Effect of ascorbic acid on microcirculation in patients with Type II diabetes: a randomized placebo-controlled cross-over study

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ABSTRACT

Manifestations of vascular disease, including microvascular changes, constitute the major part of the morbidity and mortality in diabetic patients. Oxidative stress has been suggested to play an important role in the vascular dysfunction of diabetic patients. Furthermore, epidemiological observations indicate a beneficial effect of an increased dietary intake of antioxidants. The present study tested the hypothesis that the antioxidant ascorbic acid influences microcirculatory function in patients with Type II diabetes. Patients with Type II diabetes were treated with 1 g of ascorbic acid three times a day for 2 weeks in a randomized placebo-controlled double-blind cross-over design. Microvascular reactivity was assessed by vital capillaroscopy and PRH (post-occlusive reactive hyperaemia). hs-CRP (high-sensitivity C-reactive protein), IL-6 (interleukin-6), IL-1ra (interleukin-1 receptor antagonist) and ox-LDL (oxidized low-density lipoprotein) were analysed. The results showed no significant change in microvascular reactivity assessed after 2 weeks of ascorbic acid treatment. TtP (time to peak) was 12.0 ± 3.3 s before and 11.2 ± 3.5 s after ascorbic acid (n = 17). In comparison, TtP was 11.5 ± 2.9 s before and 10.6 ± 2.8 s after placebo (not significant). IL-1ra, IL-6, hs-CRP and ox-LDL did not change significantly after ascorbic acid, neither as absolute or relative values. In conclusion, in contrast with some studies reported previously, we could not demonstrate an effect of continuous oral treatment with ascorbic acid on microvascular reactivity assessed at the level of individual capillaries. Furthermore, we found no indication of an effect on inflammatory cytokines or ox-LDL.

INTRODUCTION

Classical risk factors for atherosclerosis such as hyperlipidaemia, diabetes, hypertension and smoking have all demonstrated an association with disturbed macrovascular endothelial response. Increased oxidative stress may be a common mechanism of all these risk factors [1] and add to the initiation and progress of atherosclerosis. Evaluation of vascular function may provide a means to assess the progress of atherosclerotic disease non-invasively and at an early stage [2]. Diabetes is a chronic disease characterized by micro- and macro-vascular dysfunction [2] as well as premature and accelerated atherosclerosis [3]. Feared

Key words: ascorbic acid, microcirculation, oxidative stress, Type II diabetes, vascular disease.
Abbreviations: ACE, angiotensin-converting enzyme; AT1, angiotensin type 1 receptor; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; IL-1ra, interleukin-1 receptor antagonist; IL-6, interleukin-6; ox-LDL, oxidized low-density lipoprotein; PRH, post-occlusive reactive hyperaemia; TtP, time to peak.
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manifestations are ischaemic heart disease, nephropathy, retinopathy, stroke and peripheral arterial insufficiency, but signs of endothelial dysfunction and increased inflammatory markers have been demonstrated at a much earlier stage [4,5]. Levels of inflammatory markers have been shown to predict cardiovascular events in diabetic, as well as in non-diabetic, patients [6]. Effective preventive measures towards vascular deterioration should be of great benefit in terms of morbidity, mortality and quality of life.

Antioxidants may be expected to counteract endothelial dysfunction and microcirculatory flow if oxidative processes are involved in atherogenic progression [7]. The antioxidant ascorbic acid has been demonstrated to be independently associated with prevalence of coronary heart disease and stroke, i.e. a positive correlation between an increase in serum ascorbic acid level and a reduction in coronary heart disease and stroke prevalence [8]. Furthermore, acute administration of high doses of ascorbic acid has been shown to reduce negative effects of oxidative stress, such as smoking, on endothelial function and microvascular flow [9,10].

An involvement of oxidative stress in the disturbed endothelial function in patients with diabetes is indicated [2,11,12]. Thus a potential beneficial effect of treatment with antioxidants would be anticipated [13]. Furthermore, antioxidants might be expected to reduce the levels of inflammatory markers, [14,15].

In the present study, we tested the hypothesis that daily administration of ascorbic acid to diabetic patients improves the microvascular reactivity, levels of inflammatory markers and levels of ox-LDL (oxidized low-density lipoprotein). To the best of our knowledge, the effects of ascorbic acid treatment on the microcirculation at the level of individual capillaries have not been reported previously.

**METHODS**

**Patients**

Patients with Type II diabetes were treated with 1 g of ascorbic acid three times a day during a period of 2 weeks in order to reduce the influence of oxidative stress through a strengthened antioxidant defence. Placebo was used in a randomized cross-over design, thus allowing the patients to serve as their own matched controls. The purpose of the present study was to gain insight as to whether this antioxidant improves microcirculatory flow in individual microvessels and if it increases their functional reactivity as assessed by vital capillaroscopy after PRH (post-occlusive reactive hyperaemia).

The study was approved by the Ethics Committee at Huddinge University Hospital, Stockholm, Sweden, and was performed in accordance with institutional guidelines and in accordance with the Declaration of Helsinki (2000). All patients gave their informed consent.

<table>
<thead>
<tr>
<th>Study patients</th>
<th>Men (n)</th>
<th>Women (n)</th>
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<tbody>
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<tr>
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<tr>
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<td>1</td>
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<tr>
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<tr>
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<td>136</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
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<td>81</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Gemfibrozil</td>
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<td>1</td>
</tr>
<tr>
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</tr>
<tr>
<td>AT1 antagonist</td>
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<td>0</td>
</tr>
<tr>
<td>Management of diabetes</td>
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</tr>
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</tr>
<tr>
<td>Glibenklamide</td>
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<td>1</td>
</tr>
<tr>
<td>Metformin</td>
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<tr>
<td>Other oral antidiabetic drugs</td>
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<td>3</td>
</tr>
<tr>
<td>Insulin</td>
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<td>2</td>
</tr>
<tr>
<td>Duration of diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>⩾ 10 years</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>&lt; 10 years</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

**Study subjects and design**

Patients (n =20) with Type II diabetes mellitus were recruited from the Diabetes Day Care ward at the Karolinska University Hospital, Huddinge, Sweden. The duration of diabetes since diagnosis ranged from 1–28 years (median, 7 years; Table 1). Patients were recruited 4 weeks after discharge in order to have the patients as compliant to recommended habits as possible and with fairly stable glucose levels. They were instructed not to change the amount of fruit or juices consumed during the study period and to document their consumption. They were interviewed regarding any changes in lifestyle or medication as well as abnormal events such as disease or infection at each examination. None of the patients used additional oral vitamins, either prior to or during the study period. The pharmacological treatment of glucose regulation was heterogeneous (Table 1). Patients with β-blockers or calcium antagonists were not included, but as ACE (angiotensin-converting enzyme) inhibitors and AT1 (angiotensin type 1 receptor) antagonists are commonly included in routine treatment.
Vascular effects of ascorbic acid in diabetes

Figure 1  Randomized cross-over design of the study protocol

Patients with Type II diabetes received either 1 g of ascorbic acid three times a day or placebo, and then crossed over to the alternate treatment after a 4 week wash-out period.

in diabetes these drugs were accepted (Table 1). No vasoactive medication was used prior to examination. Patient age varied from 35–76 years (median, 54 years). Three patients discontinued participation in the study after the first treatment period (one due to unrelated illness, one due to too low visibility of microvessels and one because of a lack of time for further participation).

Half of the patients were randomized to start with 1 g of ascorbic acid three times a day (Friggs C-vitamin brustablett®; Semper Foods, Stockholm, Sweden) during a 14 day period and the other half to start with placebo (Semper Foods). All patients were examined by capillaroscopy and blood samples were collected before and after the intervention with ascorbic acid or placebo. After a 4 week wash-out period, the groups were crossed over and the protocol repeated. The patients and all members of the research team were blinded until all examinations and evaluations were finished (Figure 1).

Laboratory assessments

Blood samples were collected in connection with microcirculatory measurements on each occasion. Haemoglobin, erythrocyte volume fraction, leucocyte particle count, fibrinogen and total CO₂ were assessed, and HbA1c (glycated haemoglobin) was used as an indicator of stable metabolic control with regard to plasma glucose. Plasma glucose levels were measured at each point of microcirculatory assessment to ensure no extreme deviations at the time of examination. Lipid levels [cholesterol, HDL (high-density lipoprotein)-cholesterol and triacylglycerol (triglyceride)] were assessed by standard enzymatic assays (Boehringer Mannheim GmbH, Mannheim, Germany). hs-CRP (high-sensitivity C-reactive protein) was determined by standard procedures, and IL-1ra (interleukin-1 receptor antagonist) and IL-6 (interleukin-6) were determined by immunoassay (Quintikine HS IL-6 Immunoassay; R&D Systems, Minneapolis, MN, U.S.A.). Ox-LDL was determined using a commercial ELISA utilizing the murine monoclonal antibody mAB.4E6 (Mercodia AB, Uppsala, Sweden). Ascorbic acid levels in plasma were determined after precipitation with metaphosphoric acid as described by Kallner et al. [16].

Microcirculatory assessments

Reactivity of microvessels was studied by vital capillaroscopy. All sessions were video-recorded and evaluated further using the Capiflow® system (IM-Capiflow, Stockholm, Sweden). With this technique, blood flow velocity can be assessed continuously by a computerized dual-window cross-correlation technique which allows a continuous analysis of the velocity in a specific capillary during the registration [17].

The response to provocation was studied using PRH. TtP (time to peak) was used as the functional response variable [17]. It is possible to measure other microcirculatory variables with this method, but these constitute methodological problems in longitudinal studies [17–19].

A miniature cuff was applied to the base of the investigated finger to allow arterial occlusions. Instant release of cuff pressure results in temporary hyperaemia and TtP is thus measured as the time from the release of the occlusion to the maximal flow velocity during reactive hyperaemia. TtP was assessed after a 1 min arterial occlusion with a cuff pressure of 200 mmHg [17]. Analysis of the video photometric capillaroscopic recordings was performed using the Capiflow® system (IM-Capiflow).

Suitable capillaries with good contrast and visible signals were used at each session. Three PRHs were performed using the same capillary in each patient on all four occasions. The coefficient of variation between repeated measurements in a single capillary during a single session has been assessed to be 6 %, and the coefficient of variation between different days was <13 % when the mean of at least two TtP assessments on each occasion was used [19].

Care was taken to perform the examinations at the same temperature (ambient and digit skin temperature) and after at least 20 min of rest. The skin temperature was measured continuously with an electronic thermistor (Physitemps Instruments, Lindy, NJ, U.S.A.). The examinations were performed with the patients seated and with the arm and hand supported at heart level. Smoking, coffee, tea or a heavy meal were not allowed 2 h prior to examination. Blood pressure and heart rate were recorded on each occasion.

Statistics

The power of the study exceeded 90 % (β = 0.1, α = 0.05) based on the effects of ascorbic acid on microcirculation in an open pilot study prior to the present study.
Biochemical parameters from the 17 patients that completed the 2 week treatment with ascorbic acid and placebo

Values are means ± S.E.M. P value determined by Friedman's ANOVA. ns, not significant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Placebo</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>HbA_{1c} (%)</td>
<td>6.7 ± 0.4</td>
<td>6.6 ± 0.4</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.1 ± 0.2</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/l)</td>
<td>2.5 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>9.8 ± 1.0</td>
<td>8.4 ± 0.7</td>
</tr>
<tr>
<td>Plasma ascorbic acid (µmol/l)</td>
<td>19.9 ± 3.3</td>
<td>23.9 ± 3.2</td>
</tr>
<tr>
<td>IL-1ra (ng/l)</td>
<td>395 ± 61</td>
<td>369 ± 46</td>
</tr>
<tr>
<td>IL-6 (ng/l)</td>
<td>3.7 ± 0.6</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>2.9 ± 0.7</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>ox-LDL (units/l)</td>
<td>57 ± 4.1</td>
<td>52 ± 4.8</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the present study, we were not able to demonstrate any effect of a relatively high oral dose of ascorbic acid on the disturbed microcirculatory reactivity of patients with Type II diabetes. This plasma-soluble antioxidant might replenish endogenous antioxidant pools in patients with diabetes and thereby add to the defence against oxidative stress. This may be of particular interest in diabetic patients with their high propensity to develop micro- and macro-circulatory disease. Acute administration of ascorbic acid has been shown previously [9–11,21–23] to have beneficial effects on flow in conduit vessels and regional flow in disease states with disturbed endothelial function. However, the present randomized double-blind cross-over study using continuous oral treatment failed to demonstrate an increased responsiveness assessed at the level of individual capillaries in diabetic patients following a 2 week treatment period with ascorbic acid.

The statistical analysis showed a 99% probability that the effects of ascorbic acid and placebo were identical when a two-factor ANOVA was used. As always, this does not exclude an effect of ascorbic acid, but such an effect must have been very small and possibly clinically insignificant.

Why did this study not verify beneficial effects of ascorbic acid on vascular reactivity reported previously? Do the disparate results reflect differences in ascorbic acid administration or dosage? Studies demonstrating a positive effect of ascorbic acid on vascular reactivity have most commonly used acute arterial or intravenous administration [11,21–23], i.e. pharmacological doses, whereas oral supplements have failed to show an effect in several studies [24,25]. However, some recent studies do indicate...
a positive impact of oral ascorbic acid in diabetic patients [26–29].

The value of the very high supraphysiological doses of ascorbic acid used in some acute studies has been debated. Saturation of cells occurs and a counteractive decrease in absorption and increase in renal excretion starts at approx. 60–70 µmol/l, thereby preventing higher plasma levels [30]. Different mechanisms of action of the same antioxidant may be active depending on the actual plasma concentrations. Also, different mechanisms in the endothelium might be activated both in response to dose as well as to chronic or acute drug administration [31–33].

For example, nitric oxide is an important factor for endothelial function, which in turn has complex and varying actions, including antioxidative actions, in different biological processes in the vascular wall [12,31,34]. The possibility of a specific receptor-mediated abnormality in endothelial function in patients with Type II diabetes has been suggested [33]. In addition, a too high plasma level may actually be pro-oxidative. The required dose to obtain an optimal plasma level counterbalancing an increased oxidative stress is unknown. It is also possible that higher doses of ascorbic acid are required in certain populations, such as diabetic patients, as opposed to healthy people [13,32,35,36]. Intriguingly, epidemiological studies have suggested an inverse correlation between ascorbic acid and cardiovascular mortality [37–39].

The possibility must also be considered that the duration of treatment was too short in the present study to restore microvascular reactivity in diabetic patients. Some mechanisms of action of ascorbic acid may be fast, occurring within hours after intravascular and oral administration, as indicated by several previous studies [11,21,22,28,40,41], and the treatment period in the present study was chosen on the basis of this. Ascorbic acid is a water-soluble substance, allowing for fast interactions between circulating blood constituents and the endothelium, as is raxofelast, another water-soluble antioxidant which was shown to improve endothelial function after only 1 week of oral treatment in men with Type II diabetes [42]. Other possible effects of more long-term treatment cannot be excluded and may even be caused by different mechanisms than those of acute effects. The situation using other antioxidants, such as vitamin E, probucol or coenzyme Q, may be quite different, as these lipid-soluble substances may act and be sequestered in completely different biological compartments of importance for endothelial function.

The apparent lack of effect of ascorbic acid may reflect the multifactorial origin of the disturbed microcirculation in diabetic patients. This finding is disappointing, since diabetics are a group of patients where it is of utmost interest to improve the microcirculation. Compared with the general diabetic population, the present study group may be considered well-controlled metabolically and with a relatively low burden of risk factors (Tables 1 and 2). However, the group demonstrates an abnormal microvascular response and also a variety of factors predisposing to vascular disease. The disease has been present for many years in these patients, as diagnosis is likely to have been preceded by impaired glucose tolerance for a considerable period of time and vascular disturbances are already known to be prevalent at the time of diagnosis of Type II diabetes [43]. CRP and IL-6 have even been implicated to predict risk of developing Type II diabetes mellitus [44,45]. Furthermore, the intervention was added on top of a heterogeneous pharmacological treatment, with several drugs potentially affecting endothelial function and vasoreactivity alone or in synergy [34,46].

Despite the strong evidence for the involvement of oxidative processes in the atherosclerotic, endothelial and microvascular disease processes [31], intervention studies with antioxidants in populations usually with advanced disease states (manifest atherosclerosis) have largely been disappointing [47,48]. A hypothesis put forward to explain this discrepancy is that oxidative mechanisms might be more important or more modifiable at an earlier stage, which is supported by epidemiological studies. In diabetic patients, signs of vascular disease are often found at the time of diagnosis, indicating the presence of an insidious long-standing disease process. Thus the beneficial effect of antioxidants in such a high-risk group, with as yet not very advanced vascular disease, should be of great interest. However, in the present study, the time point of intervention may not have been early enough [4]. Studies of endothelial function with consumption of varying antioxidants in diabetes patients have shown disparate results [25,32,46], but positive ones do indicate possible in vivo effects [26,28,29,42,49].

It is not known whether endothelial dysfunction is a general phenomenon in all kinds of vessels or if there are differences between micro- and macro-vessels or in different organs. Microcirculatory disturbances in diabetic patients are known to start and to occur more frequently in the toes than in the fingers, and TtP has been shown previously to have a longer duration in toes than fingers in diabetic subjects [50]. Capillaroscopy in the present study was performed on fingers, whereas it is possible that changes in reactivity might have been more readily detected in the toes. This fact might have decreased the sensitivity of the present study.

The patients in the present study served as their own matched controls due to the cross-over design. However, the question may be asked whether these patients really had signs of pathological microvascular reactivity? In our laboratory, a group of healthy volunteers aged 21–27 years had a mean PRH of 6.3 s [19] and another group of 70 year olds (n = 25) had a mean PRH of 8.3 ± 2.8 s (Q. Lu, L. Lind, P. Henriksson and A Freyschuss, unpublished work). In contrast, patients with familiar hyperlipidaemia had a PRH of approx. 12.9 s [51]. In
comparison, our patients had significantly impaired microvascular reactivity at baseline.

Conceivable links between atherosclerosis, endothelial dysfunction and risk factors of cardiovascular disease, such as diabetes, are increased oxidative stress and inflammation [52]. IL-1α, IL-6 and hs-CRP have all been shown to co-vary with cardiovascular disease [53,54]. The assessment of ox-LDL was an attempt to indicate the level of oxidative stress in the circulation. None of these variables was affected by treatment with ascorbic acid in our present study, thereby possibly precluding a major effect of these. The effect of ascorbic acid on TtP was almost exactly the same as after placebo when a more advanced ANOVA was performed including effects of treatment, period and interaction between treatment and period. This should point to an extremely small, if any, effect of ascorbic acid on TtP.

In conclusion, we found no support in this randomized placebo-controlled cross-over study for an effect of a relatively high dose of ascorbic acid on microvascular reactivity, assessed at the level of individual capillaries, in patients with Type II diabetes after a 2 week treatment. Furthermore, no effect of ascorbic acid on the levels of inflammatory cytokines or ox-LDL was found in this high-risk patient population.

ACKNOWLEDGMENTS

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