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Oral Vitamin C and Endothelial Function in Smokers: Short-Term Improvement, But No Sustained Beneficial Effect

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OBJECTIVES To test the hypothesis that antioxidant therapy would improve endothelial function in smokers.

BACKGROUND Several studies have documented a beneficial effect of short-term oral or parenteral vitamin C on endothelial physiology in subjects with early arterial dysfunction. Possible long-term effects of vitamin C on endothelial function, however, are not known.

METHODS We studied the effects of short- and long-term oral vitamin C therapy on endothelial function in 20 healthy young adult smokers (age 36 ± 6 years, 8 male subjects, 21 ± 10 pack-years). Each subject was studied at baseline, 2 h after a single dose of 2 g vitamin C and 8 weeks after taking 1 g vitamin C daily, and after placebo, in a randomized double-blind crossover study. Blood samples were analyzed for plasma ascorbate levels and endothelial function was measured as flow-mediated dilation of the brachial artery, using high resolution ultrasound. Nitroglycerin-mediated dilation (endothelium-independent) was also measured at each visit.

RESULTS At baseline, plasma ascorbate level was low in the smokers (42 ± 21 μmol/liter; normal range, 50 to 150 μmol/liter), increased with vitamin C therapy after 2 h to 120 ± 54 μmol/liter ($p < 0.001$) and remained elevated after eight weeks of supplementation at 92 ± 32 μmol/liter ($p < 0.001$, compared with placebo). Flow-mediated dilation, however, increased at 2 h (from 2.8 ± 2.0% to 6.3 ± 2.8%, $p < 0.001$), but there was no sustained beneficial effect after eight weeks (3.9 ± 3.2%, $p = 0.26$). Nitroglycerin-mediated dilation was unchanged throughout.

CONCLUSION Oral vitamin C therapy improves endothelial dysfunction in the short term in healthy young smokers, but it has no beneficial long-term effect, despite sustained elevation of plasma ascorbate levels. (J Am Coll Cardiol 2000;35:1616–21) © 2000 by the American College of Cardiology

Smoking is a major cardiovascular risk factor and is associated with dose-dependent arterial endothelial dysfunction (1), a key early event in atherogenesis (2). The cause of endothelial dysfunction in smokers is not known, but it has been attributed to increased oxidative stress that may reduce the bioavailability of endothelium-derived nitric oxide, leading to impairment in vasodilator function (3). Previous observations have suggested that smoke-related endothelial dysfunction is potentially reversible after withdrawal from active or passive smoking (1,4). As such reversibility is incomplete, however, and rates of smoking cessation remain low (5), there is a pressing need to identify other strategies that might improve arterial health in smokers.

As smokers have low levels of antioxidant vitamin C, presumably due to increased consumption by prooxidants in cigarette smoke (6), antioxidant therapy is one such possibility. Indeed, recent human studies have shown that short-term parenteral administration of antioxidant vitamin C acutely reduces oxidative damage (7) and may improve endothelial function in smokers (8,9). The short-term beneficial effects of vitamin C on vascular reactivity have also been demonstrated in other conditions associated with endothelial dysfunction and increased oxidative stress, such as hypercholesterolemia (10), coronary artery disease (11), diabetes (12) and hypertension (13).

Much less well studied, but of greater practical relevance, are the vascular effects of longer-term supplementation with orally administered vitamin C. To our knowledge, this has not been investigated previously in smokers. We therefore aimed to examine whether a single oral dose of vitamin C improves endothelial function in asymptomatic smokers in

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Abbreviations and Acronyms

ANOVA = analysis of variance
FMD = flow-mediated dilation
HDL = high-density lipoprotein
LDL = low-density lipoprotein

the short term, and whether there are any longer-term beneficial effects after eight weeks of daily therapy with this antioxidant.

METHODS

Subjects. We studied 20 consecutive healthy volunteers who fulfilled the prespecified entry criteria: age 18 to 50 years, regular smoking history of 6 to 40 pack-years, no hypertension, no diabetes mellitus, no familial hypercholesterolemia, no clinically evident atherosclerosis, no family history of premature cardiovascular disease and not taking oral contraceptives, antioxidant vitamins or cardioactive drugs. All subjects had endothelial dysfunction at baseline, defined as flow-mediated dilation (FMD) less than the population mean for vessel size (14). On average, the baseline FMD levels were 2.0 SDs below the population mean (range, 0.2 to 3 SDs). All subjects continued their normal smoking habit for the duration of the study. This investigation was approved by the local committee on ethical practice and all subjects gave their informed consent.

Study design. Subjects were randomized to received vitamin C or matching placebo in a double-blind crossover study. To assess the short-term effect of vitamin C, each subject was studied at baseline and 2 h after a single oral dose of 2 g of vitamin C and after placebo. This dose was chosen to try to replicate the beneficial effects observed by Levine et al. (11) in their study of short-term oral vitamin C therapy in older subjects with established coronary artery disease. To assess the long-term effect of vitamin C, each subject was studied eight weeks after taking 1 g of vitamin C and after placebo. Each of these studies took place 1 to 2 h after the last dose of vitamin C. The daily dose of 1 g/d was chosen on the basis of the detailed pharmacologic studies of Levine et al. (15), demonstrating that complete plasma saturation of plasma ascorbate occurs at a dose of 1 g daily and increasing the dose above 1 g/d only activates urinary excretion mechanisms. Theoretically, at doses of >1 g daily, a risk of kidney stones, uricosuria, vitamin B_{12} deficiency, iron overload and mutagenicity exists (16). Thus, every subject was examined on six occasions (baseline, 2 h and eight weeks after vitamin C, at rebaseline after four weeks' washout and 2 h and eight weeks after placebo). In each case, subjects were fasting (other than for their study medications). One scan in one subject (2 h after vitamin C treatment) was excluded because of poor image quality. Vitamin C and the image-matched placebo preparations used in this study were provided by Blackmores Health Products (Sydney, Australia).

Ultrasound studies. All studies were performed using a 128XP/10 mainframe (Acuson; Mountain View, California) with a 7.0-MHz linear array transducer, as previously described (1,2). Brachial artery diameter was measured from B-mode ultrasound images. Scans were obtained at rest, during reactive hyperemia, again at rest and after sublingual nitroglycerin. The artery was scanned in longitudinal section 2 to 15 cm above the elbow. A resting scan was recorded, and arterial flow velocity was measured using a Doppler signal. Increased flow was induced by inflation of a pneumatic tourniquet placed around the forearm (distal to the scanned part of the artery) to a pressure of 250 mm Hg for 4.5 min, followed by release. A second scan was taken continuously for 30 s before and 90 s after cuff deflation, including a flow velocity recording for the first 15 s after the cuff was released. Thereafter, 10 to 15 min was allowed for vessel recovery, after which a further resting scan was taken. Sublingual nitroglycerin (glyceryl trinitrate spray, 400 μg) was then administered, and 3 to 4 min later the last scan was acquired.

Vessel diameter was measured by two independent observers who were "blinded" to the subject's clinical details and stage of the experiment, as previously described (1,2). Measurements were taken from the anterior to the posterior "m" line at end-diastole, incident with the R-wave on a continuously recorded ECG. For the reactive hyperemia scan, diameter measurements were taken 45 to 60 s after cuff deflation. The vessel diameter in scans after reactive hyperemia and nitroglycerin administration was expressed as the percentage relative to the average diameter of the artery in the two resting (control) scans (100%). This method has been shown previously to be accurate and reproducible for measurement of small changes in arterial diameter (17), with low interobserver error for measurement of FMD and nitrate-induced arterial dilation (2,17).

Ascorbate and serum lipoproteins. Blood samples for determination of serum lipoproteins and ascorbate levels were taken immediately before each ultrasound examination. The samples were taken 2 h after the initial 2-g dose of vitamin C and placebo, and 1 to 2 h after taking the last 1-g dose after eight weeks of therapy. The plasma levels of ascorbate were determined by high performance liquid chromatography. Fasting serum total cholesterol and triglyceride concentrations were measured using standard enzymatic methods (Boehringer Mannheim GmbH) with a fully automated analyzer (Hitachi Ltd; Tokyo, Japan). High-density lipoprotein cholesterol (HDL cholesterol) was measured after precipitation with dextran sulfate-magnesium. Low-density lipoprotein concentration (LDL cholesterol) was calculated using the Friedewald equation.

Statistical methods. Repeated-measures analysis of variance (ANOVA) for 2 × 2 crossover design was used to test

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whether the changes in study variables from baseline to 2 h and baseline to eight weeks were significantly different between vitamin C and placebo (procedure Mixed in Statistical Analysis System; SAS Institute Inc.; Cary, North Carolina). We used the Wallenstein-Fisher statistical model (18) that included fixed main effects for treatment, sequence, period and time, as well as sequence-by-time, period-by-time and treatment-by-time interaction effects. Treatment effects were calculated after taking into account any carryover effects. There were two repeated measurements for each outcome variable; the change from baseline to 2 h and the change from baseline to eight weeks. If the ANOVA revealed statistically significant treatment-by-time interaction, the Fisher least significant difference multiple comparison procedure was used to test the equality of the pairwise treatment group means. These tests were carried out as linear contrasts using the same statistical model. For correlation analysis, Pearson's correlation coefficients were calculated. Descriptive data are presented as mean ± SD (unless stated otherwise), and significance was inferred at two tailed $p < 0.05$.

With 20 subjects enrolled in a crossover trial, this study had >90% power to detect a vitamin C-related improvement of 2.5% in FMD, compared with placebo, at the $p < 0.05$ level.

RESULTS

The characteristics of study subjects (8 male, 12 female) were as follows: history of smoking, 21 ± 10 pack-years; body mass index, 24.3 ± 3.5 kg/m^2; total cholesterol, 5.0 ± 1.0 mmol/liter; LDL cholesterol, 3.0 ± 0.9 mmol/liter; HDL cholesterol, 1.42 ± 0.41 mmol/liter; triglycerides, 1.16 ± 0.66 mmol/liter; and blood pressure, 122 ± 13/70 ± 9 mm Hg.

Baseline plasma ascorbate level was 42.2 ± 20.7 μmol/liter; range was 5.0 to 83.0 μmol/liter (normal range, 50 to 150 μmol/liter) (19). Oral ingestion of 2 g of vitamin C resulted in an increase in plasma ascorbate concentration after 2 h to 120 ± 54 μmol/liter, and after eight weeks of supplementation with vitamin C 1 g daily, a sustained elevation in plasma ascorbate levels was seen, to 92 ± 32 μmol/liter ($p < 0.001$).

The mean values for FMD in each examination are shown in Figure 1. In a repeated-measures ANOVA model, the overall treatment effect was significant ($p < 0.002$), but there was a significant treatment-by-time interaction ($p < 0.01$) indicating that the effect was different at 2 h and eight weeks. After the single 2-g dose of vitamin C, FMD improved from 2.8 ± 2.0% to 6.3 ± 2.8% ($p < 0.001$). After eight weeks of supplementation with vitamin C, FMD measured 3.9 ± 3.1% and was not significantly different from placebo ($p = 0.26$).

Treatment with vitamin C had no significant short- or long-term effects on nitrate-mediated dilation, baseline vessel diameter or reactive hyperemia (the stimulus for brachial artery dilation) (Table 1).

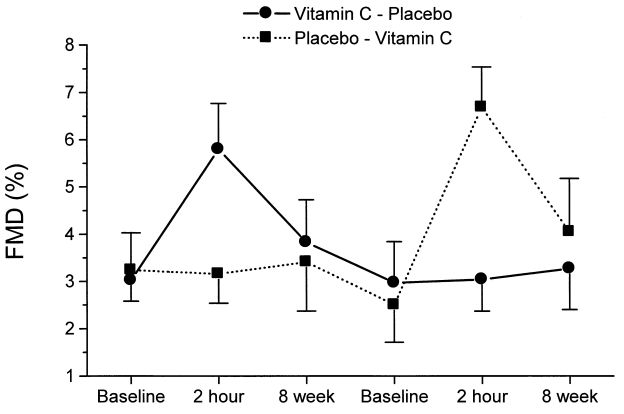

Figure 1. Values for FMD (mean ± SEM) at each visit, which improved significantly 2 h after treatment with vitamin C, but no sustained treatment effect was seen at eight weeks.

Serum lipoprotein levels were measured at baseline and at eight weeks. Treatment with vitamin C had no significant effects on serum total cholesterol, HDL cholesterol or triglycerides concentrations (data not shown).

Ascorbate level at baseline was not correlated with FMD ($r = 0.0$, $p = 0.99$); however, there was a weak correlation ($r = 0.43$, $p = 0.07$) between FMD values and plasma ascorbate concentration after the single oral dose of vitamin C. Placebo treatment did not alter any measured parameters at 2 h or at eight weeks.

DISCUSSION

Smoking remains the major modifiable risk factor for vascular disease. Although many smokers wish to quit their habit, only a minority succeed once smoking behavior is established (5). To our knowledge, no studies have previously examined the potential reversibility of smoke-related vascular disease with any long-term strategy, however, other than smoking cessation. In this randomized, placebo-controlled, crossover trial, we have found that although a single dose of vitamin C improves vascular reactivity in the short term in adult smokers with baseline endothelial dysfunction, there is no significant longer-term beneficial arterial effect.

Table 1. Effect of Oral Vitamin C Therapy on Measured Vascular Parameters

	Baseline	2 h	8 wk
FMD (%)	2.8 ± 2.0	6.3 ± 2.8*	3.9 ± 3.1
NMD (%)	15.9 ± 4.4	17.1 ± 4.9	16.2 ± 4.4
Vessel size (mm)	3.8 ± 0.47	3.8 ± 0.47	3.8 ± 0.48
Hyperemia (%)	567 ± 351	446 ± 236	693 ± 483

*$p < 0.001$ compared with placebo.
FMD = flow-mediated dilation; NMD = nitrate-mediated dilation.

Smoking and endothelial dysfunction. Previous studies have shown that cigarette smoking (1,20) and exposure to passive smoking (21) are associated with arterial endothelial dysfunction, a key early event in atherosclerosis. The exact cause of vascular dysfunction in smokers is not known, but it has been attributed to increased oxidative stress that may lead to inactivation of endothelium-derived nitric oxide by oxygen-derived free radicals (3). Nitric oxide is released by normally functioning endothelium and its release (or that of metabolite[s] closely related to nitric oxide) is responsible for endothelium-dependent arterial relaxation, which can be induced (for example) by increasing flow and shear stress (22). Preserved nitric oxide bioavailability may be a particularly important protective mechanism in the vessel wall, as nitric oxide has antiplatelet, antiadhesive and antiproliferative effects in addition to its role as a vasodilator (22).

Cigarette smoke contains large amounts of free radicals and pro-oxidants, which in biological systems can undergo redox cycling to produce reactive oxidant species (23). In addition, superoxide anion radical derived from cigarette smoke may react with endothelium-derived nitric oxide directly or via formation of peroxynitrite, a highly reactive intermediate with cytotoxic activity (24). Oxygen-derived free radicals within the vasculature may also initiate the oxidation of lipoproteins, which in turn may interfere directly with vascular function, including vessel relaxation (25). Furthermore, free radicals in cigarette smoke may increase the depletion of natural antioxidants, such as vitamin C, and thereby increase their own pro-oxidant potential. Indeed, plasma levels of ascorbate are lower in smokers compared with healthy control subjects (6).

Short-term effects of vitamin C. For these reasons, we and others have hypothesized that vitamin C therapy might improve smoke-related arterial endothelial dysfunction. Several recent studies using high dose parenteral administration of vitamin C have shown that its infusion acutely improves endothelial function in smokers (8,9), as well as in other conditions that may be associated with increased oxidative stress, such as coronary artery disease (13), hypercholesterolemia (10), diabetes (12), chronic heart failure (26) and hypertension (27). Heitzer et al. (8) measured forearm microvascular responses to acetylcholine in long-term smokers and observed a marked improvement in acetylcholine-induced vasodilation with concomitant intraarterial high dose infusion of vitamin C. Similarly, Motoyama et al. (9) observed a significant short-term improvement in brachial artery endothelial function after acute parenteral infusion of vitamin C in smokers. Concomitantly, a significant decrease was seen in the plasma levels of thiobarbituric acid–reactive substances, suggesting an acute reduction in oxidative stress (9).

To our knowledge, short-term effects of long-term oral vitamin C therapy in smokers have not been previously studied; however, Levine et al. (11) observed a significant improvement in endothelial function in patients with coronary heart disease (9 of 26 of whom were smokers), acutely after a single 2-g oral dose of vitamin C. We were able to confirm that this finding was also applicable in healthy, younger smokers, in whom ingestion of 2 g of vitamin C resulted in a threefold increase in plasma ascorbate concentration and significant improvement in FMD after 2 h.

Mechanisms of vitamin C. Several mechanisms have been proposed that may explain the short-term effects of vitamin C on endothelial function. Vitamin C is a powerful water-soluble antioxidant in human plasma (28) and it has been suggested that it may enhance endothelial function by scavenging oxygen-derived free radicals, including superoxide anion, which would otherwise interact with nitric oxide and impair its vasoactive functions (29). However, very high concentrations of vitamin C (>10 mmol/liter) are needed to prevent the interaction of superoxide with nitric oxide (30). Such high concentrations of vitamin C might be achievable by intraarterial infusions of high dose vitamin C, as used in previous studies (8–10,12,26,27), but not when given orally (30). Therefore, it has been suggested that mechanisms other than superoxide scavenging might be responsible for the improvement in endothelial function seen after oral administration of vitamin C (30).

The antioxidant activity of vitamin C is not restricted to extracellular fluids. Vitamin C is actively transported into cells and may play a role in the regulation of intracellular redox state and antioxidant defences (31), possibly via regulation of intracellular thiol species, such as glutathione. Vitamin C preserves intracellular reduced glutathione concentration (31). This may improve nitric oxide action, since depletion of glutathione has been shown to lead to decreased synthesis of nitric oxide (32), possibly through an effect on the activity of nitric oxide synthase (33). Furthermore, increased availability of reduced thiol species has been shown to stabilize nitric oxide (34) and to potentiate its vasoactive effects (35). Furthermore, data from Jackson et al. (30) support the idea that physiologic concentrations of intracellular ascorbate, reported to be in the range of 1 to 2.5 mmol/liter (36), might be sufficient to compete in the reaction between superoxide and nitric oxide. Thus, intracellular sources of superoxide that impair nitric oxide might be scavenged by concentrations of vitamin C that might be possible with short-term oral supplementation (30).

Long-term studies with antioxidants. Of much greater practical relevance in disease prevention, however, is the study of longer-term effects of vitamin C on arterial function in asymptomatic but high risk subjects. Previous long-term studies on the effects of antioxidant therapy on endothelial function have been controversial. Hornig et al. (26) studied the effects of vitamin C on conduit artery endothelial function in patients with chronic heart failure and observed a significant improvement in radial artery FMD in a subset of five patients examined after four weeks of high dose oral vitamin C supplementation (1 g twice daily). Furthermore, four-week combination therapy of oral

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vitamin C (1 g/d) and vitamin E (400 IU twice daily) has been shown to improve brachial artery FMD in children and adolescents with endothelial dysfunction due to hereditary hypercholesterolemia (37).

Recently, Gokce et al. (38) studied the effects of short- (2 h) and medium-term (30 days) oral vitamin C therapy on endothelial function in patients with established coronary artery disease. Contrary to the present study, these authors found a sustained beneficial effect on endothelial function after one month of therapy. Different study population, a different dose of vitamin C and the shorter duration of the intervention period may explain the apparently discrepant results. Regarding vitamin E therapy, Heitzer et al. (20) have documented a beneficial effect of this antioxidant on vascular endothelial function only among smokers with hypercholesterolemia (but not in those with either risk factor alone). In the present study, there were only four smokers who had LDL cholesterol levels >4.1 mmol/liter (160 mg/dl). This number of subjects was too small to perform a meaningful subgroup analysis on the effects of vitamin C therapy in hypercholesterolemic smokers.

In contrast to the positive findings reviewed above, Simons et al. (39) recently documented a lack of benefit of long-term oral vitamin E therapy on arterial endothelial function in the healthy elderly. Similarly, Gilligan et al. (40) did not observe improvement in endothelial function in hypercholesterolemic subjects after one month of therapy with a combination of antioxidant vitamin supplements (daily: beta-carotene 30 mg, vitamin C 1 g, vitamin E 800 UI), despite significant reduction in the ex vivo oxidizability of LDL. Differences in patient characteristics and antioxidant doses might explain these discrepancies. Surprisingly, however, this current study is the first report of which we are aware to describe the vascular effects of vitamin C therapy for over one month.

Vitamin C might be thought to be particularly effective in healthy young cigarette smokers, in whom ascorbate levels are known to be decreased (6). Indeed in our study, before treatment, the smokers had low plasma ascorbate levels (42 ± 21 μmol/liter) compared with previously reported normal values (50 to 150 μmol/liter) (19). After eight weeks supplementation with oral vitamin C (1 g daily), there was a sustained increase in the plasma ascorbate levels to well within the normal range (92 ± 32 μmol/liter). Despite this, however, and the encouraging vascular effects observed after 2 h, there was no significant improvement in endothelial function at eight weeks. Higher oral doses of vitamin C are unlikely to have been more effective, as bioavailability is maximal at a dose of 1 g/d; at higher doses, the extra ascorbate appears in the urine (15). The reasons for this lack of effect are unclear, but may indicate the reduction in the bioactivity of vitamin C associated with prolonged therapy.

In summary, short-term oral administration of antioxidant vitamin C markedly improved endothelial function in smokers, but there was no significant beneficial effect observable after a further eight weeks of treatment. Our results therefore suggest that oxidative stress may contribute to the endothelial impairment in smokers. The lack of sustained effect, however, implies the existence of intracellular and/or extracellular adaptive mechanisms, and therefore discourages the daily use of vitamin C for vascular protection in smokers with established endothelial dysfunction.

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