IJMS
Vol 30, No 3, September 2005

Original Article

Ascorbic Acid Improves Vascular Permeability in Experimental-Induced Diabetic Rats

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Abstract

Background: The most devastating manifestations of diabetes mellitus are vascular complications. Although there are many factors involved in the pathogenesis of diabetic vasculopathy, many studies suggest a role for glucose–induced oxidative stress. Studies in animal models, have demonstrated that the administration of antioxidants restores normal endothelial functions. The study was designed to examine the possible beneficial effects of ascorbic acid, which have antioxidant properties, on vascular permeability in the duodenum of rats with streptozotocin-induced diabetes.

Methods: Female adult rats were divided into two control and three diabetic groups. Diabetes was induced by a single injection of streptozotocin (55 mg/kg, ip). One control and two diabetic groups received ascorbic acid in drinking water (800 mg/kg). Diabetic groups received ascorbic acid either as therapeutic for 4 weeks, starting after the induction of diabetes or as combination therapy for 8 weeks starting 3-4 weeks before the induction of diabetes. Vascular permeability was estimated by measuring the extravasations of Evans blue dye and water content of duodenal tissue.

Results: As compared to the control group, diabetic animals significantly increased both Evans blue extravasations and water content by 202%. Ascorbic acid, used as treatment or in combination therapy, similarly restored these two variables to normal level.

Conclusion: The findings of this study suggest that ascorbic acid might have a role in restoring some dysfunctions of experimental diabetes.

Iran J Med Sci 2005; 30(3): 128-133.

Keywords ● Ascorbic acid ● diabetes mellitus ● vascular permeability ● rat

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Introduction

Diabetes mellitus (DM) is the syndrome of disturbed energy homeostasis, caused by an abnormal metabolism of carbohydrates, proteins and fats. It is the most common endocrine-metabolic disorder of childhood and adolescent with important consequences on physical and emotional development.[1] The most devastating complication of DM is vascular complications. Persistent hyperglycemia leads to deformation of specific glycoproteins, which may cause pathologic changes in the microvessels of vital organs.[2]

Chronic vascular complications represent the main causes of morbidity and mortality in patients with DM, and occur in both micro and macrovasculatures.[3] DM is also associated with premature microvascular disease complications such as retinopathy and nephropathy.[4] Diabetic nephropathy develops in about 35% type I DM patients that finally leads to chronic renal failure.[5]

The pathogenesis of diabetic vasculopathy is complex and differences exist between diseases affecting microvasculatures and those affecting macrovasculatures, and are primarily caused by hyperglycemia.[6] Vascular pathology involves both functional and structural changes in the endothelial layers such as altered endothelium dependent dilatation,[7] and reduced endothelial cell proliferation.[8]

Although various factors may engage in the pathogenesis of diabetic vasculopathy, numerous studies suggest a role for glucose–induced oxidative stress.[9] Hyperglycemia may increase the generation of free radicals in many ways such as glucose autoxidation,[10] autoxidative glycosilation,[11] activation of protein kinase C,[12] and increased polyol pathway metabolism with subsequent pseudohypoxia.[13] Additionally, it has been reported that potential mechanisms that contributing in endothelial dysfunction is inactivation of nitric oxide by oxygen–derived free radicals. Studies done in animal models have also demonstrated that the administration of antioxidants restore normal endothelial activity.[14,15]

Antioxidants which have been studied in clinical DM are glutathione, ascorbic acid, vitamin E and super-oxide dismutase. Ascorbic acid is a strong reducing agent acting as an antioxidant *in vitro* and *in vivo*.[16] Ascorbic acid effectively prevents peroxidative damage of human plasma lipids by scavenging oxygen-derived free radicals, and sparing other endogenous antioxidants from consumption.[17] The plasma and tissue concentration of ascorbic acid have been reported to decrease in diabetic animals and humans.[18] Thus the present study was planned to investigate the effects of ascorbic acid supplementation on permeability abnormalities in duodenal capillaries of rats with streptozotocin-induced diabetes.

Materials and Methods

The study was approved by the Ethical Committee on animal experimentation of the Rafsanjan University of Medical Sciences, Rasanjan, Iran. Adult female albino N. Mary rats weighing 250-300 g were used for this study. Animals were divided into two normal (control) and three DM groups (n=8-10 for each group). They were housed in 12 hrs light/dark cycle and free access to food and water.

Diabetes mellitus was induced by intraperitoneal injection (ip) of 55 mg/kg of streptozotocin (STZ, Upjohn, Michigan; USA). The onset of diabetes was confirmed by measuring blood glucose three days after the injection of STZ.[19] Blood samples were collected from the tip of the tail for the measurement of insulin and glucose. Animals with blood glucose levels of more than 17 mM were considered as diabetic.

Experimental procedure

Normal rats were randomly divided into control group (CNT, n=8) that received no Vit C in their drinking water, and control group Vit C treated group (CNT+Vit C, n=8) that received ascorbic acid (160mg/kg; Sigma Co. UK) in drinking water for four weeks.[9]

DM rats were also randomly divided into three groups and treated as the following. Control DM group (DM, n=8) did not receive ascorbic acid, whereas DM combination group (DM+8Vit C, n=8) received ascorbic acid (800mg/kg) in their drinking water for 8 weeks, four weeks before and four weeks after induction of diabetes as stated previously.[19] DM rats of therapeutic group (DM+4Vit C) received the same amount of ascorbic acid in their drinking water but only four weeks after induction of DM.

During the course of the study weekly body weights were recorded and the cumulative weight changes were reported. At the end of the experiment, blood samples were collected for the measurement of glucose and insulin using glucose oxidase and RIA (Insulin IRMA kit, Beckman Coulter Company, Prague, Czech Repulic) methods, respectively.

At the end of the experiment an intravenous injection of Evans blue (EB, Sigma Co. UK) was done and after ten minutes the animal was killed, the abdominal cavity was opened to take duodenal specimens.[20] Duodenum was dissected, cleaned and half of it was used for the measurement of Evans blue, and the other half was used for obtaining its water content.

Measurement of vascular permeability

Changes in capillary permeability were analyzed using Evans blue dye extravasations and water content measurement. Albumin capillary permeability was measured using the EB extravasations in animals anesthetized by ip injection of pentobarbital (55 mg/kg; Biochemic; Germany) and were injected EB dye (20 mg/kg) in the femoral vein 10 min before they were killed [20] Half of duodenum was placed in 20 ml of acetone and sodium sulfate mixture and was shacked for 24 hrs. The concentration

of E.B. dye was measured by spectrophotometry (Spectronic 20D, Belgium) at 620 nm.[21] The other half was dried at 65°C for 96 hours and water content was determined using the following formula.[22]

$$\text{Water content} = \frac{\text{(Wet weight-Dry weight)}}{\text{Wet weight}} \times 100$$

Statistic analysis

Data, presented as Mean±SEM, were analyzed by Student's t-test or one way analysis of variance followed by Tukey's tests and $p<0.05$ was considered as statistically significant.

Results

While weight gains of normal rats of control groups were significant during four weeks experiment (+40.7±1.5 g), all rats of DM groups lost weight significantly (-52.8±0.7 g; P<0.001). Compared to those rats of normal control groups, Blood glucose of all rats of three DM groups were significantly higher and the levels of their plasma insulin were significantly lower respectively. However, blood glucose and plasma insulin levels of the three DM groups were not significantly different from each other (Table 1).

Table 1: Cumulative weight changes (CWC), blood glucose and concentrations of insulin.

Groups (n=8)	CWC (g)	Glucose (mg/%)	Insulin (μU/ml)
CNT	+40.7±1.5	171±9.3	14.2±1.4
CNT+Vit C	+37.1±2.5	165±7.5	13.1±1.9
DM	-52.8±0.7 *	477±69.9 *	2.1±0.8 *
DM+8Vit C	-48.6±1.5 *	408.2±88.2 *	2.3±0.07 *
DM+4Vit C	-42.4±0.9 *	382.1±25.6 *	2.9±0.01 *

Data are presented as mean±SEM.
+ and– signs shows increase or decrease of animals body weight at the end of study as compared to that of the beginning of the experiment. CNT= normal control group CNT+ Vit C= CNT on ascorbic acid (Vit C) diet; DM= diabetic mellitus group; DM+ 8VitC= combination diet; DM+ 4Vit C= therapeutic diet. For more detail see text.
*= the presented data are significantly different from control normal group at P<0.001.

EB extravasation and water content of duodenal tissue

Extravasations of EB from duodenum of DM group was significantly increased (202%) in diabetic rats compared to the normal controls (P<0.001). Combination therapy and treatment with ascorbic acid significantly decreased EB extravasations (P<0.001). There was no significant difference among water content and Evans blue extravasations of CNT and CNT Vit C groups (Fig 1). Whereas, in DM groups water content of duodenum was significantly higher as compared to normal control groups (P<0.001; Fig 2).

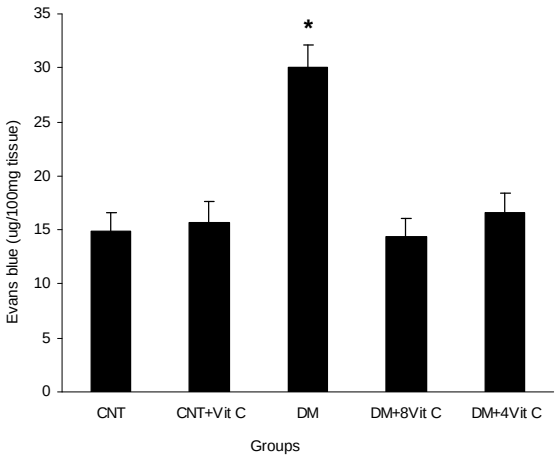

Fig.1: Extavasation of Evans blue dye (μg/100mg tissue) in different groups. CNT= normal control group; CNT+Vit C= CNT on ascorbic acid (Vit C) diet; DM= diabetic group; DM+8VitC= combination diet; DM+4Vit C= therapeutic diet.
*= Results of DM group are significantly different from other groups, P<0.001.

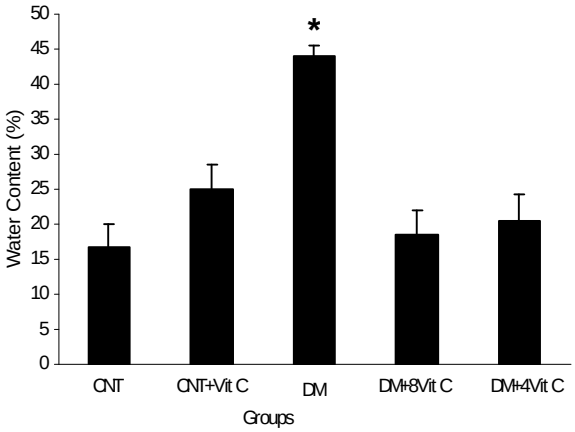

Fig.2. Water content (%) of duodenal tissues of different groups. for abbreviations see Fig 1.
*= Results of DM group are significantly different from other groups, P<0.001.

Discussion

Our results showed that the EB and fluid extravasations significantly increased in STZ-induced diabetic animals. This effect was restored by ascorbic acid supplementation either in therapeutic or combination group. So, it is legitimate to suggest that ascorbic acid can restore vascular permeability alterations in diabetic rats. Molecular and fluid movement from the vascular compartment toward the interstitium is dependent on two major physiological determinants. First, changes in the endothelial cells permeability per se through dilatation or contraction of these cells, which leads to the formation of intercellular spaces, facilitating fluid and solute movements across the capillary wall. Second, alteration in the capillary hydrostatic pressure, determined by the differ-

ence between pre and post capillary vascular resistance.[20]

The findings of the present study receive supports from a number of previous studies. The finding of Timimi and colleagues did indicate that diabetes caused nitric oxide degradation leading to abnormal vascular reactivity in humans with diabetes mellitus.[23] It was also reported that NO was quenched by advanced glycosylation end-products, which were elevated in the conditions of sustained hyperglycemia such as diabetes mellitus.[24] NO depletion has been postulated to be implicated in the increased permeability observed in diabetes.[20]

The level of body ascorbic acid depends on the interaction of dietary ascorbic acid intake and plasma concentrations of insulin and glucose. Insulin promotes the cellular uptake of ascorbic acid, whereas hyperglycemia inhibits its renal reabsorption. Therefore, in diabetes, an adequate dietary ascorbic acid intake is often associated with an unexpectedly low ascorbic acid status.[25] Diabetes also impairs the metabolism of ascorbic acid,[19] leading to the depletion of anti-oxidant defenses, which expose such patients to increased risk of cardiovascular diseases, including microangiopathy.[26,27] Ascorbic acid supplementation selectively restores the impaired endothelium-dependent vasodilation possibly by inhibition of NO degradation, which is induced by oxygen derived free radicals in diabetes.[28,29] It has been hypothesized, that glucose, under oxidative condition, reacts with proteins to form glycoproteins,[25,30] which might be involved in diabetic vasculopathy by damaging the vascular membrane proteins.[9] Therefore, it might be possible to suggest that ascorbic acid prevents protein damage by inhibiting oxidative stress, which consequently decrease vasculopathy.[30]

Other possibility is that ascorbic acid may decrease vascular permeability of DM rats and correct endothelial functions by decreasing the synthesis of cytokines such as TNF-α.[31] This may occur by inhibiting the activity of membrane neutral endopeptidase,[32] or decreasing the production of vascular endothelial growth factors.[33] The protective role of ascorbic acid has also been related to chemical properties of its molecule and increasing plasma reduced glutathione by lowering GSSG/GSH ratio.[27] An increase in plasma GSSG/GSH ratio might also contribute to the enhancement of lipid peroxidation, which might affect the integrity of plasma membrane microviscosity. The results of the present study might suggested that water leaking from tissues was not 100%, but since the control and experiment groups had been treated under a similar condition, therefore the difference could be significant. The beneficial effects of ascorbic acid seems to prevent protein and fluid leakage through vascular beds as shown in other conditions such as heat injuries,[34] hypertension,[35] and increased vascular permeability by arachidonic acid.[36]

The findings of the present study are not in agreement with a study which showed that dietary ascorbic acid supplementation, although reduced lipid peroxidation, failed to restore the impaired vascular function in small mesenteric artery of the STZ–diabetic rat.[37] The study showed that ascorbic acid was short of restoring Ach-induced endothelium-dependent relaxation in small arteries.

Serum insulin and plasma glucose in normal control group was significantly higher and lower, respectively, than those from other groups. Relative to the control group, animals in the treatment group had significantly lower body weight. This finding is consistent with the study of Palmer et al.[37] In normal rats, ascorbic acid supplementation had no effect on EB extravasations or water content in the duodenum. This might suggest that increasing plasma ascorbic acid concentration to higher-than-normal levels does not reduce the vascular permeability any further, and there is no oxidative stress in normal rats.

Conclusion

Dietary ascorbic acid supplementation seems to have some beneficial effects on capillary permeability dysfunction in experimental-induced diabetes rats. These findings may provide a new approach to examine the pathophysiology of diabetic vasculopathy and may open a new way of controlling the morbid consequence of capillary permeability in patients with diabetes mellitus.

Acknowledgement

This work was financially supported by Rafsanjan School of Medicine, Rafsanjan, Iran.

References

1 Foster DW. Diabetes mellitus in: A.S. Fauncin, Harrison's principles of internal medicine, 14th ed. Mc Graw Hill; 1998. p. 2081-20.

2 Werline S. Diabetes Mellitus in: R. Behrman, Nelson text book of pediatrics, 15th ed. W.B. Sunders; 1996. p. 1119-25.

3 Sharpe PC, Liu WH, Yue KK, et al. Glucose-induced oxidative stress in vascular contractile cells: comparison of aortic smooth muscle cells and retinal pericytes. *Diabetes* 1998; 47: 801-9.

4 Ting HH, Timimi FK, Boles KS, et al. Ascorbic acid improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest* 1996; 97: 22-8.
5 Andersen AR, Christiansen JS, Andersen JK, et al. Diabetic nephropathy in type 1 (Insulin-dependent) diabetes: an epidemiological study. *Diabetologia* 1983; 25: 496-501.
6 The diabetes control and complications trial research group: The effect of intensive treatment of diabetes on the development and progression of long-term complication of insulin–dependent diabetes mellitus. *N Engl J Med* 1993; 329: 977-86.
7 McVeigh GE, Brennan GM, Johnston GD, et al. Impaired endothelium–dependent vasodilation in patients with type 2 (non insulin–dependent) diabetes mellitus. *Diabetologia* 1992; 35: 771-6.
8 Graier WF, Grubenthal I, Dittrich P, et al. Intracellular mechanism of high D-glucose-induced modulation of vascular cell proliferation. *Eur J Pharmacol* 1995; 294: 221-9.
9 Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* 1991; 40: 405-12.
10 Hunt JV, Dean RT, Wolff SP. Hydroxyl radical production and autoxidative glycosylation. Glucose autoxidation and the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *Biochem J* 1988; 256: 205-12.
11 Wolff SP, Dean RT. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. *Biochem J* 1987; 245: 243-50.
12 Derubertis FR, Craven PA. Activation of protein kinase C in glomerular cells in diabetes. Mechanisms and potential links to the pathogenesis of diabetic glomerulopathy. *Diabetes* 1994; 43: 1-8.
13 Williamson JR, Chang K, Frangos M, et al. Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 1993; 42: 801-13.
14 Tesfamariam B, Cohen RA. Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am J Physiol* 1992; 263: H321-6.
15 Diederich D, Skopec J, Diederich A, Dai FX. Endothelial dysfunction in mesenteric resistance arteries of diabetic rats: role of free radicals. *Am J Physiol* 1994; 266: H1153-61.
16 Frei B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. *Am J Med* 1994; 97: 5S-13S.
17 Frei B, Forte TM, Ames BN, Cross CE. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. *Biochem J* 1991; 277: 133-8.
18 Som S, Basu D, Mukherjee S, et al. Ascorbic acid metabolism in diabetes mellitus. *Metabolism* 1981; 30: 572-7.
19 Amatyakul S, Chakraphan D, Chotpaibulpan S, Patumraj S. The effect of long-term supplementation of vitamine C on pulpal blood flow in streptozotocin-induced diabetic rats. *Clin Hemorheol Microcirc* 2003; 29: 313-9.
20 Chakir M, D'Orleans–Juste P, Plante GE. Neutral endopeptidase inhibition, a new approach in the exploration of diabetic vasculopathy in rats. *Eur J Pharmacol* 1995; 285: 11-8.
21 Harada M, Takeuchi M, Fukao T, Katagiri K. A simple method for the quantitative extraction of dye extravasted into skin. *J Pharm Pharmacol* 1971; 23: 218-9.
22 Lund T, Orarheim H. Mechanisms behind increased dermal inhibition pressure in acute burn edema. *Am J Physiol* 1989; 256: H940-8.
23 Timimi FK, Ting HH, Haley EA, et al. Ascorbic acid improves endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus. *J Am Coll Cardiol* 1998; 31: 552-7.
24 Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest* 1991; 87: 432-40.
25 Cunningham JJ. The glucose/insulin system and ascorbic acid: implications in insulin–dependent diabetes mellitus. *J Am Coll Nutr* 1998; 17:105-8.
26 Lindsay RM, Jamieson NS, Walker SA, et al. Tissue ascorbic acid and polyol pathway metabolism in experimental diabetes. *Diabetologia 1998;* 41: 516-23.
27 Ng LL, Ngkeekwong FC, Quinn PA, Davies JE. Uptake mechanisms for ascorbate and dehydroascoarbate in lymphoblasts from diabetic nephropathy and hypertensive patients. *Diabetologia 1998;* 41: 435-42.
28 Beckman JA, Goldfine AB, Gordon MB, et al. Oral antioxidant therapy improves endothelial function in type 1 but not type 2 diabetes mellitus. *Am J Physiol Heart Circ Physiol* 2003; 285: H2392-8.
29 Beckman JA, Goldfine AB, Gordon MB, Creager MA. Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circula*

tion 2001; 103: 1618-23.
30 Kim J, Kim KS, Shinn JW, et al. The effect of antioxidants on glycated albumin-induced cytotoxicity in bovine retinal pericytes. *Biochem Biophys Res Commun* 2002; 292: 1010-6.
31 Antoniades C, Tousoulis D, Tountas C, et al. Vascular endothelium and inflammatory process, in patients with combined type 2 diabetes mellitus and coronary atherosclerosis: the eefects of ascorbic acid. *Diabet Med* 2004; 21: 552-8.
32 Muangman P, Spenny ML, Tamura RN, Gibran NS. Fatty acids and glucose increase neutral endopeptidase activity in human microvascular endothelial cells. *Shock* 2003; 19: 508-12.
33 Obrosova IG, Minchenko AG, Marinescu V, et al. Antioxidants attenuate early up regulation of retinal vascular endothelial growth factor in streptozotocin-diabetic rats. *Diabetologia* 2001; 44: 1102-10.
34 Farstad M, Heltne JK, Rynning SE, et al. Can the use of methylprednisolone, ascorbic acid, or alpha-trinositol prevent cold-induced fluid extravasation during cardiopulmonary bypass in piglets? *J Thorac Cardiovasc Surg* 2004; 127: 525-34.
35 Rodriguez-Porcel M, Herrman J, Chade AR, et al. Long-term antioxidant intervetion improves myocardial microvascular function in experimental hypertension. *Hypertension* 2004; 43: 493-8.
36 Alvarez-Guerra M, Hannaert P, Hider H, et al. Vascular permeabilization by intravenous arachidonate in the rat peritoneal cavity: antagonism by antioxidants. *Eur J Pharmacol* 2003; 446: 199-205.
37 Palmer AM, Thomas CR, Gopaul N, et al. Dietary antioxidant supplementation reduces lipid peroxidation but impairs vascular function in small mesentric arteries of the streptozotocin-diabetic rat. *Diabetologia* 1998; 41: 148-56.