Randomized, Double-Blind, Placebo-Controlled Study of Ascorbate on the Preventive Effect of Nitrate Tolerance in Patients With Congestive Heart Failure

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**Background**—Reduced cGMP production caused by increased superoxide has been proposed as a mechanism of nitrate tolerance during continuous nitrate therapy. This study was designed to evaluate the effects of ascorbate, an antioxidant, on the development of nitrate tolerance during continuous nitrate therapy in patients with congestive heart failure.

**Methods and Results**—Twenty patients with congestive heart failure were randomized to receive intravenous infusion of nitroglycerin concomitantly with placebo (placebo group, n=10) or intravenous ascorbate (vitamin C group, n=10). After baseline measurements were obtained, dose titration was started by the infusion of nitroglycerin at a rate of 0.5 μg/kg per minute (titration period). Measurements of hemodynamic parameters and blood sampling were performed serially at 0, 6, 12, 18, and 24 hours after the titration period. At baseline, mean pulmonary artery pressure (MPAP, mm Hg), mean pulmonary capillary wedge pressure (PCWP, mm Hg), plasma vitamin E level (μmol/L), and platelet cGMP level (pmol/10⁹ platelets) were comparable in the two groups (placebo group: MPAP, 48±6; PCWP, 24±4; cGMP, 0.76±0.12; vitamin E, 18.2±1.2; vitamin C: MPAP, 49±7; PCWP, 24±4; cGMP, 0.71±0.16; vitamin E, 18.6±1.3). In both groups, at 6 hours after the titration period, MPAP and PCWP were significantly decreased (placebo group: MPAP, 26±5; PCWP, 15±4; vitamin C: MPAP, 26±4; PCWP, 16±4), and platelet cGMP was significantly increased (placebo group: 2.42±0.24; vitamin C: 2.26±0.26). However, at 18 hours after titration, in the placebo group, MPAP (44±5) and PCWP (23±4) were increased, and platelet cGMP (0.85±0.20) and plasma vitamin E levels (12.4±1.4) were significantly decreased. In contrast, in the vitamin C group, MPAP (31±6), PCWP (17±5), platelet cGMP (2.49±0.23), and plasma vitamin E levels (17.6±1.4) were maintained for 18 hours after the titration period.

**Conclusions**—These findings indicate that ascorbate, an antioxidant, may prevent the development of nitrate tolerance during continuous nitrate therapy in patients with congestive heart failure. *(Circulation. 1998;97:886-891.)*

**Key Words:** antioxidants ■ heart failure ■ nitroglycerin ■ platelets

Organic nitrates are widely used in cardiovascular medicine, but their continuous administration can result in the rapid development of tolerance. The underlying mechanisms responsible for nitrate tolerance probably are multifactorial and may include neurohormonal counterregulatory mechanisms, intravascular volume expansion, and intrinsic abnormalities such as desensitization of the target enzyme guanylate cyclase or a decrease in nitroglycerin biotransformation. Recent experimental findings have demonstrated that nitrate tolerance is associated with increased vascular production of superoxide anions. A recent study showed that tolerance was associated with an enhanced propensity for vasoconstriction due to increased endothelin expression within vascular smooth muscle. Münzel et al state that superoxide anions degrade nitric oxide derived from nitroglycerin, whereas autocrine-produced endothelin within vascular smooth muscle sensitizes the vasculature to circulating neurohor mones, such as catecholamines and angiotensin II, all of which may compromise the vasodilator potency of nitroglycerin. Ascorbate (vitamin C) is the main water-soluble antioxidant in human plasma. It is an effective scavenger of superoxide and other reactive oxygen species and plays an important role in the regulation of intracellular redox state through its interaction with glutathione. The present study was therefore designed to investigate whether ascorbate can prevent nitrate tolerance during the continuous administration of nitroglycerin in patients with congestive heart failure.

**Methods**

**Patient Population**

Twenty patients with congestive heart failure were studied after they had given written informed consent for participation. They were...
randomized to receive intravenous infusion of nitroglycerin concomitantly with either ascorbate (vitamin C group, n=10) or placebo (placebo group, n=10). All vasodilators, diuretics, and inotropic agents were discontinued at least 24 hours before the study. The clinical characteristics of the 20 patients are given in Table 1. There were no differences in age, sex, disease, or previous medications between the two groups. This study was approved by the ethics committees on human research of the University of Tsukuba and KINU Medical Association Hospital.

Study Protocol
A 7F Swan-Ganz thermodilution catheter was introduced percutaneously through the internal jugular vein and advanced into the pulmonary artery under fluoroscopic guidance, and a 23-gauge polyethylene tube was inserted into the radial artery percutaneously to measure blood pressure. A peripheral line was inserted for infusion of nitroglycerin and ascorbate or placebo, and a bladder catheter was placed for urine sampling. Hemodynamic stability (<10% variation) was ensured by two consecutive measurements performed at 30-minute intervals. After baseline measurements were obtained, dose titration was started by infusion of nitroglycerin at a rate of 0.5 μg/kg per minute (titration period). The infusion rate was doubled every 15 minutes to achieve a 30% reduction in pulmonary capillary wedge pressure (PCWP). After achievement of the desired hemodynamic response, ascorbate (55 mg/kg per minute) or placebo was infused, and a bladder catheter was placed for urine sampling. Hemodynamic stability (<10% variation) was ensured by two consecutive measurements performed at 30-minute intervals. After baseline measurements were obtained, dose titration was started by infusion of nitroglycerin at a rate of 0.5 μg/kg per minute (titration period). The infusion rate was doubled every 15 minutes to achieve a 30% reduction in pulmonary capillary wedge pressure (PCWP). After achievement of the desired hemodynamic response, ascorbate (55 mg/kg per minute) or placebo was infused, and the infusion rate of nitroglycerin (average, 1.5±0.5 μg/kg per minute) was maintained for 24 hours. Hemodynamic measurements and blood sampling were repeated 0, 6, 12, 18, and 24 hours after start of the nitroglycerin infusion concomitant with ascorbate or placebo. Blood samples were drawn from the right atrium for determination of blood pressure. A peripheral line was inserted for infusion of nitroglycerin and ascorbate or placebo, and a bladder catheter was placed for urine sampling. Hemodynamic stability (<10% variation) was ensured by two consecutive measurements performed at 30-minute intervals. After baseline measurements were obtained, dose titration was started by infusion of nitroglycerin at a rate of 0.5 μg/kg per minute (titration period). The infusion rate was doubled every 15 minutes to achieve a 30% reduction in pulmonary capillary wedge pressure (PCWP). After achievement of the desired hemodynamic response, ascorbate (55 mg/kg per minute) or placebo was infused, and the infusion rate of nitroglycerin (average, 1.5±0.5 μg/kg per minute) was maintained for 24 hours. Hemodynamic measurements and blood sampling were repeated 0, 6, 12, 18, and 24 hours after start of the nitroglycerin infusion concomitant with ascorbate or placebo. Blood samples were drawn from the right atrium for determination of platelet cGMP and plasma vitamin E level. The study protocol is summarized in Fig 1.

Hemodynamic Measurements
PCWP and pulmonary arterial pressure were determined with the Swan-Ganz catheter connected to a pressure transducer (BSM-8301, AP-800P; Nihon Kohden). Systolic and diastolic arterial pressures were determined with the 23-gauge polyethylene tube connected to the pressure transducer. The zero pressure reference level was taken at midchest level. Cardiac output was determined in triplicate by the thermodilution technique. Heart rate was continuously monitored on lead II of the ECG. All measurements were performed with the patient at the supine position.

Preparation of Platelets for cGMP Assay
Blood samples were drawn into syringes containing 5 mmol/L EDTA and a cGMP phosphodiesterase inhibitor (10−3 mol/L 2-O-propoxyphenyl-8-azapurin-6-one dissolved in 1% triethanolamine). Platelet-rich and platelet-poor plasma were prepared immediately after blood sampling by centrifugation at 2000g for 20 minutes. Platelet-rich plasma was centrifuged further at 2500g for 10 minutes, and the supernatant was discarded. The pellet was suspended in modified Tyrode’s solution (containing 0.35% bovine serum albumin and 5 mmol/L HEPES, pH 7.35) to obtain a final platelet count of 2 to 3×10^10 platelets/μL. The samples were stored frozen at −70°C until analysis.15

Platelet cGMP Assay
Trichloroacetic acid (0.5 mL at a final concentration of 6%) was added to 1 mL of the platelet preparation. After centrifugation at 2500g for 20 minutes, trichloroacetic acid was extracted four times from the supernatant with water-saturated ether. The aqueous phase then was assayed for cGMP using a commercially available radiomunnoassay kit (Yamasa Shoyu).16 The results are expressed in picomoles per 10^10 platelets. The coefficients of variation averaged 3.4% for intra-assay error and 11.9% for interassay error.

Measurement of Vitamin E
Vitamin E (α-tocopherol) content in plasma was estimated using the high-performance liquid chromatography method of Thompson and Hatina.17

Statistical Analysis
Results are expressed as mean±SD for hemodynamic parameters and mean±SEM for platelet cGMP and vitamin E levels. Differences among the test days were analyzed by repeated-measures ANOVA with Bonferroni’s test, and differences between the two groups were analyzed with the Student’s t test. Findings of P<.05 were considered statistically significant.

Results
Heart Rate and Blood Pressure
Heart rate did not change during the study in either group, and there was no difference in heart rate between the two groups (Fig 2A).

Systolic blood pressure was decreased significantly in the two groups during the titration period (vitamin C group, 143±20 to 124±18 mm Hg; placebo group, 142±20 to 120±18 mm Hg). During prolonged infusion, systolic blood pressure was significantly decreased 6 hours after nitroglycerin
initiation in both groups. The effect was maintained for 24 hours after titration in the vitamin C group. However, in the placebo group, systolic blood pressure began to increase 18 hours after titration, and there was a significant increase in systolic blood pressure compared with that at 0 hours after titration (18-hour systolic blood pressure: vitamin C group, 121 ± 620 mm Hg; placebo group, 141 ± 619 mm Hg) (Fig 2B).

Pulmonary Artery Pressure and PCWP
Mean pulmonary artery pressure (MPAP: vitamin C group: 45 ± 8 to 24 ± 7 mm Hg, placebo group: 46 ± 6 to 25 ± 5 mm Hg) and mean PCWP (vitamin C group: 23 ± 5 to 17 ± 6 mm Hg, placebo group: 24 ± 5 to 16 ± 4 mm Hg) were significantly decreased during the titration period in both groups. These effects were maintained for 12 hours after the titration period in the two groups. However, at 18 hours after the titration period, MPAP and PCWP began to increase in the placebo group, whereas the levels in the vitamin C group were maintained for 24 hours after titration (MPAP: vitamin C group: 31 ± 6 mm Hg, placebo group: 44 ± 5 mm Hg; PCWP: vitamin C group: 17 ± 5 mm Hg, placebo group: 23 ± 4 mm Hg) (Fig 3).

Platelet cGMP Level
Platelet cGMP level was significantly increased during the titration period in both groups (vitamin C group: 0.74 ± 0.15 to 2.13 ± 0.22 pmol/10^9 platelets; placebo group: 0.78 ± 0.13 to 2.24 ± 0.20 pmol/10^9 platelets, mean ± SEM). The elevated platelet cGMP level was maintained for 12 hours after the titration period in both groups. However, in the placebo group, platelet cGMP level was decreased 18 hours after the titration period, whereas platelet cGMP level in the vitamin C group was maintained for 24 hours after the titration period (vitamin C group: 2.49 ± 0.23; placebo group: 0.85 ± 0.20) (Fig 4).

Plasma Vitamin E Level
There was no difference in plasma vitamin E level between the two groups at baseline (vitamin C group: 18.2 ± 1.2 μmol/L; placebo group: 18.6 ± 1.3 μmol/L, mean ± SEM). During the titration period, plasma vitamin E level was not changed in either group. At 6 or 12 hours after the titration period, plasma vitamin E levels were not changed in either group, and there was no difference in plasma vitamin E level between the two groups. At 18 hours after the titration period, plasma vitamin E level in the vitamin C group was not changed, but the value in the placebo group was significantly decreased (vitamin C group: 17.6 ± 1.4 μmol/L; placebo group: 12.4 ± 1.4 μmol/L) (Fig 5).

Discussion
This placebo-controlled, double-blind study demonstrated that ascorbate, a water-soluble antioxidant, maintained vasodilation and intracellular production of cGMP during prolonged infusion of nitroglycerin in patients with congestive heart failure. These findings suggest that ascorbate may prevent the development of nitrate tolerance during continuous nitrate tolerance.
Mechanisms of Nitrate Tolerance

Although the phenomenon of nitrate tolerance was first described during the early part of this century, it was not considered clinically important until later research demonstrated that nitrate tolerance limited the efficacy of nitrates in patients with ischemic heart disease and congestive heart failure. The mechanisms of nitrate tolerance remain poorly defined and are likely multifactorial. Several mechanisms of this phenomenon have been proposed. Nitrate tolerance is thought to be due to the inability of vascular tissue to respond to nitroglycerin. Many previous studies have proposed four possible mechanisms of nitrate tolerance: (1) desensitization of the target enzyme guanylate cyclase, (2) increase in phosphodiesterase activity, (3) intracellular sulf-hydryl group depletion, and (4) impaired nitroglycerin bio-transformation.

Effect of Ascorbate on Prevention of Nitrate Tolerance

Ascorbate (vitamin C) is the main water-soluble antioxidant in human plasma. It effectively scavenges superoxide and other reactive oxygen species, and it plays an important role in the regulation of intracellular redox state through its interaction with glutathione. Several large epidemiological studies have suggested that dietary intake of vitamin C and plasma vitamin C concentration is inversely associated with the risk of ischemic heart disease. Recently, ascorbate has been reported to reverse endothelial vasomotor dysfunction in the brachial circulation of patients with coronary artery disease and to improve endothelial dysfunction in chronic smokers. In an experimental animal study, Bassenge and Fink demonstrated that ascorbate prevented nitrate tolerance in the dilatation of coronary arteries and production of platelet cGMP. We recently reported effects of ascorbate on the prevention of nitrate tolerance in normal volunteers. The present study provides the first evidence that the development of nitrate tolerance in patients with congestive heart failure may be prevented by supplementation with an antioxidant, ascorbate. We demonstrate that ascorbate prevented the attenuation of hemodynamic effect and reduced production of cGMP during the continuous administration of nitroglycerin in patients with congestive heart failure. These findings strongly support the theory that increased the production and activity of oxygen-derived free radicals contribute to the development of nitrate tolerance in patients who receive long-term therapy with organic nitrates.

Figure 5. Changes in plasma vitamin E (α-tocopherol) level. ○, Vitamin C group values. □, Placebo group values. Values are mean±SEM. *P<.01 vs. 0 hour in the prolonged infusion. †P<.01 vs. vitamin C group.

Other Studies of the Prevention of Nitrate Tolerance

Because nitrate tolerance has the potential to limit the therapeutic efficacy of nitrates in patients with congestive heart failure, there has been an extensive effort to develop effective strategies to prevent this phenomenon. Some studies have found that the concomitant administration of ACE inhibitors and nitroglycerin reversed or prevented nitrate tolerance, but other studies have failed to confirm these findings. Although it is difficult to explain the differences in the efficacy of ACE inhibitors between these studies, the use of higher doses of ACE inhibitors may be required to inhibit angiotensin II formation. Münzel and Bassenge reported that high-dose enalapril reversed nitrate tolerance in vivo. However, they did not evaluate the intracellular production of cGMP. Therefore, more information is needed to determine the clinical usefulness of ACE inhibitors and diuretics in the prevention of nitrate tolerance.

Recently, the preventive effect of hydralazine on nitrate tolerance in patients with congestive heart failure was demonstrated by Gogia et al and Elkayam. Münzel et al showed in their study that hydralazine could inhibit the enzyme system responsible for the increase in superoxide anions in nitrate tolerance.

Study Limitations

There are some limitations in the present study. First, we measured platelet cGMP level to evaluate the intracellular production of cGMP. The in vivo effects of nitroglycerin on the intracellular production of cGMP in vascular smooth muscle cells can be evaluated only through biopsy. Nitroglycerin activates soluble guanylate cyclase in platelets, and the increased level of platelet cGMP inhibits platelet adhesion. Platelets contain predominantly the soluble guanylate cyclase. Therefore, platelets are an appropriate material for the clinical measurement of intracellular cGMP. In a previous study, we demonstrated that platelet cGMP level can be used as an indicator of the effects of nitroglycerin and the development of nitrate tolerance. Second, we did not investigate the effects of other antioxidants, such as vitamin E and β-carotene.
Vitamin E and β-carotene may both favorably influence cardiovascular risk, but there are several important differences between these naturally occurring antioxidants. Vitamin C is water soluble and present in most body fluids; vitamin E and β-carotene are both lipid soluble, and the concentrations of these compounds in plasma and specific cellular compartments differ. The primary mechanisms of these antioxidants also are distinct. Thus, the beneficial effects observed in this study cannot necessarily be assumed to be obtainable with other antioxidants. More recently, we reported the preventive effect of vitamin E on nitrate tolerance in healthy volunteers and patients with ischemic heart disease. Further studies are required to clarify the possible beneficial effects of antioxidants on nitrate tolerance in patients with congestive heart failure.

Conclusions

Our findings suggest that continuous nitrate therapy results in a lack of hemodynamic effect and that combination therapy with ascorbate is potentially useful for the prevention of nitrate tolerance during continuous nitrate therapy in patients with congestive heart failure. Further studies are required to clarify the possible beneficial effects of antioxidants on the development of nitrate tolerance during continuous nitrate therapy.

References

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