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ScienceDirect
Microvascular Research 74 (2007) 32–38
MVR Microvascular Research
www.elsevier.com/locate/ymvre

Long-term effects of oral vitamin C supplementation on the endothelial dysfunction in the iris microvessels of diabetic rats

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Received 10 November 2006; revised 27 February 2007; accepted 15 March 2007
Available online 23 March 2007

Abstract

The current study was aimed to investigate effects of long-term supplementation of vitamin C on the iris microcirculation 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 rats (CON), STZ-induced diabetic rats (STZ), and STZ rats supplemented with vitamin C (STZ-vitC). For supplementation of vitamin C, ascorbic acid (1 g/l) was added into the drinking water. The experiments were performed at different periods (8, 12, 24 and 36 weeks) after injection of STZ. Blood glucose, tissue lipid peroxidation and plasma vitamin C were measured. To examine the endothelial function, leukocyte adhesion to the venular endothelium was evaluated in the iris post-capillaries by means of counting the number of leukocytes labeled with rhodamine 6G. Blood flow perfusion in the iris was monitored using a laser Doppler flow meter. In the STZ rats, hyperglycemia was induced with an increase in HbA_{1c} and lipid peroxidation but with a decrease in the plasma vitamin C level which improved by vitamin C supplementation. The number of adherent leukocytes increased significantly, associated with reduction in the iris blood flow perfusion, at 8, 12, 24 and 36 weeks after injection of STZ. In the STZ-vitC rats, the iris blood flow perfusion was significantly increased in comparison with the STZ rats, while the leukocyte adhesion was decreased at 24 and 36 weeks. The statistical analysis shows that the leukocyte adhesion decreased with increase in the iris blood flow perfusion in STZ and STZ-vitC rats. In conclusion, vitamin supplementation suppressed leukocyte adhesion and thus endothelial dysfunction, associated with increase in iris blood flow perfusion in diabetes. The antioxidant vitamin C may be a therapeutic agent for preventing diabetic retinopathy.

Keywords: Ascorbic acid (Vitamin C); Diabetes; Fluorescence videomicroscopy; Iris microcirculation; Leukocyte adhesion; Retinopathy; Streptozotocin (STZ)

Introduction

Diabetic retinopathy is a major complication that causes the most suffering in diabetic patients. In diabetes, interaction between leukocytes and vascular endothelium may be enhanced markedly in the ocular microcirculation, which may induce endothelial dysfunction in the retina. In fact, Schroder et al. (1991) showed the appearance of capillary occlusion with leukocyte in the retinopathy of diabetic rats. According to Bandello et al. (1994) such capillary non-perfusion occurred in the iris microvessels of diabetic patients as well. Increase in leukocyte adhesion might be a critical factor in the early retinopathy through decrease in retinal flow and increases in cytokine expression and vascular endothelial growth factor (Antonia et al., 2002; Abiko et al., 2003).

Vitamin C can improve the endothelial dysfunction induced during diabetes (Ting et al., 1996; Solzbach et al., 1997; Kugiyama et al., 1998; Gokce et al., 1999; Rayment et al., 2003; Haidara et al., 2004). Moreover, vitamin C can scavenge

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0026-2862/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.mvr.2007.03.002

superoxide and other reactive oxygen species (ROS) and also protect lipid against peroxidation (Martin and Frei, 1997; Carr et al., 2000). Effects of vitamin C on diabetes-induced endothelial dysfunction have been evaluated experimentally in the brain (Jariyapongskul et al., 2002; 2003), renal (Delles et al., 2004; Yusuksawad and Chaiyabutr, 2006) and other organs (Zanardo et al., 2003; Amatyakul et al., 2003). There are very few studies regarding in vivo effects of vitamin C on the iris tissue intimately connected with diabetic retinopathy.

Intravital observation of the microcirculation in the retina has been made for clinical assessment of the degree of retinopathy (Yuan et al., 2000; Algenstaedt et al., 2003; Ramsay et al., 2003). Iris angiopathy is of clinical significance in that the first sign of diabetic vascular damage can be seen in the iris earlier than in the retina. Its incidence increases with the duration of diabetes, independent of age (Demeler, 1979). Thus, vascular changes of the iris may be alternative of the retinopathy.

In previous studies (Jariyapongskul et al., 2006; Patumraj et al., 2006), we developed a fluorescence videomicroscopic technique that enables us to observe the iris microcirculation directly and continuously. The purpose of this study is to investigate long-term effects of oral vitamin C supplementation on the endothelial dysfunction in the iris microvessels of diabetic rats, using our fluorescence videomicroscopy. As a diabetic model, we used streptozotocin (STZ)-induced diabetic rats. We measured leukocyte adhesion in the venules to evaluate the endothelial dysfunction at 8–36 weeks after diabetic induction. The iris blood flow perfusion was measured using laser Doppler flowmetry to examine the correlation between leukocyte adhesion and iris blood flow perfusion in the rats without and with vitamin C at different time-points.

Materials and methods

Diabetic induction

Ninety five male Wistar-Furth rats (200–250 g b.w.) were divided randomly into diabetic ($n=62$) and non-diabetic ($n=33$) group. Diabetes was induced by a single intravenous injection of STZ (55 mg/kg b.w., Sigma, USA). The STZ was freshly prepared by dissolving in citrate buffer (pH 4.5, Sigma, USA) and immediately injected into the tail vein and the same volume of citrate buffer (pH 4.5) was injected by the same route to non-diabetic control rats. The diabetic condition was defined as a plasma glucose concentration ≥ 250 mg/dl.

Supplementation of vitamin C

Rats was supplemented with vitamin C (L-ascorbic acid, 99%, Sigma, USA), starting 48 h after STZ injection. The vitamin C was prepared daily by dissolving in drinking tap water at a concentration of 1 g/l. The experimental rats had free access to the drinking water with vitamin C.

Experimental protocol

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

At the termination of the experiments, the animals were anesthetized with sodium pentobarbital (60 mg/kg b.w., i.p.) and a tracheotomy 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 and diastolic blood pressure using a pressure transducer (Nihon Kohden, Japan).

Iris blood flow perfusion measurement

The iris blood flow perfusion was measured using a laser Doppler flow meter 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 of about 1 mm distance. Eight different measurements were performed at each time, and the mean of iris-blood flow perfusion was determined for each animal.

Intravital fluorescence microscopy

The iris microcirculation was observed under an intravital fluorescence videomicroscope. After the left eye was retracted, the ocular was suffused with a drop of sterile saline solution. Then, a piece of thin transparent glass (2×2 mm^2 in area) was placed on the left ocular. It was fixed to the skin around the left eye. The animal was moved to the stage of the fluorescence videomicroscope. The FITC-dextran (MW 150,000; 15 mg/kg b.w.) was used in conjunction with blue light excitation for labeling plasma. Rhodamine 6G (0.3 mg/ml, Sigma, USA) was used in conjunction with the green light for staining mitochondria in leukocytes. Both the fluorescent tracers were injected i.v. through the cannulated femoral veins and were visualized in parallel by switching two different filters. The epifluorescent image was observed under a low light throughout the experiment period, using $\times20$ objective lens and a silicon intensified television (SIT) camera (Dage, USA). The videotapes recorded in each experiment were played back flame-by-flame for further analysis of image processing, using a software (Global lab image II).

Leukocyte adhesion

Leukocyte adhesion in the iris post-capillary venules was measured based on rhodamine video images. During the playback of the video tape, adherence of leukocytes to the endothelium of the post-capillary venules (20 to 50 μm in diameter) was examined. Diameters of post-capillary venules were measured from FITC videoimage. Adherent leukocytes were defined in each vessel as cells remaining stationary for equal or longer than 30 s (Jariyapongskul et al., 2002). The number of adherent cells was manually counted and expressed as the number of cells/field of view (cell/0.01 mm^2). For a correlation between the present expression of leukocyte adhesion and the conventional expression in single vessels, see Appendix.

Metabolic indices

Metabolic indices determined in this study were the levels of blood glucose (BG), blood glycosylated hemoglobin (HbA_{1c}) and plasma vitamin C. All the indices were determined by collecting blood sample from the femoral artery at the end of each experiment. Blood glucose was determined by using a glucometer (Advance Glucometer, Boehringer Mannheim, Germany). The plasma was kept at −80 °C for the determination of plasma vitamin C levels as measured by the enzyme-assisted spectrophotometric method (Liu et al., 1982).

The level of lipid peroxidation in the left eye was assayed by measuring the reaction products between malondialdehyde (MDA) and thiobarbituric acid (TBA) as follows. The rat eye was homogenized with 1.15% KCl in 0.1 M phosphate buffer, pH 7.4 and washed. After resuspending in distilled water, the lipid peroxidation was allowed to react with TBA at 95 °C for 60 min. The reaction mixture was cooled to a room temperature. The reaction product was extracted with *n*-butanol and the formation of the pink chromogen was measured at 532 nm (Ohkawa et al., 1979).

Statistical analysis

All data of leukocyte adhesion and iris blood flow perfusion were divided in sub-groups with every 10 AU in the iris blood flow (i.e. <20, 20–30, 30–40, 40–50, 50–60, and >60 in STZ and STZ-vitC rats), where AU indicates an arbitrary unit. Using the means ± SD of the data calculated in each sub-group, we examined the correlation between leukocyte adhesion and iris perfusion to discuss their difference in STZ and STZ-vitC rats.