that patients with retinopathy showed significantly increased MDA level compared to diabetics without retinopathy and healthy controls, using the TBA test. The degree of reactive oxygen species (ROS) occurred could be estimated by the assessment of its main product, malondialdehyde or MDA (Halliwell B et al, 1993).

Currently, there is a great interest in the potential contribution of increased oxidative stress to the development of complications in diabetes. Increased presence of ROS has also been implicated in the pathogenesis of IDDM (Santini SA, 1997). It has been suggested that long-term exposure of body tissues to elevated blood glucose can result in diabetic patients suffering from oxidation (Wolff SP et al, 1987). Oxidative stress can produce major interrelated derangement of cell metabolism, leading to the peroxidation of cellular membrane lipids as well as the increased oxidative modification of amino acids and DNA (Halliwell B et al, 1991).

There are many suggestions regarding the origins of oxidative stress in diabetes, including free radical reactions related to glycation of proteins, consumption of NADPH through the polyol pathway, glucose autooxidation, hyperglycemia-induced pseudohypoxia, and activation of protein kinase C (Vanderjagt DJ, 2000). From the results of our studies, hyperglycemia leads to non-enzymatic glycosylation of proteins, HbA_{1c}, was found to increase

in STZ-rats. The serum levels of MDA correlated best with glycosylated hemoglobin. Increased lipid peroxides suggesting increased free radical activity is associated with pathogenic implications. Under normal circumstances the extent of lipid oxidation is largely controlled by antioxidant concentration in the surrounding medium which is usually sufficiently high to prevent propagation of oxidative free radical reactions by oxygen-derived free radicals in blood. In tissue, there is, however, a greater likelihood that localized deficiencies of antioxidants would allow lipid oxidation to occur. (Rimm EB et al, 1993). This has led to a huge interest in dietary antioxidants and their protective role in diabetic complications.

Interestingly, vitamin C supplementation can reduce MDA values in 16, 24, and 36 weeks of STZ-Vit C rats compared with STZ-rats at the same aged match group. We present evidences that continued vitamin C supplementation in diabetic rats revealed a highly significant reduction in MDA levels.

Vitamin C is the most important water-soluble antioxidant and has the potential to scavenge the superoxide and hydroperoxyl radicals, which are typical physiological forms of ROS. It has been documented for its effective scavenging superoxide and other reactive oxygen species and protection lipid against peroxidation. Several studies show that dietary antioxidants, vitamin C, can prevent propagation of oxidative free radical reactions (Beyer RE, 1994).

II The antioxidant effects of vitamin C supplementation on leukocyte-endothelial interaction

In our experiment, the exhibition of marked enhancement of leukocyte adhesion and transmigration was observed through intravital fluorescence microscope by using rhodamine 6 G to label leukocyte.

In the present study, the iris postcapillary venules with the diameter of 20-50 μm were chosen for consequently observing the leukocyte-endothelium interaction using an intravital fluorescence microscopy on 8, 12, 16, 24 and 36 weeks after STZ injection. Our results demonstrated that the density of leukocyte adherence per field of view were significantly increased in STZ-rats at all monitored time compared with control rats.

The peripheral polymorphonuclear leukocyte (PMN) is one of the main inflammatory cells. PMN-EC adherence resulted in the formation of a microenvironment between the PMN and the EC (Harlan JM, 1987). Under this scheme, adhesion occurs between mutually "activated" leukocytes and endothelium particularly in the small vessels and caused capillary obstruction and occlusion. The harmful of these phenomenon is that contact of circulating leukocytes with the vascular endothelium promotes a cascade of events leads to further

leukocyte activation. Once activated, PMNs release ROS and mediator of proteolytic tissue degradation, contributing to oxidative stress, subsequent inflammation, and causing surrounded endothelial cells even more damaged (Semdly LA et al, 1986).

Recent studies indicated that both glucose (Schroder S, 1991 ; Kim JA et al, 1995), and advanced glycation end-products may damage endothelium and caused the expression of adhesion molecules (Baumgartner-Parzer SM. et al, 1995 ; Schmidt AM et al, 1995). Non-enzymatic glycation of proteins may also interfere with leukocyte behavior. Massuda et al, (1990) have demonstrated that glycosylated protein separated from the serum of diabetic rats is capable of decreasing membrane fluidity of control leukocyte which may alter leukocyte function such as leukocyte migration. Even a small reduction in leukocyte deformability would likely increase leukocyte retention in capillaries. The observation of less deformable leukocytes has also been extended to both IDDM and NIDDM patients (Ernst E and Matrai A, 1986 ; Pecsvarady Z et al 1994). Sannomiya P et al (1997), demonstrated that aminoguanidine, an inhibitor of advanced glycation end products formation, prevented the decreased leukocyte rolling and migration in alloxan-diabetic rats.

In the present study, vitamin C supplementation had the effect to reduce the number of leukocyte adhesion to endothelium of postcapillary venule in 24- and 36-week STZ-Vit C rats, but not equal to the control values. Zanardo RO et al (1998) have previously demonstrated that vitamin C corrected the reduced cell migration in alloxan-diabetic rats as well. Raised levels of leukocyte adhesion have been demonstrated in diabetic rats. Besides, it had been prognostic importance in the development of diabetic complications. For instance, the role of leukocytes in the pathogenesis of proliferative diabetic retinopathy has been suggested (Schroder S, 1991; Forrester JV, 1993). Especially, greater leukocyte adhesion in diabetic venules was able to reason by dysfunctional diabetic endothelium. Because of several studies suggested that nitric oxide (NO) was reduced in the endothelium of diabetic vessels. In addition, the effects of NO not only on vascular smooth muscle, but also Kubes et al (1991) reported that inhibition of nitric oxide production resulted in a 15-fold increase in leukocyte adherence to cat mesenteric venules. Additionally, NO was also reported to reduce leukocyte adhesion in an acute model of canine myocardial ischemia and reperfusion (Lefer et al, 1993). The increased oxidative stress due to hyperglycemia has been reported for its effects on decreasing nitric oxide activity and synthesis (Jariyapongskul et al 2002, Sridulyakul et al 2003). From our all results, therefore, we suggested that the increase in generation of oxygen derived free radicals (demonstrated by MDA levels) is the major contributors to induce increasing leukocyte adhesion in diabetic rats. Since ROS might cause the decrease in NO,

therefore, the activation of adhesive molecule were occurred. Through this matter, it explains the reason why could improve or prevent leukocyte adhesion.

III. The antioxidant effects of vitamin C supplementation on iris blood-flow perfusion

By using laser doppler flowmetry, mean iris blood-flow perfusion of STZ rats was monitored. The results showed that iris blood-flow in STZ-rats was significantly reduced by 33.64 %, 50.46 %, 57.95, 57.60 % and 56.12 compared with 8, 12, 16, 24, and 36 weeks control rats, respectively.

Interestingly, our results have demonstrated that the reduced iris blood flow could be prevented by vitamin C supplementation. The mechanisms responded for this reduction of iris blood flow in diabetes has not yet been clarified. However, as we have mentioned that there was an imbalance between endothelium-derived vasodilators (nitric oxide, prostacyclin) and endothelium-derived vasoconstrictors (endothelin) reported well in diabetic rats (Ward KK et al., 1989, Cameron NE et al., 1994) This imbalance is believed to result from the increased in oxygen-derived free radicals. Moreover, there is an evidence showed that increase vasoconstrictor could lead to reduce local organ blood flow (Cotter MA et al., 1995) Besides, Helmke BP et al (1997) have the other point of view that the leukocyte adhesion in postcapillary venules may restrict organ blood flow by inducing vasoconstriction in the neighboring arterioles. In our study, increasing leukocyte adhere to the vascular endothelium were found in iris postcapillary venules in streptozotocin-induced diabetic rats. Therefore, the reduction of organ blood flow including iris blood flow is the result from leukocyte adhesion and imbalance between endothelium- vasoconstrictor and vasodilator. However, both of them share the same basis initiated primarily by the oxygen derived free radicals inducing endothelium dysfunction. Such that is the reason why the vitamin C supplementation could be used as an antioxidant in order to prevent reduction in iris blood flow.

The effects of long-term supplementation of antioxidant; vitamin C on vascular structure

In our study, microaneurysms (Figure 12.) were able to observe especially 24 and 36 weeks. It was suggested that the distension of the vascular wall at sites of microaneurysm formation may be resulted from the lack of arterial control of blood flow in which it has already compromised by loss of smooth muscle cell or pericytes.

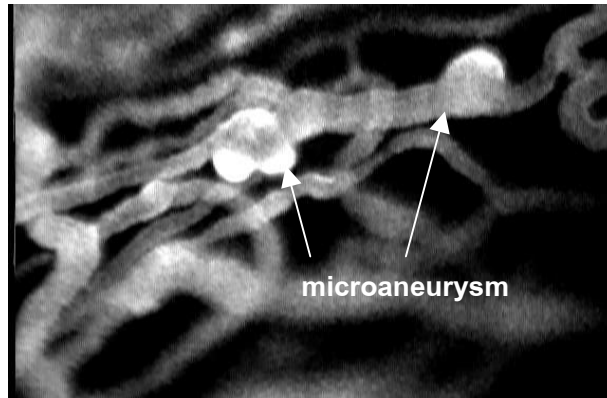


Figure 12. Demonstrate the abnormality of arteriolar vascular wall. Microaneurysm was observe only in occurred in the group of STZ.

Relationship on iris blood-flow perfusion and leukocyte endothelial cell interaction

One of our major objective was to determine whether the increased leukocyte adhesion were contribute to the decrease in iris blood-flow perfusion or not. The increased leukocyte adhesion to the capillaries have been noted to occur in diabetic patients and diabetic animal model. In addition, those findings have been suggested to the important for the development of diabetic angiopathy, since both reduced blood flow and increased leukocyte adhesion may contribute to the formation of nonperfused cappillaries, which are believed to be major contributors for the progression of diabetic angiopathy and result to the with increase in capillary permeability and angiogenesis.

In our study, we have identified both two changes of iris blood-flow perfusion and leukocyte adhesion as early as 8 weeks of diabetic induction. In addition, the result also indicated that increased leukocyte adhesion was correlated to the decrease in iris blood-flow perfusion significantly. As which the correlation between leukocyte adhesion and iris blood-flow perfusion can be characterized by the linear regression showed in Figure 11., $y = -0.192x + 17.019$, $r = -0.292$, $p < 0.034$. Interestingly, the correlation was more clarified and supported by the results of STZ-Vit C, $y = -0.1579x + 13.259$, $r = -0.554$, $p < 0.001$. Several reports have suggested that increase in leukocyte / monocyte adhesion was a critical factor in early retinopathy causing decrease in retinal blood flow and increase in cytokine expression, as well as vascular endothelial growth factor (Miyamoto K et al, 1999; Joussen AM et al, 2002). Miyamoto K. et al

reported that increased leukostasis been shown in animal models of diabetes with short duration of disease. Multiple reports have suggested that the increase in leukostasis was associated with leukocyte or endothelial cell activation and increased expression of adhesion molecules on both cell types (Miyamoto K et al, 1999; Lo SK et al, 1993).

Accordingly, the results demonstrated the correlation between increased leukocyte adhesion and decrease in iris blood-flow perfusion was significantly characterized by the linear regression. Therefore, would like to hypothesize the simple reason explained such correlate as that once hyperglycemia induced endothelial dysfunction, may be through the decrease of nitric oxide activity, as described previously therefore, more adhesive molecules of both leukocyte and endothelial cell were expressed. As when leukocyte adhesion occluded midperipheral vessels then large areas of capillary nonperfusion were developed and finally it may contribute to hypoxic development as a consequence of ischemia. The more leukostasis causes the vascular occluded, the more capillaries become no flow, and results to less of iris blood-flow perfusion.

The data demonstrates that the presence of diabetes are necessary for measurable effect on iris blood-flow perfusion can occur in diabetes, not founded relationship in control rats. Because of the supplementation of vitamin C could prevent both the increase in leukocytes adhesion and decreases in iris blood-flow perfusion in diabetic rats. One might simply say that the prevention of leukostasis probably via the inhibition of expression of adhesive molecule (Rayment SJ et al, 2003) may be the therapeutic tool for preventing hypoxic condition in diabetic iris including retina.

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Output

1. Jariyapongskul A, Rungjaroen T, Kasetsuwan N, Patumraj S , Hideyuki N. Leukocyte adhesion and iris blood flow perfusion in streptozotocin-induced diabetic rats: role of vitamin C (in preparation)
2. เสนอผลงานแบบโปสเตอร์เรื่อง ผลของวิตามินซีต่อการทำงานของเอนโดทีเลียมในหลอดเลือดขนาดเล็กบริเวณม่านตาในหนูเบาหวาน: โดยวิธีอินทราไวทอลฟลูออเรสเซนไมโครสโคปี้ ประชุมเพื่อเสนอผลงานวิจัย “นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส” สำนักงานกองทุนสนับสนุนการวิจัย วันที่ 9-11 มกราคม 2547 ณ โรงแรมเฟลิกซ์ ริเวอร์แคว กาญจนบุรี (Abstract)
3. เสนอผลงานแบบปากเปล่าเรื่อง “Effect of vitamin c on changes of endothelial function of iris microcirculation in diabetic rats :intravital fluorescence microscopic study” ในการประชุมเพื่อเสนอผลงานวิจัย “นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส” สำนักงานกองทุนสนับสนุนการวิจัย วันที่ 14 -16 มกราคม 2547 ณ โรงแรมเฟลิกซ์ ริเวอร์แคว กาญจนบุรี (Abstract)

4. เสนอผลงานแบบปากเปล่าเรื่อง “Leukocyte adhesion and iris blood flow perfusion in streptozotocin-induced diabetic rats: role of vitamin C” ในการประชุม The 6th Asian Congress for Microcirculation (ACM'05) วันที่ 23-26 กุมภาพันธ์ 2548 เมืองโตเกียว ประเทศญี่ปุ่น (Abstract)

Leukocyte adhesion and iris blood flow perfusion in streptozotocin-induced diabetic rats: role of vitamin C

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Leukocyte adhesion and iris blood flow perfusion in streptozotocin-induced diabetic rats: role of vitamin C

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Abstract. Effects of long-term supplementation of antioxidant vitamin C on the iris microcirculation was investigated in streptozotocin (STZ)-induced diabetic rats. Diabetes was induced in male Wistar Furth rats by intravenous injection of STZ (55 mg/kg b.w.). The rats were divided into three groups: control (CON), STZ rats and vitamin supplemented STZ rats (STZ-vitC). Ascorbic acid (1g/L) was added into drink water for supplementation of vitamin C. The experiments were performed at 8, 12, 24 and 36 weeks after injection of STZ. Blood glucose, tissue lipid peroxidation (MDA) and plasma vitamin C were measured. To examine the endothelial function, leukocyte adhesion to the venular endothelium was evaluated by counting leukocytes labeled with rhodamine 6G. The iris blood flow perfusion was monitored using a laser doppler flowmeter. In the STZ-rats, hyperglycemia was induced with increase in the HbA1c, increase in the lipid peroxidation and decrease in the plasma vitamin C. Their abnormal levels were improved by supplementing vitamin C in STZ. The number density of adherent leukocytes was increased significantly at 8, 12, 24 and 36 week after injection of STZ, while the iris blood flow perfusion was decreased. In the STZ-vitC rats, the iris blood-flow perfusion was significantly increased while the leukocyte adhesion was decreased at 24 and 36 weeks. The iris blood-flow perfusion was related with leukocyte adhesion. It is concluded that long-term supplementation of vitamin C could prevent the endothelial dysfunction in diabetes through decrease in leukocyte adhesion and increase in iris blood flow perfusion. The antioxidant vitamin C might be a great therapeutic agent for preventing diabetic retinopathy.

Keywords. diabetes, fluorescence videomicroscopy, iris microcirculation, leukocyte adhesion, retinopathy, STZ-induced diabetic rat, vitamin C,

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1. Introduction

Diabetic retinopathy is one of major complications that cause most suffering in diabetic patients. In diabetes, interaction between leukocyte and endothelium may be enhanced markedly. In fact, Schroder et al,1991. showed the appearance of capillary occlusion with leukocyte in the retinopathy of diabetic rats (1). Recently, Bandello et al. observed phenomena of capillary non-perfusion in the iris microvessels of diabetic patients, suggesting that these vaso-occlusive processes were not limited to retina alone (2). Certainly, leukocyte adhesion may be closely connected with blood flow perfusion in diabetes. However, there are very few studies on the relation between leukocyte adhesion and blood flow perfusion in the iris microvasculature.

According to Giugliano et al (1996), vitamin C was reduced significantly in intracellular concentration under diabetic hyperglycemic condition, while glutathione and vitamin E were decreased. Since vitamin C is a water soluble antioxidant in human plasma (3). It is capable of scavenging reactive oxygen species and sparing other endogeneous antioxidant *in vivo*. In our previous study (4), we demonstrated that daily supplementation of vitamin C could improve the diabetic-induced endothelial dysfunction in STZ-rats. In particular, long term supplementation of vitamin C could decrease leukocyte adhesion to the cerebral post-capillary venules in diabetic-rats. However, there is no data available for effects of vitamin C on the iris microcirculation in diabetes.

In this paper, we designed an experiment for elucidating effects of vitamin C on leukocyte adhesion and iris blood-flow perfusion using streptozotocin (STZ)-induced diabetic rats. We visualized leukocytes flowing in the iris microvasculature under a fluorescence videomicroscope. By counting the number of leukocytes adhering to the venular endothelium, we evaluated the leukocyte adhesion. We also evaluated the iris blood flow perfusion using a laser Doppler flowmeter. We analyzed both parameters of leukocyte adhesion and iris blood-flow perfusion in normal and STZ rats with and without supplementing vitamin C at different periods after injection of STZ.

2. Materials and methods

2.1. Diabetic induction

Ninety five male Wistar-Furth rats weighing 200-250 g were divided randomly into diabetic (n =62) and non diabetic (n=33) group. 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 injected into the tail vein and same volume of citrate buffer pH 4.5 was injected by the same route to non-diabetic control animal. After 24 hours of STZ injection and prior to each experiment, the glucose level was verified using a glucometer (Advance Glucometer, BOEHRINGER MANNHEIM, Germany). The diabetic condition defined as a plasma glucose concentration ≥ 250 mg/dl.

2.2. Vitamin C supplementation

Supplementation of the rats with vitamin C (L-ascorbic acid, 99%, Sigma chemical Co., USA) started 24 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 (4).

2.3. Experiments

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

On the day of experiments, the rat was anesthetized with sodium pentobarbital (60 mg/kg BW, i.p.) and a thecheotomy was performed. It was ventilated mechanically with room air and supplemental oxygen. A catheter was inserted into a femoral vein for injection of fluorescence tracers, and a femoral artery was cannulated for measurement of systolic (SBP) and diastolic blood pressure (DBP) using a pressure transducer connected to a polygraph system (Nihon Kohden, Japan).

2.3.1. Iris blood flow-perfusion measurement

The iris blood flow-perfusion was measured using a laser Doppler flowmetry with a 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 measurements were performed at each time, and the mean of iris-blood flow perfusion was then determined for each animal .

2.3.2 Intravital fluorescence microscopy

The iris microcirculation was observed under an intravital fluorescence videomicroscope. After preparing the eye, briefly, after the left eye was retracted such ocular was suffused with a drop of sterile saline solution. Afterthat, a piece of thin transparent glass with 2×2 mm² was placed on the left ocular. The glass was fixed to the skin arround the left eye. The animal was moved to the stage of the fluorescence videomicroscope. The FITC-dextran (MW 150,000; 15 mg/kg BW) in conjunction with blue light excitation was used for labeling plasma. The other florescent dye is rhodamine 6G (0.15 mg/kg BW) in conjunction with green light was used for staining mitochondria, especially in leukocyte. The both fluorescent tracers were injected intravenously through the cannulated femoral vein; These can be used, in parallel, by switching between two different filter blocks. The epifluorescent image was observed under a low light in real-time throughout the experiment period, using x20 objective lens and a SIT video-camera (DAGE Co. USA). Simultaneously, the image was monitored. The videotapes recorded in each experiment were played back flame-by-flame for further analysis of image processing, using a software called Global lab image II.

2.3.3. 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 the iris post-capillary venules were observed under a fluorescence videomicroscope and recorded in real time throughout the experimental period. The emission wavelength of R6G lies between 530 and 540 nm.

During playback of the videotape, the number of leukocyte adherence to endothelium of the post-capillary venule (20 to 50 μ m in diameter) were counted using the software (called Global lab image). To measure the diameter of post-capillary venule, FITC-labeled dextran (MW 150,000) suspension in physiological saline solution (0.2 ml of 15mg/kg b.w.) was injected into the femoral vein for providing an immediate contrast between plasma and the interstitium.

We regarded the leukocyte as adherent one when it remained stationary for equal or longer than 30 second (5). The number of adherent cells were manually counted and expressed as the number of cell per field of view (6).

2.4. Changes in systemic parameters

2.4.1 Blood glucose, and plasma vitamin C

The parameters for metabolic changes were blood glucose (BG), blood glycosylated hemoglobin (HbA_{1c}) and plasma vitamin C. All these parameters were determined at the end of each experiment by collecting blood sample from the femoral artery. Blood glucose was determined by using a glucometer (Advance Glucometer, Boehringer Mannheim, Germany). The plasma was kept at -80 °C for determination of plasma vitamin C level and was measured using enzyme-assisted spectrophotometric method (5).

2.4.2. Free radical by products: Malondialdehyde (MDA)

The level of lipid peroxidation in the left eye was assayed by measuring the reaction products between malondialdehyde (MDA) and thiobarbituric acid (TBA). Briefly, rat eye was homogenized and washed . After resuspending in distilled water ,lipid peroxidation was allowed to react with TBA at 95° C for 60 minutes. The reaction mixture was cooled to room temperature and the reaction product was extracted with n-butanol and the formation of a pink chromogen is measured at or close to 532 nm (6).

2.5. Statistical analysis

Results were expressed as mean $\pm$ standard deviation of mean (SD). 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.

3. Results

3.1. Changes in systemic parameters

The blood glucose levels was significantly elevated in STZ rats (397.12 ± 65.48 mg/dl to 463.37 ± 106.52), compared with non diabetic control rats (83.5 ± 11.86 to 100.9 ± 11.51 ; $p < 0.001$). Table 1. shows the level of blood glucose, plasma vitamin C, and tissue MDA in all the monitored time points. The level of vitamin C in diabetic rats was significantly decreased ($P < 0.01$), showing the concentration approximately 60% smaller than that in control rats. This reduction in plasma vitamin C returned up to the control level after the supplementation of vitamin C. Plasma

To determine the antioxidant effect of vitamin C on the eye, tissue MDA, product of lipid peroxidation was used. The tissue MDA was found to accumulate in the STZ-rats higher than control rats in all monitored time points (8, 12, 24, 36 weeks). The eye of rats supplemented with vitamin C (8-36 weeks) showed a 19.57-44.82 % decrease in eye MDA level. These results indicated that the beneficial effects of vitamin C were mediated through their antioxidant actions.

Table1. Systemic parameters (blood glucose, plasma vitamin C, tissue MDA, systolic pressure, diastolic pressure in control,diabetic with and without vitamin C supplementation. Values are expressed as mean $\pm$ SE

	BG (mg/dl)	VIT C (μ mol/L)	MDA (nmol/100mg wet wt.)
CON 8 W	83.5 ± 11.8 (n = 10)	-	58.3 ± 27.7 (n = 7)
12 W	97.0 ± 13.8 (n = 12)	44.5 ± 2.1 (n = 5)	72.9 ± 21.4 (n = 10)
24 W	106.8 ± 10.4 (n = 13)	43.5 ± 8.1 (n = 8)	70.4 ± 31.3 (n = 11)
36 W	100.9 ± 11.5 (n = 11)	44.89 ± 2.93 (n = 5)	73.6 ± 22.9 (n = 10)
STZ 8 W	408.6 ± 97.8 (n = 9) ^{***}	-	103.3 ± 25.9 (n = 9) [*]
12 W	397.1 ± 65.4 (n = 8) ^{***}	23.0 ± 0.9 (n = 8) ^{***}	107.1 ± 35.7 (n = 6) [*]
24 W	425.0 ± 64.4 (n = 7) ^{***}	21.4 ± 1.8 (n = 8) ^{***}	137.5 ± 37.8 (n = 6) ^{***}
36 W	463.0 ± 106.5 (n = 8) ^{***}	15.9 ± 2.0 (n = 5) ^{***}	145.6 ± 28.8 (n = 11) ^{***}
STZ-vit C 8W	413.6 ± 94.0 (n = 8) ^{NS}	-	67.7 ± 29.0 (n = 6) ^{NS}
12 W	431.4 ± 83.2 (n = 10) ^{NS}	43.6 ± 3.92 (n = 5) ^{NS}	84.1 ± 13.4 (n = 6) ^{NS}
24 W	380.2 ± 68.9 (n = 11) ^{NS}	39.4 ± 2.04 (n = 5) ^{##}	84.9 ± 13.9 (n = 10) ^{##}
36 W	279.3 ± 77.0 (n = 8) ^{##}	38.5 ± 2.0 (n = 5) ^{##}	80.8 ± 20.8 (n = 8) ^{##}

BG = blood glucose; VITC = plasma vitamin C; MDA = tissue malondialdehyde.

^{***} $p < 0.001$ as compared to CON; ^{**} $p < 0.01$ as compared to CON; ^{*} $p < 0.05$ as compared to CON ;

^{##} $p < 0.01$ as compared to STZ ; ^{NS} not significant different as compared to STZ

3.2 Leukocyte-endothelial cell interaction

All the leukocytes counted as adherent one remained stationary for 30 second. The leukocyte adhesion was counted per field of view totally of postcapillary venule (diameter: 20-50 μm).

The present videomicroscopic visualization showed clear images of leukocyte adhering to the endothelium of postcapillary venules in control (non-diabetes), STZ-rats with and without vitamin C supplementation in all the monitored time points. Fig.1. shows image of leukocyte adhesion (strained by rhodamine-6G) at 36 weeks of control, STZ-rats with and without vitamin C supplementation. The number of leukocyte adhesion appear dramatically increased in STZ-rats but very few leukocyte adhesion on the endothelial lining in control and STZ-rats with supplementation of vitamin C .

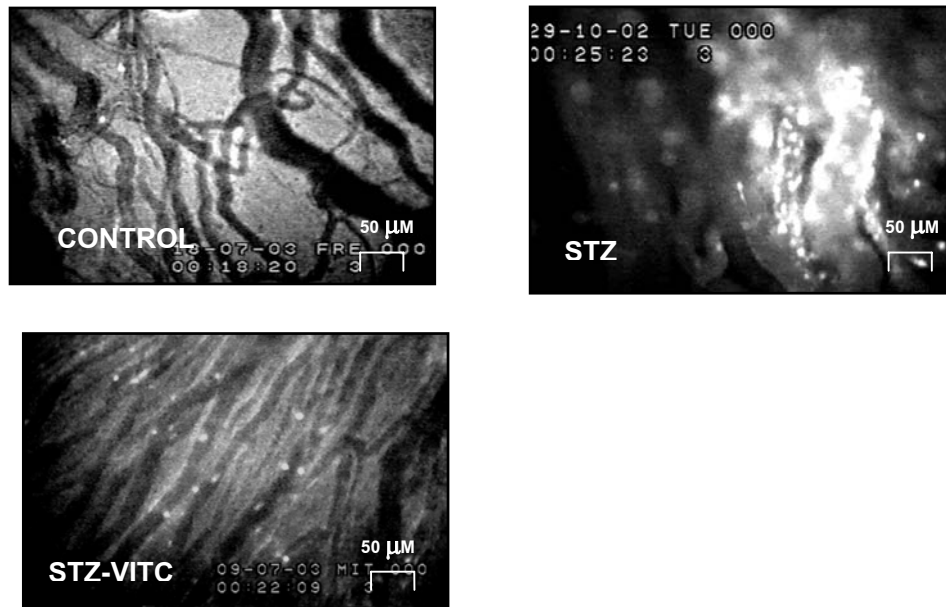


Fig.1 shows image of leukocyte adhesion (strained by rhodamine-6G) at 36 weeks of control

2.3. Iris blood flow perfusion

By using laser Doppler flowmetry, the regional iris blood flow perfusion was measured at 8 points of the iris's area. Table 2 shows the mean values of iris blood flow perfusion. The means of iris blood flow perfusion in STZ rats were significantly reduced to 33.64 %, 50.46 %, 57.60 % and 56.12 %, respectively, compared with control rats on 8, 12, 24 and 36 weeks. There appeared significant difference between STZ and STZ-vit C on 24 and 36 weeks.

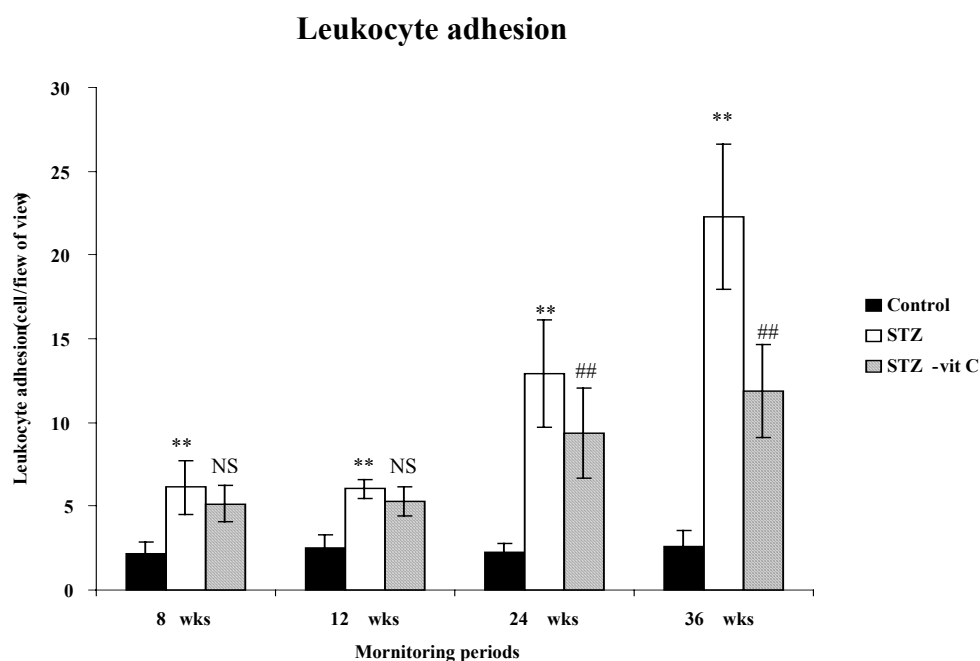


Fig. 2. LA = Leukocyte adhesion; ** $p < 0.01$ as compared to CON; * $p < 0.05$ as compared to CON ; ## $p < 0.01$ as compared to STZ ; NS not significant different as compared to STZ

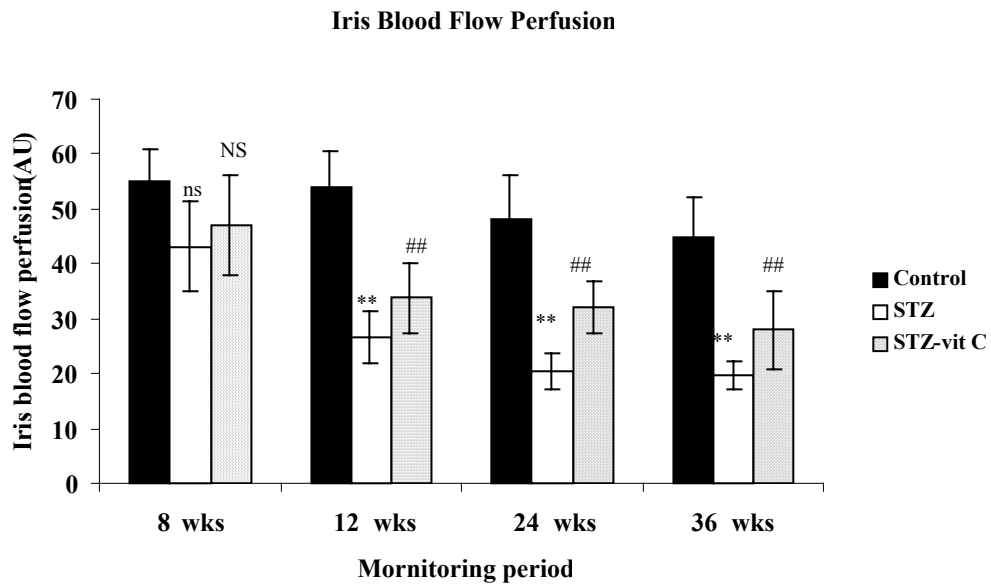


Fig. 3. AU = arbitrary unit ** $p < 0.01$ as compared to CON; * $p < 0.05$ as compared to CON; ## $p < 0.01$ as compared to STZ; ns not significant different as compared to CON; NS not significant different as compared to STZ

2.4 Relationship between the iris blood flow perfusion and leukocyte-endothelial cell interaction

Linear regression analysis was used to examine the relationship on the iris blood-perfusion and leukocyte-endothelial cell interaction. The obtained relationship was shown in Fig. 4. The iris blood flow perfusion values and leukocyte adhesion (cells per field of view of postcapillary venule) in control and diabetic rats were plotted at all the monitored time points. The two parameters, iris blood flow perfusion (x) and leukocyte adhesion (y), were correlated as follows:

$$y = 0.0015x + 1.18 \quad (r = 0.060, P < 0.696) \quad \text{for control rat,}$$

$$y = -0.192x + 17.0 \quad (r = 0.292, p < 0.034) \quad \text{for STZ-rat}$$

$$y = -0.158x + 13.3 \quad (r = 0.554, p < 0.001) \quad \text{for STZ-vitC rat.}$$

It is interesting to note that any effect on the iris blood flow perfusion appeared in diabetic rats, but not in control rats. This indicates that the presence of diabetes is necessary for measurable effect on the iris blood flow perfusion to occur. The present relationship between the iris blood flow perfusion (x) and leukocyte adhesion (y) showed that: 1) In control (non-diabetic) rats appeared no effect on the iris blood flow perfusion, This indicates that the presence of diabetes is necessary for measurable effect on the iris blood flow perfusion to occur; 2) In diabetic rats, the iris blood flow perfusion was reduced when leukocyte adhesion was raised. This tendency was lowered in diabetic rats with vitamin C, compared to diabetic rats without vitamin C.

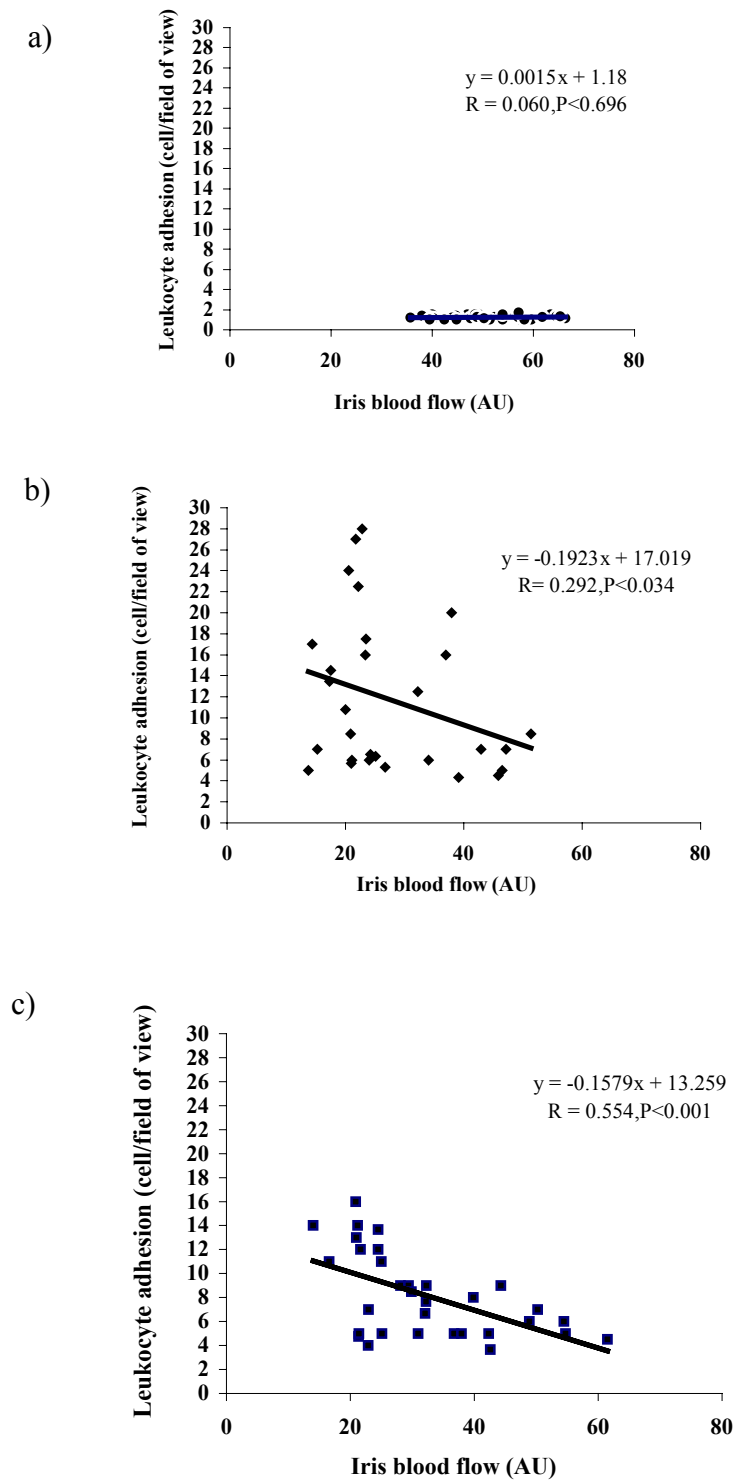


Fig.4. Correlation of leukocyte adhesion and iris blood flow perfusion in the postcapillary venule from 8-36 week of a) Control, b) STZ-rat, c) STZ with vitamin C supplementation.

Discussion

Hyperglycemia may lead to elevated production of oxidative stress by a number of mechanisms, including the production of oxidants by vascular endothelium and leukocytes. The present study shows increased oxidative stress (MDA levels) in the eye of diabetic rats which can be inhibited significantly by the supplementation of antioxidant, vitamin C.

Vitamin C is an important water-soluble antioxidant, and has a potential to scavenge superoxide and hydroperoxyl radicals. Up to now, a number of documents have showed its effectiveness for scavenging superoxide and other reactive oxygen species and protection lipid against peroxidation (7,8). In the present study, we showed its effectiveness for reducing lipid peroxidation (MDA) levels in STZ-induced diabetic rats. In fact, vitamin C supplementation could reduce MDA levels significantly at 24 and 36 weeks after STZ injection. Currently, there is a great interest in the potential contribution of increased oxidative stress to the development of complications in diabetes. It has been suggested that long-term exposure of body tissues to elevated blood glucose can result in diabetic patients suffering from oxidation (9). Oxidative stress can produce major interrelated derangement of cell metabolism, leading to the peroxidation of cellular membrane lipids (10). The extent of lipid oxidation is controlled by antioxidant concentration in the surrounding medium, so that the level may be usually sufficiently high for the propagation of oxidative free radical reactions in blood, but deficit of antioxidants in tissues may allow lipid oxidation to occur (11). This has led to a substantial interest in dietary antioxidants and their protective role in diabetic complications.

In the present study, we monitored the iris blood-flow perfusion in STZ-induced diabetic rats with and without supplementation of vitamin C, using laser doppler flowmetry. The measured iris blood-flow was reduced significantly at 8, 12, 24, and 36 weeks after STZ injection, compared with control rats. Moreover, the supplementation of vitamin C increased the iris blood-flow perfusion significantly at 24 and 36 weeks. It is to be noted that reduction in iris blood-flow perfusion in diabetes might be suppressed by administration of vitamin C.

In general, vitamin C could prevent the impairment endothelium-dependent vasodilation. This mechanism has been elucidated both by intravenous infusion (12,13,14) and by oral administration (15,16). In hyperglycemia, reactive oxygen species (ROS) is induced to increase, associated with increase in MDA level. The ROS increases leukocyte-endothelial interaction through inducing endothelial dysfunction, which may lead to decreasing the iris blood-flow perfusion. It is, therefore, reasonable to assume that once ROS is decreased by vitamin C as an antioxidant, endothelial dysfunction should be prevented.

In the present study, we visualized the behavior of leukocytes in microvessels by rhodamine 6G to label leukocyte for examination the leukocyte-endothelium interaction under a fluorescence videomicroscope. Choosing the iris postcapillary venules with 20-50 μm in diameter, we continuously observed the cells flowing through the venules. Our result demonstrated that the number density of leukocyte adhering to venule was significantly increased in STZ-induced diabetic rats at different periods after STZ injection, compared with control rats. A number of factors influences the leukocyte adhesion to endothelium, including oxygen-derived free radicals. According to recent studies, high blood glucose may damage endothelium (1,17), and also cause the expression of adhesion molecules (18,19). Our study indicated that vitamin C is effective for reducing leukocyte adhesion to venular endothelium in diabetes.

It has been noted that increases in leukocyte adhesion to microvessels occur in diabetic patients and diabetic animal models. Such increased levels of leukocyte adhesion may be of

prognostic importance in the development of diabetic complications. In fact, leukocytes played a role in the pathogenesis of proliferative diabetic retinopathy (1,20). Especially, enhanced adhesion of leukocyte was considered a reason for dysfunction of endothelium induced in diabetes. There are several studies to indicate that nitric oxide (NO) was reduced in the endothelium of diabetic vessels. Kubes et al (1991) reported that inhibition of NO production resulted in a 15-fold increase in leukocyte adhesion to cat mesenteric venules (21). Moreover, NO reduced leukocyte adhesion in an acute model of canine myocardial ischemia and reperfusion (22). There are *in vivo* studies to report that increased oxidative stress due to hyperglycemia might be effective for decreasing NO activity and synthesis (23). Our result provides a clue to understanding why antioxidant could improve or prevent leukocyte adhesion. The increase in generation of oxygen-derived free radicals (demonstrated by MDA levels) may be a major contributor increasing leukocyte adhesion in diabetic rats. Considering decrease in NO by ROS, adhesive molecules might be activated.

Both increased leukocyte adhesion and reduced blood flow appear in diabetic patients and diabetic animal models. It is reasonable to suppose that the both factors should be essential for the development of diabetic angiopathy. An aim of the present study was to seek for relation between leukocyte adhesion and blood flow perfusion in the iris microvasculature. By identifying both changes of iris blood-flow perfusion and leukocyte adhesion as early as 8 weeks after diabetic induction, we could relate the leukocyte adhesion with the iris blood flow perfusion. As it is shown in Fig.4, the correlation between leukocyte adhesion and iris blood-flow perfusion were expressed by the linear regression. Especially, the obtained relationship showed a good correlation for STZ-induced diabetic rats supplemented vitamin C.

There are several reports to indicate that increase in leukocyte adhesion was a critical factor in the early retinopathy through decrease in retinal blood flow and increase in cytokine expression and vascular endothelial growth factor (24,25). Miyamoto K. et al (1999) showed how leukostasis was increased in animal models with diabetic diseases during short periods. Such increase in leukostasis might be associated with leukocyte or endothelial cell activation and increased expression of adhesion molecules (24,26). Our result showed that increase in leukocyte adhesion could be closely related with decrease in iris blood flow perfusion. Presumably, once hyperglycemia induced endothelial dysfunction, it might decrease NO activity, which resulted in more expression of adhesive molecules in both leukocytes and endothelial cells. Once leukocyte adhesion occludes mid-peripheral vessels, non-perfusion may be induced at large areas of capillary network, which leads to hypoxic development as a consequence of ischemia. It is likely that the more leukostasis causes microvascular occlusion, the more capillaries become non-perfused, which results in less blood-flow perfusion in the iris microvasculature.

In conclusion, the present experiment provided an *in vivo* evidence to indicate that lipid peroxidation levels (MDA) was induced in the diabetic eye and was decreased by supplementing vitamin C. Moreover, the leukocyte adhesion was related with the iris blood flow perfusion. The supplemented vitamin C could prevent both increases in leukocytes adhesion and decreases in iris blood-flow perfusion in diabetic rats. A therapeutic tool for preventing hypoxic condition in diabetic iris, including retina may be the prevention of leukostasis, which may be possible by inhibiting the expression of adhesive molecules (27).

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