Vitamin C Affects Thrombosis/Fibrinolysis System and Reactive Hyperemia in Patients With Type 2 Diabetes and Coronary Artery Disease

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OBJECTIVE — To examine the effect of vitamin C on forearm vasodilatory response to reactive hyperemia and on plasma level of plasminogen activator inhibitor 1 (PAI-1), von Willebrand factor (vWF), tissue plasminogen activator (tPA), antithrombin III (ATIII), proteins C and S, and factors V (fV) and VII (fVII) in patients with both type 2 diabetes and CAD.

RESEARCH DESIGN AND METHODS — A total of 39 patients with type 2 diabetes and CAD were divided into two groups and received vitamin C (2 g/day) or no antioxidant for 4 weeks. Forearm blood flow was determined using venous occlusion gauge-strain plethysmography at baseline and after treatment. Forearm vasodilatory response to reactive hyperemia (RH%) or nitrate (NTG%) was defined as the percent change of flow from baseline to the maximum flow during reactive hyperemia or after administration of nitrate, respectively. Biochemical markers were determined by enzyme-linked immunosorbent assay (ELISA) or other standard methods.

RESULTS — RH% was significantly increased after treatment with vitamin C (from 62.4 ± 7.2 to 83.1 ± 9.3%, $P = 0.024$) but remained unaffected in the control group. Vitamin C decreased plasma levels of fV (from 143 ± 5.4 to 123 ± 6.03%, $P = 0.038$), vWF (from 133.5 ± 14.5 to 109.5 ± 11.4%, $P = 0.016$), and tPA (from 12.3 ± 0.99 to 8.40 ± 0.60 ng/ml, $P = 0.001$), whereas these levels remained unaffected in the control group. The changes in RH%, vWF, and tPA were significantly greater ($P = 0.028$, 0.036, and 0.007, respectively) in the vitamin C–treated group than in the control group. Levels of ATIII, proteins S and C, fVII, and PAI-1 remained unchanged in all groups.

CONCLUSIONS — Short-term treatment with high doses of vitamin C improved RH% and decreased plasma levels of tPA and vWF in patients with type 2 diabetes and CAD.

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In the last decade, our knowledge of diabetes has evolved and new disorders such as oxidative stress (1), endothelial dysfunction (1,2), and impaired fibrinolytic activity (3) have been suggested to explain, at least in part, the pathophysiology of the observed coronary atherothrombosis. Each of these abnormalities, which play important roles in the development and progression of cardiovascular disease, also provides new targets for treatment.

It has been shown recently that patients with diabetes have lower serum levels of vitamin C than nondiabetic subjects, and this is believed to be an important factor contributing to the increase of oxidative stress status and endothelial dysfunction in diabetes (4). The underlying mechanisms of vitamin C deficiency in diabetes are unclear. The reduced renal reabsorption of vitamin C induced by hyperglycemia, the competition between glucose and vitamin C for the uptake into certain cells and tissues, and a possible secondary depletion due to increased oxidative stress have been proposed (4). However, the role of vitamin C treatment in patients with type 2 diabetes with or without advanced atherosclerosis is controversial (2). Antioxidant treatment depresses the expression of proinflammatory cytokines in vitro (5) and improves endothelial function in patients with coronary artery disease (CAD) (6), but its effect on endothelial function and thrombosis/fibrinolysis system in patients with type 2 diabetes and underlying coronary atherosclerosis is unknown.

The purpose of this study was to investigate the impact of short-term oral administration of vitamin C on forearm vasodilatory response to reactive hyperemia (RH%) and on plasma levels of plasminogen activator inhibitor 1 (PAI-1), von Willebrand factor (vWF), tissue plasminogen activator (tPA), antithrombin III (ATIII), proteins C and S, and factors V (fV) and VII (fVII) in patients with both type 2 diabetes and CAD.

RESEARCH DESIGN AND METHODS

Patients

A total of 39 patients with type 2 diabetes were recruited in this study. All subjects were randomly selected from the registry of the Cardiology Department in Hip-

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Abbreviations: ATIII, antithrombin III; CAD, coronary artery disease; ELISA, enzyme-linked immunosorbent assay; fV, factor V; fVII, factor VII; NTG%, forearm vasodilatory response to nitrate; PAI-1, plasminogen activator inhibitor 1; RH%, forearm vasodilatory response to reactive hyperemia; tPA, tissue plasminogen activator; vWF, von Willebrand factor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

pokration Hospital, and all had been patients admitted to our clinic from 1 January to 31 December 2001. The protocol was performed in the Cardiology Department of Hippokration Hospital in Athens University Medical School during the first 6 months of the year 2002. Type 2 diabetes was defined in accordance with the National Diabetes Data Group criteria (7). All subjects had angiographically documented CAD, with at least one coronary stenosis >50%. All patients were treated with sulfonylurea plus diguanides, had duration of diabetes of 11.3 ± 1.4 years, were normotensive (blood pressure <140/90 mmHg), and had HbA_{1c} <7.0% at the beginning of the protocol. Exclusion criteria were tobacco use within the past 5 years, use of antioxidant vitamin supplements or hormone replacement therapy during the past year, and laboratory evidence of hepatic or hematologic abnormalities. Subjects were required to have had no incidents of coronary unstable syndrome for ≥3 months before they were recruited. These patients were randomly divided into two groups and received 2 g/day vitamin C 2 ($n = 19$) or no antioxidant treatment ($n = 20$) for 4 weeks. Baseline characteristics of the participants are shown in Table 1.

Table 1—*Baseline characteristics*

	Vitamin C treatment group	Control group	*P* value
n (men/women)	19 (17/2)	20 (17/3)	
Age (years)	63.3 ± 2.7	67.4 ± 2.1	0.444
Duration of diabetes (years)	11.4 ± 1.1	11.5 ± 1.8	0.870
BMI (kg/m^2)	27.6 ± 0.61	26.5 ± 0.53	0.214
Baseline flow 1 (ml · 100 ml^{-1} · min^{-1})	4.28 ± 0.36	4.29 ± 0.32	0.857
Maximum hyperemia flow (ml · 100 ml^{-1} · min^{-1})	6.8 ± 0.44	6.04 ± 0.42	0.270
RH% (%)	51.8 ± 4.7	43.8 ± 4.7	0.158
Baseline flow 2 (ml · 100 ml^{-1} · min^{-1})	3.94 ± 0.23	4.08 ± 0.31	0.938
Maximum flow after nitrate (ml · 100 ml^{-1} · min^{-1})	6.56 ± 0.33	6.42 ± 0.44	0.406
NTG% (%)	66.3 ± 6.75	60.8 ± 6.9	0.563
Vitamin C (μmol/l)	39.9 ± 2.0	39.5 ± 2.1	0.667
AT III (%)	82.3 ± 3.2	84.6 ± 3.3	0.422
protein C (%)	103.7 ± 5.1	87.9 ± 8.8	0.695
protein S (%)	96.2 ± 4.6	89.8 ± 6.0	0.268
fV (%)	144.6 ± 5.7	127.7 ± 10.3	0.822
fVII (%)	100.3 ± 8.8	71.9 ± 9.0	0.202
Von Willebrand factor (%)	133.5 ± 14.5	141.0 ± 27	0.888
PAI-1 (IU/l)	3.06 ± 0.36	2.18 ± 0.40	0.202
tPA (ng/ml)	12.3 ± 0.99	12.2 ± 1.3	0.621
Total cholesterol (mg/dl)	217 ± 12	215 ± 9	0.749
HDL (mg/dl)	37.3 ± 2.0	36.1 ± 2.2	0.496
Fasting glucose (mg/dl)	131 ± 17	158 ± 14	0.379
Triglycerides (mg/dl)	145 ± 10	141 ± 13	0.550

Data are means ± SE. There were no significant differences between the two groups.

All participants underwent a complete history (including dietary habits) and physical examination, including electrocardiography, and 12-h fasting blood samples were collected. In the present study, all participants were Greek, were inhabitants of Athens, and followed a more western European diet than a typical Greek-Mediterranean diet. Gauge-strain plethysmography was performed at the beginning of the study and after 4 weeks of treatment. Patients were asked to discontinue any vasoactive drugs for 2 days before each measurement. At the second visit, subjects were asked to receive the last dosage of intervention compound 12 h before the beginning of the protocol. The protocol was approved by the institutional ethics committee, and informed consent was given by each subject.

Forearm blood flow measurements

All measurements were performed between 1000 and 1300. The participants were recommended to refrain from eating or drinking any vasoactive agent such as coffee or alcohol for at least 12 h before the study. Subjects rested in a supine position, in a dark quiet room under constant temperature (22–25°C) for 30 min before the study. Forearm blood flow was measured using gauge-strain plethysmography (EC-400; D.E. Hokanson, Bellevue, WA), as previously described (8,9). The forearm blood flow output signal was transmitted to a personal computer (Hokanson NIVP3 software; D.E. Hokanson). Forearm blood flow was finally calculated as the percent change of arm volume per 100 ml tissue per minute. RH%, induced by 4-min ischemic occlusion of the distal forearm, was defined as the percent change of flow from baseline to the maximum flow during reactive hyperemia. Forearm vasodilatory response to nitrate (NTG%) was defined as the percent change of flow from baseline to the maximum flow after sublingual administration of 0.8 mg nitroglycerine, as previously described (8,9). RH% was considered an index of endothelium-dependent dilation, whereas NTG% was considered an index of endothelium-independent dilation, as previously established (8,9).

Biochemical measurements

Venous blood samples were collected before plethysmography was performed. After centrifugation at 3,500 rpm at 4°C for 15 min, plasma or serum was collected and stored at −80°C until assayed. Routine chemical methods were used to determine serum concentrations of total cholesterol, HDL, triglycerides, and glucose. Serum level of vitamin C was measured using high-performance liquid chromatography (ImmunDiagnostik, Bensheim, Germany). Plasma levels of thrombosis/fibrinolysis system were determined as follows: vWF (BC von Willebrand Reagent; Dade Behring, Marburg, Germany), PAI-1 activity (Berichrom PAI; Dade Behring), tPA antigen (ELISA kit for tPA, Asserachrom; Diagnostica Stago, Asniéres, France), ATIII activity (Chromogenic determination of ATIII autoanalyzer method, Berichrom ATIII [A]; Dade Behring), fV (coagulation factor V activity; Dade Behring), fVII (coagulometric method for determination of factors II, VII, and X activities; Dade Behring), protein C (reagents for the determination of

protein C activity; Dade Behring), and protein S (clotting assay of protein S by STA analyzers, Staclot Protein S kit; Diagnostica Stago).

Statistical analysis

The statistical analysis was performed using a personal computer and SPSS 9.0 statistical software package (SPSS, Chicago, IL). All values were expressed as mean ± SE. Mann-Whitney *U* test was used to evaluate the differences in variables between the two groups at baseline and to compare the change of each variable between the two groups after treatment. Changes in the examined parameters by the treatment were assessed by ANOVA for repeated measurements. Statistically significant differences were determined as those with $P < 0.05$.

RESULTS

Effects of vitamin C on forearm blood flow

At baseline, there were no significant differences in dietary habits or any of the examined parameters between the vitamin C treatment group and the control group (Table 1). Serum vitamin C levels were significantly increased in the vitamin C treatment group (from 39.9 ± 2.0 to 79.9 ± 3.6 μmol/l, $P = 0.001$) but remained unchanged in the control group (from 39.5 ± 2.1 to 40.0 ± 2.2 μmol/l, $P = 0.755$).

RH% was significantly improved in the vitamin C treatment group (from 51.8 ± 4.72 to 72.5 ± 6.57%, $P = 0.024$) but remained unaffected in the control group (from 43.75 ± 4.65 to 41.8 ± 4.5%, $P = 0.444$). This change in RH% in the vitamin C treatment group (20.7% [95% CI 38.4–2.98], $P = 0.024$) was significantly different ($P = 0.028$) compared with the change in the control group (−1.95% [3.27 to –7.17], $P = 0.444$).

Baseline forearm blood flow, maximum flow during reactive hyperemia, maximum flow after nitrate administration, as well as NTG% remained unaffected in both the vitamin C treatment group (4.28 ± 0.36 to 3.9 ± 0.18, $P = 0.420$; 6.8 ± 0.43 to 6.88 ± 0.30, $P = 0.895$; and 6.56 ± 0.33 to 6.42 ± 0.28 ml · 100 ml tissue^{-1} · min^{-1}, $P = 0.626$; as well as 66.3 ± 6.8 to 59.2 ± 5.44%, $P = 0.393$; respectively) and the control group (4.29 ± 0.32 to 4.4 ± 0.31, $P = 0.586$; 6.04 ± 0.42 to 6.33 ± 0.50, $P = 0.266$; 6.5 ± 0.47 to 6.73 ± 0.56 ml · 100 ml tissue^{-1} · min^{-1}, $P = 0.382$; as well as 58.6 ± 6.9 to 61.6 ± 7.3%, $P = 0.579$; respectively).

RH% was similarly increased in those with baseline vitamin C concentrations ≤41 μmol/l (from 45.2 ± 6.5 to 72.9 ± 9.4%, $P = 0.032$, $n = 9$) compared with those with vitamin C concentration >41 μmol/l (from 57.8 ± 6.4 to 72.2 ± 9.6%, $P = 0.046$, $n = 10$). This change was not significantly different between the two groups ($P = 0.560$).

Effects of vitamin C on thrombosis/fibrinolysis system

Plasma levels of fV were significantly decreased in the vitamin C treatment group (−21% [–1.32 to −40.8], $P = 0.038$) but remained unchanged in the control group (−7.98% [20.4 to −36.4], $P = 0.661$) (Fig. 1). However, this decrease in fV plasma levels was not significantly different between the two groups ($P = 0.596$). Plasma levels of vWF were also decreased in the vitamin C treatment group (−24% [−5.2 to –42.7], $P = 0.016$) but not in the control group (−6.88% [25.2 to –38.9], $P = 0.665$) (Fig. 1). This decrease in vWF plasma levels in the vitamin C treatment group was significantly greater than in the control group ($P = 0.036$). Plasma levels of tPA were decreased in the vitamin C treatment group (−3.858 ng/ml [–1.89 to –5.825], $P = 0.001$) but not in the control group (1.33 ng/ml [5.96 to –3.31], $P = 0.542$) (Fig. 2). This change of tPA in the vitamin C treatment group was significantly greater compared with the change in the control group ($P = 0.007$). In the vitamin C treatment group, there was no significant change in protein S (−5% [13.9 to –23.9], $P = 0.552$), protein C (−7.5% [0.82 to –15.9], $P = 0.074$), fVII (−9.58% [9.34 to –28.5], $P = 0.296$), ATIII (−3.71 [2.55 to –9.97], $P = 0.226$), and PAI-1 (−0.573 IU/l [0.417 to –1.56], $P = 0.235$) (Fig. 1).

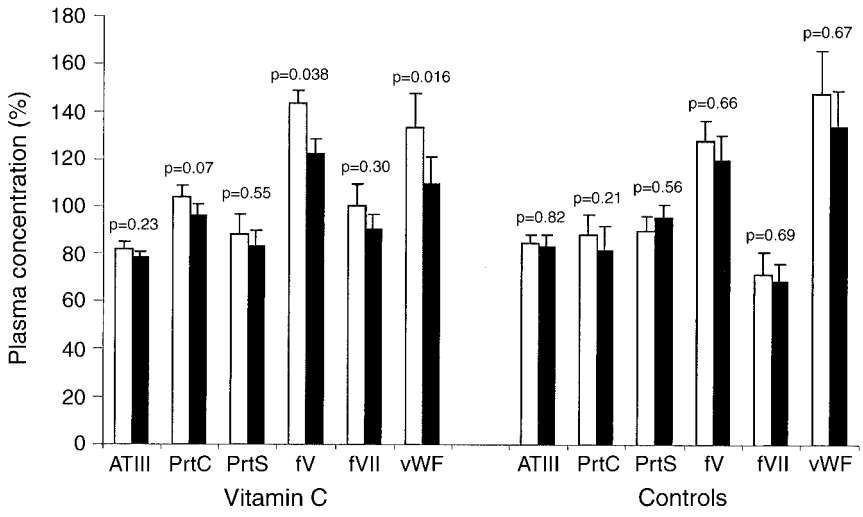

Figure 1—*Effects of vitamin C on plasma levels of vWF, ATIII, protein C (prtC), protein S (prtS), fV, and fVII in type 2 diabetic patients with CAD. Plasma levels of fV and vWF were significantly decreased only in the vitamin C treatment group (P < 0.05 for both) but remained unaffected in the control group. The decrease of vWF levels was significantly greater in the vitamin C treatment group compared with the control group (P = 0.036). Levels of ATIII, prtS, prtC, and fVII remained unchanged in both groups. ■, before treatment; □, after treatment. Data are expressed as means ± SE.*

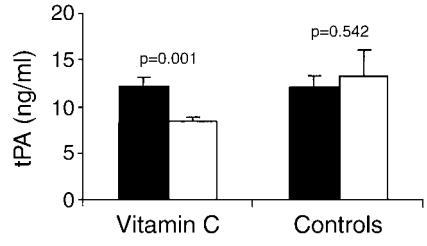

Figure 2—*Effects of vitamin C on plasma levels of tPA in type 2 diabetic patients with CAD. Plasma tPA levels were significantly decreased only in the vitamin C treatment group but remained unaffected in the control group. The decrease of tPA levels was significantly greater in the vitamin C treatment group compared with the control group (P = 0.007). ■, before treatment; □, after treatment. Data are expressed as means ± SE.*

Similarly, in the control group, no significant difference was observed in protein S (5.7% [26.8 to −15.4], $P = 0.556$), protein C (−6.57% [4.28 to –17.4], $P = 0.212$), fVII (−2.97% [12.9 to –18.9], $P = 0.692$), ATIII (−0.857% [7.24 to –8.96], $P = 0.823$), and PAI-1 (0.446 IU/l [1.35 to –0.457], $P = 0.303$) (Fig. 1). Baseline level of vitamin C did not affect the response to vitamin C treatment in the vitamin C treatment group.

CONCLUSIONS — In this study, we examined the effects of vitamin C on endothelial function and on thrombotic/fibrinolytic markers in patients with type 2 diabetes and underlying CAD. Short-term treatment with high doses of vitamin C improved RH% and significantly decreased plasma levels of tPA and vWF in patients with type 2 diabetes and coronary atherosclerosis.

Endothelium in type 2 diabetes and CAD: the role of vitamin C

It is well known that diabetes is associated with endothelial dysfunction (2). Different mechanisms are implicated in the pathogenesis of endothelial dysfunction in type 2 diabetes, such as hypertension, obesity, aging, and insulin resistance (2). It has been proposed that the basic mechanism of endothelial dysfunction in type 2 diabetes is the decreased production and increased oxidative inactivation of nitric oxide (NO) by reactive oxygen species (2,10). It has been shown that hyperglycemia inhibits production of NO by blocking endothelial NO synthase activation and by increasing the production of reactive oxygen species, especially superoxide anion (O^{2-}), in endothelial and vascular smooth muscle cells (2). Superoxide anion directly quenches NO production by forming the toxic peroxynitrite ion, which uncouples endothelial NO synthase by oxidizing its cofactor, tetrahydrobiopterin, and causes endothelial NO synthase to produce O^{2-} (2).

The role of antioxidant treatment in patients with type 2 diabetes is controversial. It has been shown that acute intrabrachial infusion of vitamin C restores endothelial function in patients with type 2 diabetes (11) or during experimental hyperglycemia in healthy subjects (12), whereas chronic supplementation of vitamin C did not improve endothelial function in type 2 diabetic patients (13). Vitamin C may improve endothelial function in patients with CAD (6), but its effect in type 2 diabetic patients with CAD is unknown. It was found that 4 weeks of treatment with vitamin C (2 g/day) improves RH% in type 2 diabetic patients with CAD. This might be due to the antioxidant effect of vitamin C, which protects, NO deactivation by reactive oxygen species (12) and protects the endothelium against oxidative damage by scavenging damaging free radicals.

Thrombosis/fibrinolysis in type 2 diabetes: the role of vitamin C

Type 2 diabetes has been associated with increased blood coagulability (14). Patients with type 2 diabetes have impaired fibrinolytic capacity as expressed by the endothelium-derived components of thrombosis/fibrinolysis such as vWF, tPA, and PAI-1. Furthermore, a significant increase of clotting factors such as fV and fVII is observed in type 2 diabetic patients (14), whereas altered plasma levels of anticoagulant molecules such as ATIII and proteins S and C have also been reported (14). In this study, we evaluated the effect of short-term treatment with vitamin C on thrombosis/fibrinolysis system in patients with type 2 diabetes and CAD.

Vitamin C and endothelium-derived components of thrombosis/fibrinolysis system

vWF is a glycoprotein stimulating platelet adhesion to the subendothelium and a good marker of endothelial integrity (15). This molecule is mainly produced by endothelial cells (16), and it has been proposed as a marker of both endothelial injury and activation. Increased levels of vWF have been associated with CAD and unstable coronary syndromes (14). Plasma level of vWF is increased in patients with diabetes, mainly as a result of endothelial cells oxidative injury, but it is unknown whether antioxidant treatment may prevent this phenomenon.

Increased plasma levels of PAI-1 have also been associated with CAD and unstable coronary syndromes (16). There is strong evidence that PAI-1 plasma level is a predictor for future events in patients with CAD because it modulates fibrinolysis and cell migration (16). A decrease in PAI-1 level was reported after chronic vitamin E administration in type 2 diabetic patients (17). It has also been suggested that vitamin C may attenuate the increase in PAI-1 plasma levels caused by acute phase response (18).

tPA, an endothelium-derived component of the fibrinolysis system, was recently found to be a predictor of CAD and myocardial infarction (16), presumably because the higher antigen levels reflect increased inactive bound tPA–PAI-1 complexes. There is strong evidence indicating that increased oxidative stress is accompanied by impaired fibrinolysis and a parallel increase in PAI-1 and tPA plasma levels (19), whereas it was reported that vitamin C and tPA levels are inversely correlated (20). Furthermore, previous studies reported no effect of low-dose antioxidant treatment with vitamins E and C on tPA plasma levels (21), whereas higher doses of vitamin E led to rather contradictory results (19,22).

In the present study, 4 weeks of treatment with high doses of vitamin C (2 g/day) significantly decreased plasma levels of vWF and tPA in patients with type 2 diabetes and CAD. This finding may be the result of the antioxidant capacity of vitamin C, which protects endothelial cells from oxidative damage.

Vitamin C and liver-derived components of thrombosis/fibrinolysis system

ATIII is a broad-spectrum serine protease inhibitor that inhibits the activity of multiple serine proteases of the coagulation pathway (14). The effect of diabetes on ATIII plasma levels is controversial (14). Reactive oxygen species are capable of inhibiting ATIII, increasing the hypercoagulability state observed in diabetes (23). However, we did not find any effect of antioxidant treatment with vitamin C on plasma levels of ATIII in patients with type 2 diabetes and CAD.

fVII is a plasma vitamin K–dependent glycoprotein that plays an important role in the initiation of tissue factor–induced coagulation. An increase in fVII coagulant activity has been proposed as an independent risk factor for CAD despite the observed discrepancies in the literature (3). Increased dietary intake of low-dose antioxidant vitamins does not seem to influence plasma levels of fVII (21). These findings are consistent with the results of the present study, in which 4 weeks of treatment with 2 g/day vitamin C in patients with type 2 diabetes did not significantly change plasma levels of fVII.

fV is a clotting factor that is controlled

by protein C; proteins C and S are also vitamin K–dependent proteins produced by hepatocytes, circulating in inactive forms until the clotting system is activated (14). Proteins S and C deactivate factor Va, having anticoagulant effects. In patients with diabetes, fV is increased, whereas plasma levels of protein C and protein S are decreased (14). However, the exact mechanism of this effect is unknown. This is the first study examining the effect of antioxidant treatment on plasma levels of fV, and proteins S and C. We have found that 4 weeks of treatment with antioxidant vitamin C (2 g/day) slightly decreased plasma levels of fV, but the change in the vitamin C treatment group was not significantly different from that in the control group. Vitamin C had no effect on levels of ATIII and proteins S and C in patients with type 2 diabetes and CAD.

The main limitations of the present study were the relatively small sample size and the small percentage of female participants. However, the observed changes in RH% and components of thrombosis/fibrinolysis system were statistically significant. Furthermore, all participants were patients with type 2 diabetes and CAD; therefore, extrapolations to patients with type 2 diabetes without macroangiopathy should be made with caution.

In conclusion, we examined the effect of vitamin C supplementation on endothelial function and thrombosis/fibrinolysis system in patients with type 2 diabetes with CAD. We found that short-term high-dose administration of vitamin C led to an improvement of RH% and reduced plasma levels of endothelium-derived components of thrombosis/fibrinolysis system, such as vWF and tPA in these patients.

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