Asian Biomedicine Vol. 1 No. 2 August 2007

Original article

Long-term oral vitamin C administration improves cerebral microvascular vasodilatory impairment in diabetes: in vivo evidence using diabetic rats

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Background: Many clinical reports have indicated that ascorbic acid (vitamin C) improves vasodilatory impairments in patients with diabetes mellitus, but there is very little *in vivo* evidence to demonstrate its effectiveness on the brain.
Objective: To investigate long-term effects of oral vitamin C administration on the cerebral microvascular vasodilation in diabetes, using streptozotocin (STZ)-induced diabetic rats.
Materials and methods: Diabetes was induced in male Wistar Furth rats by a single intravenous injection of STZ (55 mg/kg b.w). Ascorbic acid (vitamin C) was administered in drinking water (1g/l). The rats were divided into control and diabetic groups with or without administration of vitamin C. The cerebral microcirculation was observed at different times (12, 24 and 36 weeks) after vitamin C supplementation, using fluorescence videomicroscopy. Responses of cerebral arterioles to acetylcholine (ACh), adenosine-5 diphosphate (ADP) and nitroglycerine (NTG) were studied by measuring diameters of cerebral arterioles before and after topical application on the cortical surface.
Results: The vasodilatory responses of cerebral arterioles to ACh and ADP were significantly decreased in diabetic rats, compared with non-diabetic (control) rats. The response to NTG was not altered in diabetic rats, indicating that the vasodilatory impairment involves at the endothelium. The impaired endothelium-dependent vasodilation was prevented by long-term vitamin C administration.
Conclusion: Long-term oral vitamin C administration might be of clinical relevance in improving cerebral microvascular vasodilatory impairment in diabetes.

Keywords: Ascorbic acid (vitamin C), cerebral microcirculation, diabetes, fluorescence videomicroscopy, vasodilation.

In diabetes mellitus, the microvascular beds of various organs are frequently altered in morphology and function [1]. In the brain, microcirculatory disorders in diabetes are caused by the loss of autoregulation (or vasodilatory impairment) in diabetes. This may be due to dysfunction of cerebral microvessels in response to various stimulations [2-4]. The microvascular vasodilatory response is linked to the functions of endothelial cells (EC) and vascular smooth muscle cells (VSMC). Endothelium-dependent vasodilation may be related to nitric oxide (NO) release from EC, while the endothelium-independent one is due to VSMC guanylate cyclase activation. Endothelial dysfunction may lead to the impairment of microvascular vasodilation.

A number of studies have shown impairment of endothelium-dependent vasodilation in large and resistant arteries of diabetic animals. Miyata et al. [6] demonstrated similar impairments in the aorta and mesenteric arteries of two genetic diabetes-prone rats. Tesfamariam et al. [7] demonstrated a decreased endothelium-dependent vasodilation in an isolated abdominal aorta to acetylcholine (ACh) and adenosine-5 diphosphate (ADP) in alloxan diabetic rabbit after 6 weeks of diabetes.

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Recently, much attention has been paid to roles of oxidative stress in the pathogenesis of various vascular damages during diabetes [8-10]. Many clinical studies have indicated that antioxidant vitamin C (ascorbic acid) could improve impairment of endothelium-dependent vasodilation in patients [11-13]. However, there is very little *in vivo* evidence to demonstrate the effectiveness of oral vitamin C administration in the brain in diabetes.

The purpose of this paper is to investigate long-term effects of oral vitamin C administration on diabetes-induced impairment of cerebral micro-vascular vasodilation, using diabetic rats. As a diabetic model, we used streptozotocin (STZ)-induced diabetic rats. Responses of cerebral arterioles to different vasodilators (ACh, ADP and nitroglycerine (NTG)) were examined by topical application on the cortical surface. Diameter changes of cerebral arterioles were measured in control and diabetic groups at different time-points (12, 24 and 36 weeks) after vitamin C administration, using fluorescence videomicroscopy.

Materials and methods

This study was conducted according with the guiding principles for the care and use of animals in the field of physiological sciences (published by the Physiological Society of Japan) and the guideline for experimental animals suggested by The National Research Council of Thailand (1999).

Diabetic rat model

Seventy-two male Wistar Furth rats (200-250g b.w.) were used for this study. The rats were divided into control (n=36) and diabetic groups (n=36). Diabetes was induced by a single intravenous injection of streptozotocin (STZ) (Sigma, St. Louis, USA) (55 mg/kg b.w.). The STZ was dissolved into citrate buffer (pH 4.5, Sigma, St Louis, USA) immediately before injection and injected intraveneously for the experiemental group, while the control group received the same amount of saline. After 48 hours of STZ injection and prior to each experiment, the glucose level was verified using a glucometer (Advance Glucometer, Boehringer Mannheim, Mannheim, Germany). Diabetes was defined by the glucose concentration $\geq$ 300 mg/dl.

Vitamin C administration

Administration of vitamin C was started 24 hours after adminstration of STZ or saline solution. Vitamin C (L-ascorbic acid 99 %, Sigma, St. Louis, USA) was added to drinking water at a concentration of 1g/L [14], which was prepared daily. Non-diabetic rats without and with administration of vitamin-C were indicated by CON (n=18) and CON-vitC rats (n=18), respectively, while diabetic rats with and without administration of the vitamin C were indicated by STZ (n=18) and STZ-vitC rats (n=18), respectively.

Animal preparation

Experiments were started at 12, 24 and 36 weeks after injection of STZ or saline solution. The rats were anesthetized with pentobarbital sodium (60mg/kg b.w, i.p). After a tracheotomy was performed, the rats were ventilated mechanically with an air-oxygen mixture.

A catheter was inserted into a femoral vein for injection of fluorescence tracers and a femoral artery was cannulated for measurement of blood pressure and analysis of blood gas. The blood pressure was monitored throughout the experiment using a pressure transducer (Nohon Kohden, Tokyo, Japan). The blood gas pressure and pH were measured using a blood gas analyzer (Model 278, Ciba Corning, England). Throughout the experiment, the blood gas pressure and pH levels were maintained at a physiological range (PCO_2=35-45 mmHg, PO_2 =100-120 mmHg, and pH=7.35-7.45).

Metabolic measurement

Blood glucose (BG) and levels of triglycerides (TG), cholesterol (CHOL) and vitamin C (VITC) in plasma were measured in blood samples collected from a femoral artery at the termination of each experiment. The BG was measured using the glucometer. The blood sample was centrifuged and the plasma was collected for the measurement of metabolic indices. One part of plasma (0.5 ml) was stored at -80^{o}C, and the level of plasma VITC was measured using enzyme-assisted spectrophotometry [15]. Another part was used for the measurement of plasma CHOL and TG at RIA Laboratory Co. Ltd (Bangkok, Thailand).

Intravital microscopic observation

A craniotomy was performed to expose the anterior cerebral cortex. The dura was opened using a micro-needle. A stainless metal frame with a circular glass window (7 mm diameter) was fixed to the cranial bone [16]. An artificial cerebrospinal fluid (CSF) (composition: NaCl=118.0, KCl=4.0, $MgSO_4$=1.2,

$CaCl_2$=1.5, NaH_2PO_4=1.2, $NaHCO_3$=25.0, Glucose 5.0 in mM) was infused into the cranial space. The cranial window was connected to an infusion pump through microtubing, and the artificial CSF was infused during 30 minutes after the surgical procedure.

A fluorescence microscopic system (Nikon, Tokyo, Japan) equipped with a SIT videocamera (C2400-08, Hamamatsu Photonics, Japan), video timer (VTG-55, FOR.A, Tokyo, Japan) and videorecorder (Sony SVHS, Tokyo, Japan) was used for intravital observation of the cerebral microcirculation. A 20X objective lens was used, and the video images were recorded on videocassettes for the analysis.

Rhodamine-labeled dextran was used to visualize microvascular morphology, respectively [17]. Rhodamine isothiocyanate (RITC) (40,000 MW, Sigma, St. Louis, USA) was dissolved in a phosphate buffered saline pH 7.4 at a concentration of 25 mg/mL, and 0.3 mL of the solution was injected intravenously.

Microvascular response measurement

We used three kinds of vasoactive substances: acetylcholine (ACh), adenosine-5 diphosphate (ADP) and nitroglycerine (NTG). The ACh, ADP and NTG were mixed in the artificial CSF at the concentration of 10^{-7} M, 10^{-6} M and 10^{-6} M, respectively. Each solution was superfused over the cerebral cortical surface for 5 minutes, using an infusion pump with the rate of 1.5 ml/min. The cerebral microvascular response was continuously recorded before and after topical application of each solution. The response to each solution was examined in arterioles with the third order (diameter: 20 to 40 μm). Their diameters were measured based on the RITC image before and 5 minutes after the topical application of each solution.

To express the vasodilatory response to vasoactive substances, we introduced the percent change of diameter as follows:

$$D = \{(d-d_o)/d_o\}x100\ (\%), \quad (1)$$

where d and d_o represent diameters of an arteriole after and before the application of ACh, ADP and NTG, respectively.

Data analysis

Results were expressed as means standard error of mean (SEM). Statistical analysis was made using two-way ANOVA, followed by Student's unpaired t-test. A probability (P) less than 0.05 was considered to be significantly different.

Results

Changes in metabolic indices

The BG level was elevated significantly after the injection of STZ. **Table 1** shows the levels of BG,

Table 1. Metabolic indices measured in normal and diabetic rats at different time-points (12, 24 and 36 weeks) after vitamin C administration (BG=blood glucose; VITC=plasma vitamin C; CHOL=plasma cholesterol; TG=triglyceride; MAP= mean arterial pressure). Values are expressed as means±SEM.

	Number of rats	BG (mg/dl)	VITC (μg/l)	CHOL (mg/dl)	TG (mg/dl)	MAP (mmHg)
CON 12w	6	98.5 ± 2.99	43.3 ± 2.6	72.0 ± 6.2	92.2 ± 21.6	85 ± 550
24w	6	104.7 ± 2.0	43.6 ± 1.2	66.8 ± 3.1	66.0 ± 6.3	95 ± 371
36w	6	100.0 ± 2.1	44.3 ± 3.4	71.2 ± 6.0	79.8 ± 14.2	99 ± 2
CON-vitC						
12w	6	100.0 ± 2.4	54.5 ± 1.2	78.5 ± 13.4	47.0 ± 6.5	89 ± 4
24w	6	101.0 ± 1.5	56.9 ± 5.4	75.5 ± 8.9	78.3 ± 11.1	95 ± 1
36w	6	101.1 ± 2.1	57.5 ± 5.3	81.0 ± 6.9	86.0 ± 22.7	97 ± 4
STZ 12w	6	400.0 ± 31.5**	23.0 ± 0.9**	73.7 ± 9.9^{ns}	94.3 ± 25.0^{ns}	129 ± 3**
24w	6	384.3 ± 31.5**	21.4 ± 2.8**	95.8 ± 9.3*	145.6 ± 34.5*	136 ± 9*
36	6	396.5 ± 23.3**	14.1 ± 2.0**	157.8 ± 25.2**	154.2 ± 37.1**	126 ± 3**
STZ-vitC						
12w	6	381.2 ± 15.8^{NS}	45.0 ± 4.4$^{\#\#}$	72.7 ± 6.5^{NS}	50.5 ± 6.2^{NS}	104 ± 8$^{\#\#}$
24w	6	359.8 ± 23.4^{NS}	38.1 ± 2.2$^{\#\#}$	63.0 ± 3.7$^{\#\#}$1	67.2 ± 13.2$^{\#}$	111 ± 1$^{\#\#}$
36w	6	292.7 ± 19.4**	38.5 ± 2.8$^{\#\#}$	74.0 ± 5.9$^{\#\#}$	54.2 ± 11.6$^{\#}$	112 ± 3$^{\#\#}$

*P<0.05, compared with CON-rats; **P<0.01, compared with CON-rats; $^{\#}$P<0.05, compared with STZ-rats; $^{\#\#}$P<0.01, compared with STZ-rats; ^{ns} not significantly different, compared with CON-rats; ^{NS} not significantly different, compared with STZ-rats.

plasma VITC and MAP measured in control and diabetic rats at different observation periods (12, 24 and 36 weeks) after vitamin C administration.

A significant reduction in level of plasma VITC was observed in diabetic rats ($P<0.01$). The VITC level was approximately 60 % lower than that of control rats. This reduction returned up to the control level after the administration of vitamin C. The long-term administration of vitamin C decreased BG in STZ-diabetic rats. After 36 weeks of vitamin C, the reduction in BG level was approximately 25 % greater than that without it. The MAP in STZ-diabetic rats was significantly higher than that in control rats. This elevation was decreased by approximately 10 % by administration of vitamin C.

Arteriolar responses to Ach, ADP and NTG

The cerebral arterioles started to dilate approximately 2 minutes after superfusion of each substance, reaching a steady state within 5 minutes. After the cortical surface was washed out with artificial CSF, the diameter returned to the baseline within 2-3 minutes. **Figure 1** shows an example to demonstrating the baseline and vasodilated state of a cerebral arteriole in response to ACh.

Arteriolar diameters were measured just before and 5 minutes after the application of each substances (ACh, ADP and NTG), and the percentage change (D) of vasodilation from the baseline was calculated using eq (1). **Figure 2** shows the averaged percent change of vasodilation over all arterioles measured after different periods (12, 24, and 36 weeks) of vitamin C administration in control and diabetic rats.

The vasodilatory responses to ACh and ADP were greatly reduced in STZ-rats, compared with CON-rats. The reduction were improved at all time-periods after vitamin C administration, attaining levels up to 70-85 % improvement of CON- or CON-vitC rats. These results indicated that diabetes impairs the endothelium-dependent vasodilation, and the impairment can be prevented by the long-term administration of vitamic C.

The response to NTG was not different among the four groups of CON, CON-vitC, STZ, and STZ-vitC rats, but it was different from responses generated by ACh and ADP. These results suggest that diabetes impairs the endothelium-dependent vasodilation, but does not impair the endothelium-independent vasodilation due to the activity of vascular smooth muscle.

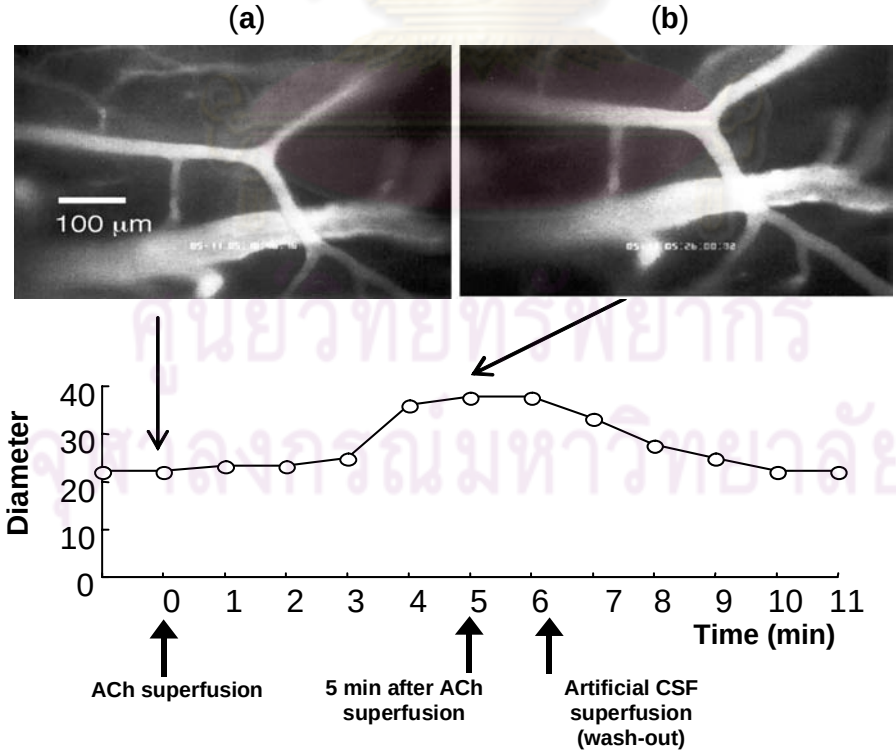

Fig.1 An example of vasodilatory responses of a cerebral arteriole to ACh (10^{-7}M) (Upper: rhodamine-visualized arterioles just before (**a**) and 5 min after (**b**) the initiation of ACh superfusion on the cortical surface; Lower: time-course of the arteriolar diameter).

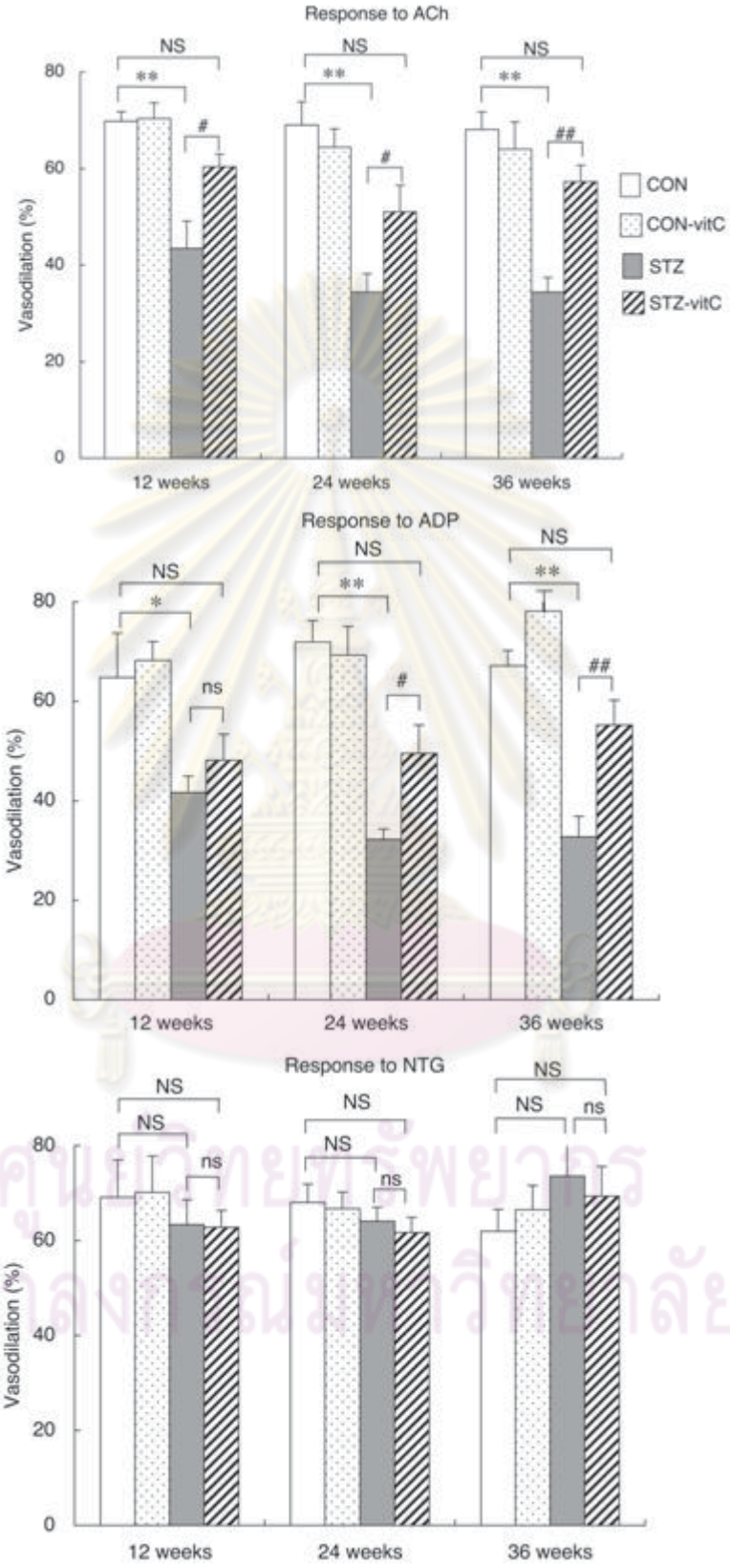

Fig. 2 Averaged percent change of vessel diameter (vasodilation) in response to ACh (10^{-7}M) **(a)**, ADP (10^{-6}M) **(b)** and NTG (10^{-6}M) **(c)** for all arterioles measured on different times (12, 24 and 36 weeks) after vitamin C administration, in control and diabetic rats (CON, CON-vit C, STZ, STZ-vit C). Values are expressed as mean±SEM in the percent change from the baseline. **P<0.01, compared with CON-rat; #P< 0.05, compared with STZ-rat; ##P< 0.01, compared with STZ-rat; *P< 0.05, compared with CON-rat; NSnot significantly different, compared with the control.

Discussion

We have examined the responses of rat cerebral arterioles to three kinds of vasoactive substances (ACh, ADP and NTG). ACh and ADP are endothelium-dedpendent vasodilators with different receptors, while NTG is an endothelium-independent vasodilator. The vasodilatory responses to ACh and ADP depend on the function of EC, but the response to NTG is promoted directly through the activation of VSMC. When each vasoactive substance was superfused inside the cranial space, cerebral arterioles dilated, reaching a steady state within 5 minutes (**Fig. 1**). The arteriolar vasodilatory response to each substance, measured by changes in diameter from the baseline, showed around a 60-70 % increase over control level. In STZ-induced diabetic rats, the responses of cerebral arterioles to both ACh and ADP were significantly decreased, compared with those in control rats. The response to NTG was not different between STZ-diabetic and control rats. The present results imply that the vasodilatory impairment could be caused by dysfunction of EC and moreover that it is not directly related to the cell receptors. The vasodilatory impairment during diabetes was improved after long-term vitamin C oral administration (**Fig. 2a and 2b**).

A number of factors may be involved in the dysfunction of endothelial cells during diabetes, including dysfunction of cell receptors or decrease in NO release/synthesis. A possible mechanism responsible for the EC dysfunction may be the decrease in the endothelium-derived NO, perhaps through the accumulation of oxygen-derived free radical [18, 19]. In fact, the generation of oxygen-derived free radical (oxidative stress) may play an important role in the pathology of diabetic vascular complication [20]. There are experimental and clinical data to indicate that increase in oxidative stress is responsible for endothelial dysfunction in diabetes mellitus [21-23].

We [3, 10, 24, 25] used STZ (55 mg/kg body weight) to experimentally induce diabetes in rats. STZ causes oxidative stress in addition to causing diabetes [26]. When STZ is transported into the islet b-cell, it may damage the cells in the pancreas. In rats, both hyperglycemia and hyperlipidemia are induced as shown in **Table 1.** This may lead to an excess of oxygen-derived free radicals in diabetes mellitus [27]. It is thought that high elevation of total cholesterol increases LDL-cholesterol, which increases the oxidation of LDL in diabetes, compared to oxygen-derived free radicals [28]. In the present experiment, we administered vitamin C in rats 24 hours after STZ injection. Some cases showed a spontaneous recovery to normal levels of blood glucose and body weight. Since the half life of STZ is very short and less than 15 min, the STZ-induced oxidative stress could not have affected the present vitamin effect [10, 29]. However, STZ may cause unwanted toxicities in the animal model. In the near future, we will develop an alternative approach by using genetically induced diabetic animal models.

In human diabetes, vitamin C has been reported to have a rapid effect on cells [30]. In the present strain of diabetic rats that we used, vitamin C had long-term effects on the cerebral microvascular vasodilatory impairment. In diabetes, plasma and tissue ascorbic acid are reduced in humans and animals [31, 32]. In diabetes with hyperglycemia, reactive oxygen species (ROS) may be continuously formed throughout the duration of the diabetic state. In the present experiment, vitamin C was continuously administered in an effort to decrease blood glucose in diabetic rats, which may have improved ascorbic acid transport into the endothelial cells. Therefore, a balance of the ROS-generating and the antioxidant systems may have been achieved, leading to significant recovery in diabetic rat administered vitamin C. This processes may explain the week-long delay in the vitamin C action on cerebral vasodilation in diabetic rats.

In conclusion, diabetes-induced impairment of cerebral microvascular vasodilation could be prevented in rats by long-term vitamin C administration. In diabetes, the endothelium-independent vasodilation may act via the cGMP pathway, but there may be other possible mechanisms, possibly involving nerve endings or other as yet unidentified neurotransmitters/factors, that could use other pathways. Long-term oral vitamin C administration may be of clinical relevance in improving cerebral microvascular vasodilatory impairment in diabetes, suggesting “long-term therapy”, as proposed by Ellis, et al. [33].

Acknowledgement

The authors express their thanks to Dr. Takashi Yamakawa for his valuable discussion at at the National Cardiovascular Center of Japan. The authors have no conflict of interest to declare.

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