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The effects of vitamin C and nitric oxide synthase inhibition on coronary flow and oxidative stress markers in isolated rat heart

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Abstract. The aim of this study was to assess the effects of vitamin C (ascorbic acid) on coronary flow and oxidative stress markers with or without non-specific inhibition of nitric oxide synthase by N^{ω}-nitro-L-arginine monomethyl ester (L-NAME) in isolated rat hearts. The hearts of male Wistar albino rats (n = 12, age 8 weeks, body mass 180–200 g) were retrograde perfused according to the Langendorff technique at gradually increased constant perfusion pressure (40–120 cm H_2O). Coronary flow, nitrite outflow, superoxide anion production, and index of lipid peroxidation (by measuring thiobarbituric acid reactive substances) in coronary effluent were determined. The experiments were performed during control conditions and in presence of vitamin C (100 μM) alone or vitamin C (100 μM) + L-NAME (30 μM). Administration of vitamin C induced only increase of nitrite levels, while vitamin C + L-NAME induced significant decrease of coronary flow above autoregulatory range, i.e. especially at higher coronary perfusion pressure (CPP) values, accompanied with similar dynamic in nitrite outflow. Vitamin C + L-NAME also induced significant decrease in TBARS production. The results of our study show no significant effects of vitamin C administration either on ROS levels or on coronary flow in isolated rat heart.

Key words: Vitamin C — Nitric oxide — Coronary flow — Oxidative stress

Introduction

Vitamin C (ascorbic acid) is the most potent water-soluble antioxidant in human plasma (Frei et al. 1990). Due to its remarkable redox properties, ascorbate (major form of vitamin C at physiological pH) is one of the most efficient antioxidants in cells and biological fluids (Rose and Bode 1993; Vergely et al. 2001). It readily scavenges free radicals such as superoxide anion radical, hydroxyl radical, and hypochlorous acid (Jeserich et al. 1999), regenerates vitamin E from its tocopheroxyl radical and spares intracellular glutathione (Carr and Frei 2000). This latter function may be particularly important with respect to endothelial vasodilator function because glutathione has been implicated in enhanced endothelium-derived nitric oxide (NO) synthesis and stabilization of NO through the formation of S-nitrosothiols (Carr and Frei 2000). Vitamin C has also been implicated in the release of NO from S-nitrosothiols (Scorza et al. 1997).

The effects of ascorbic acid on the bioactivity of endothelium-derived NO have been extensively studied in humans and to a lesser extent in experimental animals (Tomasian et al. 2000). It was demonstrated that ascorbic acid reverse endothelial dysfunction in patients with numerous diseases (Levine et al. 1996; Ting et al. 1996; Motoyama et al. 1997; Hornig et al. 1998; Taddei et al. 1998; Teramoto et al. 2004; de Sousa et al. 2005; Grebe et al. 2006; Hernández-Guerra et al. 2006; Cangemi et al. 2007). These remarkably consistent findings prompt studies to elucidate the mechanism of this beneficical effect of ascorbic acid on the bioactivity of endothelium-derived NO.

Cellular damage caused by reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-) or hydroxyl radicals (OH^-) is a significant causal factor involved in heart diseases, especially during myocardial ischemia-reperfusion (Vergely et al. 2001). Many authors have provided extensive evidence that free radicals are produced and released from the ischemic heart and that their production is especially intensive in the reperfusion period (Blasig et al. 1994; Dhalla

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et al. 2000; Guaiquil et al. 2004). Rapid restoration of the blood flow increases the level of tissue oxygenation which causes the second burst of ROS generation that leads to the reperfusion injury (Maxwell and Lipp 1997). One of the possible mechanisms of ROS-mediated cardiovascular diseases is lowering the levels of vasodilatory substances produced in the endothelium (Ajay and Mustafa 2006), among which NO is the most important (Gewaltig and Kojda 2002).

The aim of this experimental study was to assess the effects of vitamin C on the coronary flow, oxidative stress markers and nitrites with or without non-specific nitric oxide synthase (NOS) inhibition by N^{ω}-nitro-L-arginine monomethyl ester (L-NAME) in isolated rat hearts.

Materials and Methods

Isolated rat heart preparation

The hearts (total number $n = 12$, 6 hearts for each experimental group; discarded hearts were not included in the total number of the hearts) excised from Wistar male albino rats, 8 weeks old, with body mass of about 200 g (obtained from Military Medical Academy, Belgrade, Serbia) were perfused with Langendorff apparatus (Hugo Sachs Elektronik-Harvard Aparatus GmbH, March-Hugstetten, Germany). After short-term ether narcosis, animals were killed by cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986, UK), with heparin premedication as an anticoagulant. After urgent thoracotomy and rapid heart arrest by superfusion with ice-cold isotonic saline, the hearts were rapidly excised, isolated, the aortas were cannulated and retrograde perfused according to the technique for constant pressure conditions. The composition of the non-recirculating Krebs-Henseleit perfusate was as follows (in mmol/l): NaCl 118, KCl 4.7, $CaCl_2 \times 2H_2O$ 2.5, $MgSO_4 \times 7\ H_2O$ 1.7, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, pyruvate 2, equilibrated with 95% O_2 + 5% CO_2 and warmed to 37°C (pH 7.4). All hearts were electrically paced (5 V, 320 bpm) by the electric stimulator (Hugo Sachs Elektronik-Harvard Aparatus GmbH) and constant left ventricular draining through the dissected mitral valve was performed.

Physiological assay

After the heart perfusion had been set up, a 30 min period was allowed for stabilization of the preparation. Stabilization of preparation was performed at basal coronary perfusion pressure of 60 cm H_2O. In order to test coronary vascular reactivity, all hearts were challenged by short-term occlusions (5–30 s), as well as by bolus injection of 5 mmol/l adenosine (60 µl at a flow rate of 10 ml/min in order to elicit maximal coronary flow). The hearts were discarded (about 25%) if the flow did not increase by 100% over the control value (for both tests). After the equilibration period, coronary perfusion pressure was lowered to 50 and 40 cm H_2O and then gradually increased to 70, 80, 90, 100, 110 and 120 cm H_2O in order to establish coronary autoregulation. When the flow was considered as stable at each value of perfusion pressure, samples of the coronary effluent were collected. Properly performed control experiments included studying of the coronary perfusion pressure/coronary flow relationship twice in the absence of any drug. It was essential to confirm that the used preparation was stable and that the responses to the first and the second run of changes in perfusion pressure did not differ substantially. After performing the control experimental protocol, the hearts were perfused with ascorbic acid (100 µM) and with ascorbic acid (100 µM) plus an inhibitor of NO synthesis, (30 µM of L-NAME) for minimum. Test period started immediately after control experiments in order to avoid time-dependent adverse effects. Drug administration was performed until stable flow was reached but not less then 5 minutes at each perfusion pressure. Data (coronary flow, levels of superoxide anion radical, nitrites and index of lipid peroxidation) obtained during control experimental protocol was compared with data obtained after vitamin C or vitamin C + L-NAME perfusion.

Biochemical assays

Samples of the coronary venous effluent were collected after the stabiliation of flow at each value of gradually increased perfusion pressure. Spectrophotometric method was used for determination of nitrite, superoxide anion radical and index of lipid peroxidation.

Nitrite determination

Nitric oxide decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrite was determined and used as an index of nitrite oxide production by the spectrophotometric method using the Griess's reagent. 0.5 ml of perfusate was precipitated with 200 µl of 30% sulfosalicylic acid, vortexed for 30 min and centrifuged at 3000 × *g*. Equal volumes of the supernatant and Griess's reagent, containing 1% sulfanilamide in 5% phosphoric acid/0.1% napthalene ethylenediamine-dihydrochloride was added and incubated for 10 min in the dark and read at 543 nm of wavelength. The nitrite levels were calculated by using sodium nitrite as a standard (Green et al. 1982).

Superoxide anion radical determination

The level of O_2^- was measured using Nitro blue tetrazolium (NBT) reaction in TRIS-buffer with coronary venous efflu-

ent and read at 530 nm of wavelength. Krebs-Hensenleit solution was used as a blank probe (Auclair and Voisin 1985).

Index of lipid peroxidation (Thiobarbituric acid reactive substances – TBARS)

The degree of lipid peroxidation in coronary venous effluent was estimated by measuring of TBARS using 1% Thiobarbituric acid (TBA) in 0.05 NaOH incubated with coronary effluent at 100°C for 15 minutes and read at 530 nm. Krebs-Hensenleit solution was used as a blank probe (Ohkawa et al. 1979).

Drugs

The provider of L-NAME, spectrophotometric assay kit (used for nitrite determination), napthalene ethylenediamine-dihydrochloride as well as sulfosalicylic acid was from Sigma-Aldrich Chemie GmbH, while vitamin C (ascorbic acid) was purchased from Galenika AD (Belgrade, Serbia). Sulfanilamide, phosphoric acid, NBT, Tris-Buffer and TBA were purchased from Merck KGaA (Darmstadt Germany).

Statistical analysis

Values are expressed as means ± S.E.M. Statistical analysis was performed by multifactorial analysis of variance for repeated measurements between subject factors as well as Bonferroni's test; p values less than 0.05 were considered to be significant.

Results

Coronary flow

Coronary flow increased proportionally to coronary perfusion pressure in the whole range of perfusion pressure studied both in control and study groups. During control conditions coronary flow varied in range from 2.68 ± 0.51 ml/min/g weight of tissue (wt) at 40 cm H_2O to 9.65 ± 0.93 ml/min/g wt at 120 cm H_2O (Fig. 1, left). Vitamin C did not induce significant change in coronary flow (Fig. 1, left). On the other hand, vitamin C + L-NAME induced significant decrease of coronary flow above autoregulatory range, i.e. at higher coronary perfusion pressure (CPP) values (Fig. 1, right).

Nitrite outflow

During control conditions nitrite outflow varied from 0.63 ± 0.18 nmol/min/g wt at 40 cm H_2O to 2.71 ± 0.74 nmol/min/g wt at 120 cm H_2O and was parallel with CPP- coronary flow curve (Fig. 2, left). Vitamin C induced significant increase in nitrite outflow (0.93 ± 0.21 nmol/min/g wt at 40 cm H_2O to 3.72 ± 1.09 nmol/min/g wt at 120 cm H_2O) (Fig. 2, left). Vitamin C + L-NAME induced significant reduction of the nitrite outflow from 18% at 40 cm H_2O to 37% at 120 cm H_2O (Fig. 2, right).

Superoxide anion production

During control conditions superoxide anion radical production varied from 9.92 ± 1.43 nmol/min/g wt at 40 cm H_2O to

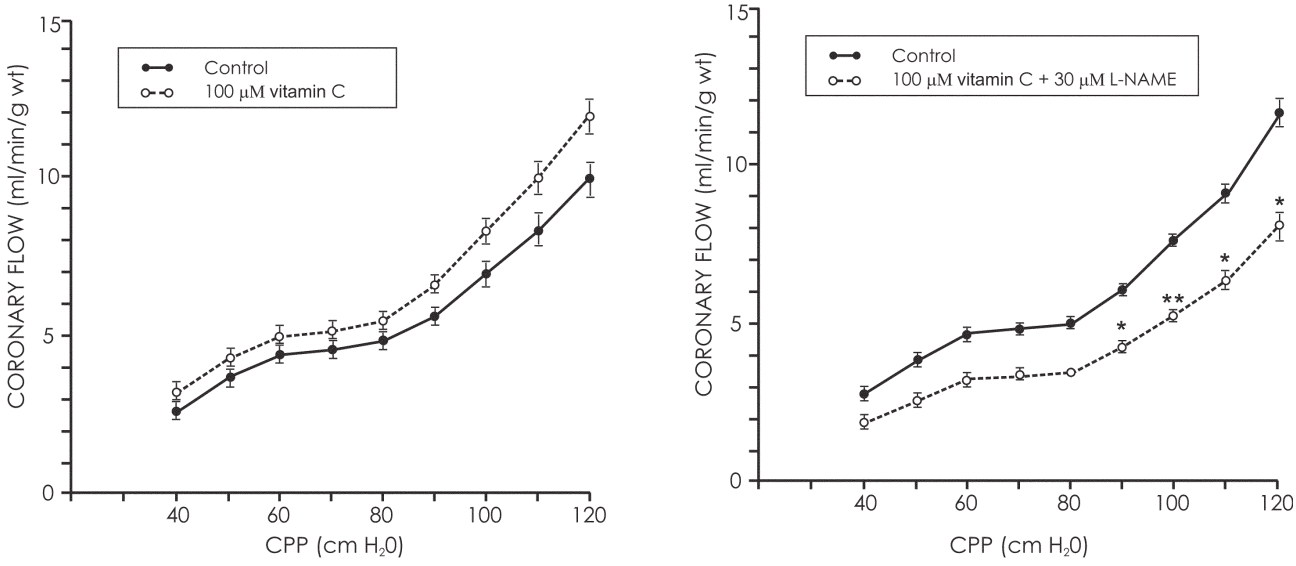

Figure 1. Coronary flow values (ml/min/g wt) at different coronary perfusion pressure (CPP) values (cm H_2O) in vitamin C-treated group (left) and in vitamin C+L-NAME-treated group (right). * $p < 0.05$, ** $p < 0.01$.

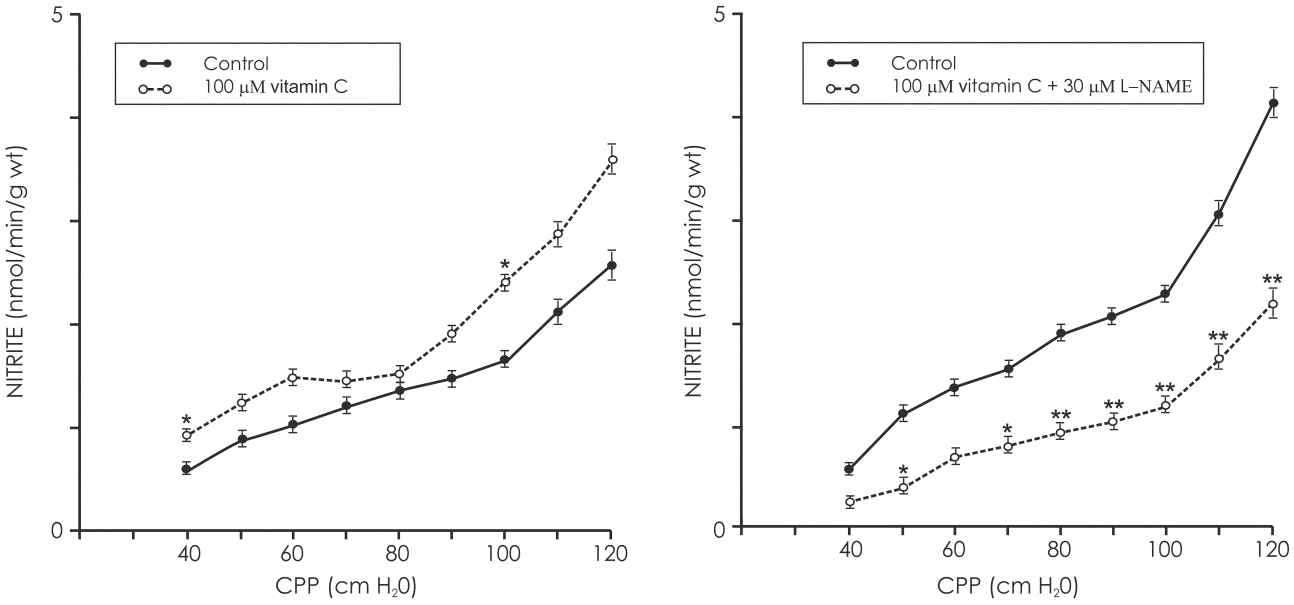

Figure 2. Nitrite outflow (nmol/min/g wt) in coronary venous effluent at different coronary perfusion pressure (CPP) values (cm H_2O) in vitamin C-treated group (left) and in vitamin C+L-NAME-treated group (right). * $p < 0.05$, ** $p < 0.01$.

28.89 ± 1.34 nmol/min/g wt at 120 cm H_2O and was parallel with CPP- coronary flow curve (Fig. 2, left). Vitamin C did not induce significant changes in O_2^- levels. (Fig. 3, left). Vitamin C + L-NAME also did not change significantly superoxide anion production compared with control values (Fig. 3, right).

Index of lipid peroxidation (TBARS production)

During control conditions TBARS production varied from 0.91 ± 0.26 μmol/min/g wt at 40 cm H_2O to 2.46 ± 1.35 μmol/min/g wt at 120 cm H_2O and was parallel with CPP- coronary flow curve (Fig. 4, left). Vitamin C did not

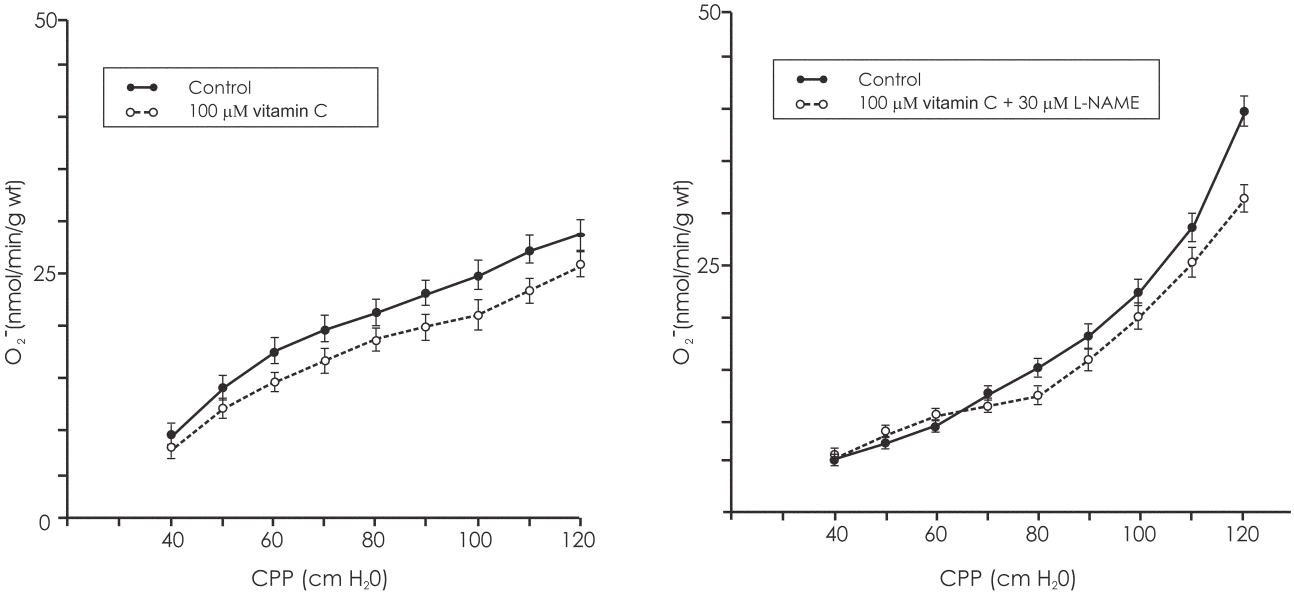

Figure 3. Superoxide anion production (nmol/min/g wt) in coronary venous effluent at different coronary perfusion pressure (CPP) values (cm H_2O) in vitamin C-treated group (left) and in vitamin C+L-NAME-treated group (right).

change significantly TBARS production at any CPP-value (Fig. 4, left). On the other hand, vitamin C + L-NAME significantly decreased TBARS production compared with control values (from 8% at 40 cm H_2O to 37% at 120 cm H_2O (Fig. 4, right).

Discussion

In this study, the vascular effects of acute vitamin C or vitamin C + L-NAME (NOS inhibitor) administration in the isolated rat hearts at various CPP values were studied. The coronary flow, production of nitrites (as a marker of NO) and production of oxidative stress markers (superoxide anion radical and TBARS) were estimated. Data (coronary flow, levels of NO_2^-, O_2^- and TBARS) obtained at each CPP value after vitamin C or vitamin C + L-NAME administration were compared with data obtained in the control conditions. The differences in the levels of investigated parameters in certain groups of animals in the control conditions are due to reason of biological diversity.

After administration of vitamin C alone, the only statistically significant change in investigated parameters was found in NO (NO_2^-) levels (the increase of NO_2^- levels), while administration of vitamin C + L-NAME induced the decrease of three parameters – coronary flow, NO_2^- and TBARS. The presented data are different to folic acid-induced effects on the same experimental model (Djuric et al. 2007). In contrast to vitamin C, which induced only the rise in NO_2^- levels, folic acid induced the significant increase in both NO_2^- levels and coronary flow, and decreased O_2^- levels. TBARS also increased after folic acid administration, which is a bit contradictory to changes of superoxide production (Djuric et al. 2007). Furthermore, effects of in vitamin C + L-NAME administration were significantly different than effects of folic acid + L-NAME administration which induced no significant changes in any of the investigated parameters. The results regarding TBARS levels are unexpected since O_2^- levels were unchanged after vitamin C + L-NAME administration.

Given the importance of superoxide anion radical as a mechanism of endothelial dysfunction in atherosclerosis and the role of lipid peroxidation as a mechanism of impaired bioactivity of endothelium-derived NO in atherosclerosis (Tomasian et al. 2000; Kawashima and Yokoyama 2004), many authors assumed that ascorbic acid exerts its beneficial effects by scavenging superoxide anion and inhibiting lipid peroxidation (Levine et al. 1996; Ting et al. 1996). Another suggested mechanism by which ascorbic acid improves bioactivity of endothelium-derived NO is by influencing cellular redox state (Tomasian et al. 2000). There is evidence that ascorbic acid spares intracellular glutathione from oxidation thus increasing its availability which is important in both NO experimental models (Ghigo et al. 1993; Gorren et al. 1997; Zaidi et al. 2005) and in human subjects (Kugiyama et al. 1998; Prasad et al. 1999). Furthermore, there is evidence that vitamin C may have direct effects on activity of NOS, possibly through an effect on tetrahydrobiopterin (Heller et al. 1999; Huang et al. 2000; Förstermann and Münzel 2006). Heller et al.

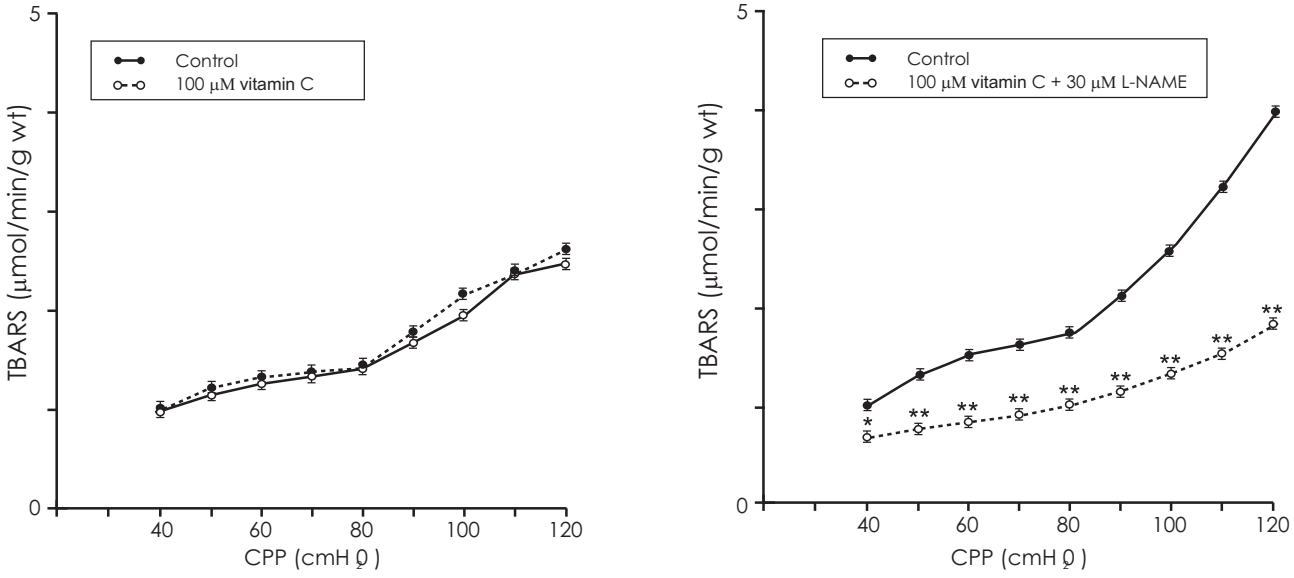

Figure 4. TBARS production (µmol/min/g wt) in coronary venous effluent at different coronary perfusion pressure (CPP) values (cm H_2O) in vitamin C-treated group (left) and in vitamin C+L-NAME-treated group (right). * $p < 0.05$, ** $p < 0.01$.

(1999) investigated the effects of vitamin C (0.1–100 mM) on the synthesis of NO by cultured human coronary artery and umbilical vein endothelial cells, and found that vitamin C increases the production of both citrulline, the byproduct of NO synthesis, and cyclic GMP, a marker of NO bioactivity. The authors implicated modulation by vitamin C of the availability or affinity of tetrahydrobiopterin to NOS as a possible mechanism for the observed increase in endothelium-derived NO synthesis. This vitamin C effect on NO production is evident at physiologically relevant extracellular and intracellular concentrations of ascorbic acid (Heller et al. 1999; Huang et al. 1999), while it was suggested that the superoxide anion scavenging mechanism might explain improved NO action only in studies that involved intra-arterial infusion of supraphysiological concentrations of ascorbic acid (Heitzer et al. 1996; Ting et al. 1996; Solzbach et al. 1997; Taddei et al. 1998), not following acute or chronic administration of physiological doses of ascorbic acid (Levine et al. 1996; Motoyama et al. 1997; Chambers et al. 1999). The results of our study (the absence of vitamin C effects on either O_2^- and TBARS levels or coronary flow) suggest that a mechanism other than O_2^- scavenging or flow-mediated NO increase was responsible for the increase in NO levels in isolated heart. One of the possible mechanisms may be the increase of dimeric form of NO synthase.

Although administration of vitamin C increased levels of NO (NO_2^-), coronary flow was not increased compared with flow in the control conditions. On the other hand, administration of vitamin C + L-NAME induced the fall in NO_2^- level which was followed by the fall in coronary flow. These effects are probably fully caused by L-NAME, since we previously showed that addition of the L-NAME alone induces significant fall in levels of nitrites and coronary flow (Jakovljevic and Djuric 2005). Furthermore, the decrease of TBARS level by vitamin C + L-NAME addition could reflect rather negative NO effect (production of peroxynitrites), especially after increasing perfusion pressure. It is demonstrated also by "opening scissors" between control and vitamin C + L-NAME TBARS levels and respective coronary perfusion pressure.

The limitations of our study are: 1) the heart rate and heart contractility were not registered; 2) the concentrations of DHA and OH^- were not measured in coronary venous effluent; 3) the real time measurement of various free radicals in tissue bath solutions was not performed; 4) concentration-response curves for vitamin C and L-NAME could not be obtained at all CPP values in this experimental model, taking into consideration long duration of such experiments and a great possibility to cause the heart injury (usual L-NAME concentration for the isolated heart was found from 0.3 μM to 300 μM and for vitamin C from 10 μM to 100 μM).

Although it was suggested that a loss of vitamin C tissue availability might impair its ability to counterbalance increased ROS production, as observed during post-ischemic reperfusion in the heart (Haramaki et al. 1998; Guaiquil et al. 2004) and that administration of exogenous antioxidants would improve recovery (Shuter et al. 1990), the results of our study clearly showed no significant effects of vitamin C administration either on ROS levels or on coronary flow in isolated rat heart.

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