Ozonized autohemotransfusion does not affect arterial vasodilation in patients with peripheral arterial disease

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A Coppola, L Coppola, C Luongo, et al. Ozonized autohemotransfusion does not affect arterial vasodilation in patients with peripheral arterial disease. *Int J Angiol* 2007;16(1):7-11.

Ozonized autohemotransfusion has been used as a complementary therapy in patients with peripheral arterial disease (PAD). To determine whether ozone therapy could acutely modify artery vasodilatory capacity, a flow-mediated dilation test was performed at the brachial artery level before and after an ozonized autohemotransfusion in 16 patients with PAD, mean ($\pm$ SD) age 55 ± 1.8 years, and 14 healthy volunteers matched for age, sex and body mass index. Before ozonized

autohemotransfusion, the mean baseline diameter of the brachial artery was higher in PAD patients than in healthy subjects (4.6 ± 0.54 mm versus 3.6 ± 0.54 mm, $P < 0.001$) while mean flow-mediated brachial artery dilation and percentage of increase in flow were significantly lower in PAD patients than in controls ($6.3 \pm 6.1\%$ versus $11.8 \pm 2.4\%$, $P < 0.02$; $433 \pm 61\%$ versus $580 \pm 46\%$, $P < 0.02$, respectively). No significant changes were observed after ozonized autohemotransfusion, indicating that ozonized autohemotransfusion does not modify endothelium-dependent ischemia-induced vascular reactivity.

Key Words: *Flow-dependent vasodilation; Ozone therapy; Vascular patients*

Ozonized autohemotransfusion has been used as a complementary therapy with the aim of improving tissue oxygenation in patients with critical ischemia of the lower limbs. Although large randomized trials supporting this approach are still lacking, a number of small clinical studies (1-5) agree there are beneficial effects of such therapy: longer pain-free intervals between *claudicatio intermittens* episodes and a decrease in the amputation ratio.

In previous studies (6-8), we demonstrated that in patients with occlusive peripheral arterial disease (PAD), the reinfusion of autologous blood after ozonization in a glass box tended to correct the altered hemostatic-hemorheological parameters (platelet aggregation, whole blood viscosity and erythrocyte filterability) and shifted the O_2 saturation curve to the right, thus improving blood flow and the release of O_2 from hemoglobin into the tissues. We also demonstrated that autohemotransfusion with ozonized blood per se does not significantly influence the fibrinolytic balance (9).

The mechanisms involved in these effects, however, have not yet been completely explained.

At variance with the reported beneficial effects of ozonized autohemotransfusion is the toxic role attributed to superoxides and other free radicals. In particular, short-term exposure to ozone air pollution (enough to increase O_3 concentration to $5 \mu\text{g}/\text{m}^3$) is associated with a significant increase of risk for acute myocardial infarction (10).

It is well known that flow-mediated dilation of large arteries counteracts neurogenic and myogenic vasoconstriction, increases conductance during exercise and maintains shear stress within physiological limits. This mechanism is endothelium-dependent and is mediated by normal activity of the L-arginine-nitric oxide pathway (11,12). It has been shown (13) that endothelium-dependent arterial dilation is impaired with advancing age, in patients with coronary artery disease, and in children and adults with risk factors for atherosclerosis. These factors include hypercholesterolemia, diabetes mellitus, hyperhomocysteinemia, hypertension and smoking (14-17). The impairment occurs before anatomical plaque formation becomes evident; thus, it has been considered a possible early event in atherogenesis.

On the other hand, it has been demonstrated that nitric oxide, reacted with a superoxide to form peroxynitrite, potentially acquires high toxicity. Peroxynitrite modifies the tyrosine residues in proteins that produce nitrotyrosines. Nitration of structural proteins, including neurofilaments and actin, can disrupt filament assembly – with functional and pathological consequences (18). Evidence has also been presented for an association between oxidative stress (with enhanced vascular superoxide anion production) and nitrate tolerance (19).

Considering that the oxidizing capability of ozone on a variety of biological molecules has been widely demonstrated

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(20-22), the present study was designed to verify the hypothesis that ozonized autohemotransfusion can acutely impair flow-mediated arterial vasodilation.

METHODS

Subjects

The present study enrolled 16 outpatients, mean age ($\pm$ SD) 55 ± 1.8 years (range 45 to 64 years), diagnosed with PAD, from the Oxygen-Ozone Therapy Service, Department of Anesthesiology, Reanimation and Intensive Therapy, Second University of Naples (Naples, Italy) (Table 1). After informed, written patient consent, study inclusion criteria were absence of diabetes, obesity, liver failure and/or kidney failure; absence of anticoagulant, fibrinolytic or vasoactive therapy taken during the three weeks preceding commencement of the study; absence of estrogen replacement therapy; and no smoking for at least two years. The diagnosis of PAD was based on the presence of lower limb symptoms (intermittent claudication, non-healing wounds, gangrene and eventual amputation) and/or an Ankle-Brachial Index value less than 0.9 (23). Fourteen volunteers, matched for sex, age and body mass index (Table 1), were recruited from the medical and paramedical staff of the Department of Geriatrics and Metabolic Diseases, Second University of Naples, after informed consent and had a Doppler examination of their legs to exclude subclinical vascular problems.

All subjects (PAD patients and healthy subjects) were on a normal sodium and isocaloric diet (55% carbohydrates, 20% protein and 25% fat) and were under appropriate metabolic and hemodynamic control.

Other clinical and metabolic characteristics of the study groups are presented in Table 1. The protocol was approved by The Ethics Committee of The School of Medicine, Second University of Naples.

Study protocol

Each subject was familiarized with the procedure by undergoing a simple (nonozonized) autohemotransfusion one day before the ozone testing. Between 08:00 and 10:00 on the day of the experiment, each of the 30 subjects (healthy volunteers and PAD patients), after an overnight fast, was instrumented for free-flowing blood sampling and ozonized autohemotransfusion. An 18-gauge Teflon catheter was inserted in a large antecubital vein in each subject's left arm. The vein was kept patent using a very slow (20 drops/min) saline (150 mM NaCl) infusion. The subject then rested supine for at least 20 min in a quiet, air-conditioned room maintained at a constant temperature (22°C to 24°C). Before and immediately after the ozonized autohemotransfusion, brachial artery diameter and flow velocity – at rest and after the induction of flow-mediated dilation – were measured in each subject by high-resolution ultrasound echography in the same manner as Celermajer et al (13). Venous blood samples were drawn at the same time and were used for measuring the plasma concentrations of malondialdehyde (MDA), a marker of lipid peroxidation.

Autohemotransfusion

An ozonized autohemotransfusion consisted of the reinfusion (5 mL/min) of 150 mL autologous blood that was exposed to an $\text{O}_2\text{-O}_3$ mixture, infusing the blood sample with 3600 μg ozone in a glass box, regulated by a Multiossigen Medical 93 instrument (Multi Tech, Italy). As a safety measure, all blood

ozonizations were carried out in special rooms with an appropriate system of gas evacuation-filtration as indicated by European Commission rules (98/79/EC). Instrumentation and pharmacological equipment for cardiovascular emergencies and reanimation were on hand. The autohemotransfusion was performed by a physician specialist in anesthesiology and expert in reanimation-resuscitation.

Measurements of brachial artery hemodynamics

The upper right arm was used for the ultrasound test. Measurements were performed while subjects rested supine for at least 20 min. Vascular imaging and induction of brachial arterial hyperemia were performed using a procedure described by others (13,24). A blood pressure cuff was placed just below the axilla, and the resting blood pressure was measured. A 7.5 MHz vascular ultrasound probe was then placed over the brachial artery longitudinally, approximately 5 cm above the antecubital crease, and held in place by an assistant. To take the largest artery diameter at each time point, the transducer position was adjusted by small mediolateral (transversal) movements. To assure measurement from the same cross section of the brachial artery, no longitudinal movements of the probe were allowed. Measurements were taken from the anterior to the posterior M line at the end of diastole, incident with the R wave of the electrocardiogram. Flow was calculated using Doppler flow velocity and vessel diameter.

After taking baseline measurements of flow velocity and brachial artery diameter, the blood pressure cuff was inflated to 50 mmHg above systolic pressure, thus arresting the flow of blood through the artery. After 5 min, the cuff was rapidly deflated and a second scan was taken at the peak increase in brachial artery flow, 15 s to 60 s after cuff deflation. Scans were repeated each minute for 5 min. Reactive hyperemia was calculated as the maximum flow recorded in the first 20 s after cuff deflation divided by the flow during the baseline scan.

Laboratory determinations

Plasma glucose, urea and total cholesterol were determined enzymatically using commercially available kits and a Kodak Ektachem 500 Analyzer (Eastman Kodak Company, USA).

The hematological parameters (hematocrit, hemoglobin and blood cell count) were evaluated using a model JT2 Coulter Counter (Beckman Coulter Inc, USA). Plasma fibrinogen concentration was measured by following the clotting method of Clauss (25) using a Clot 2S semiautomatic analyzer and a fibrinogen kit, both purchased from Radim Diagnostics, Italy.

MDA plasma levels were measured spectrophotometrically at 532 nm as thiobarbituric acid reactants, using the method of Waravdekar and Saslaw (26).

Statistical analysis

Data are presented as means $\pm$ SD. The nonparametric Mann-Whitney rank sum test was used to assess differences between parameters in the two groups (ie, PAD patients and healthy subjects) at baseline. ANOVA for repeated measures followed by a two-tailed paired *t*-test was used for differences before and after the ozonized autohemotransfusion and the induction of flow-mediated brachial artery dilation. A value of $P < 0.05$ was considered significant. The SigmaStat statistical program (Systat Software Inc, USA) was used for all analyses.

TABLE 1
Clinical and laboratory baseline characteristics of the study participants

	Healthy subjects, n=14	PAD patients, n=16	P
Sex (male/female)	7/7	8/8	NS
Age, years	54±4.5	55±1.8	NS
Body mass index, kg/m ²	25.7±1.1	26.3±0.9	NS
Blood pressure, mmHg			
Systolic	139±4.3	142±8.1	NS
Diastolic	77±3.1	75±6.2	NS
Plasma glucose, mmol/L	5.0±0.2	5.2±0.3	NS
Total cholesterol, mmol/L	5.3±0.4	5.4±0.3	NS
Hematocrit value, %	40.8±2.9	40.6±2.2	NS
Hemoglobin, g/L	138±15	136±19	NS
Fibrinogen, mmol/L	8.38±1.8	8.6±2.1	NS
Malondialdehyde, µmol/L	0.07±0.01	0.19±0.06	<0.001
Measures of vascular function			
Baseline brachial diameter, mm	3.6±0.5	4.6±0.5	<0.001
Baseline flow, mL/m	93±9	99±15	NS
Flow-mediated dilation, %	11.8±2.4	6.3±6.1	<0.02
Hyperemia (% increase in flow)	580±46	433±61	<0.02

Results presented as mean ± SD unless otherwise noted. For statistical analysis, the nonparametric Mann-Whitney rank sum test was used to assess differences between the two groups. NS Nonsignificant; PAD Peripheral arterial disease

Figure 1 Malondialdehyde (MDA) plasma levels before and after ozonized autohemotransfusion. PAD Peripheral arterial disease

RESULTS

Characteristic data of PAD patients and healthy subjects participating in the present study are shown in Table 1. There were no significant differences in demographic, biometrical and hematological parameters between the two groups. PAD patients had higher MDA plasma levels than the healthy subjects ($P<0.001$).

The baseline brachial artery diameter was greater in PAD patients than in healthy subjects (4.6 ± 0.54 mm versus 3.6 ± 0.54 mm, $P<0.001$). Flow-mediated brachial artery dilation and percentage increase in flow were significantly lower in PAD patients than in normal controls ($6.3\pm6.1\%$ versus $11.8\pm2.4\%$, $P<0.02$; $433\pm61\%$ versus $580\pm46\%$, $P<0.02$, respectively). Dilation was correlated inversely with resting vessel diameter ($r=-0.657$; $P<0.005$). There were no significant

TABLE 2
Measures of vascular function before and after ozonized autohemotransfusion

	Before	After	P
Healthy subjects			
Baseline brachial diameter (mm)	3.6±0.5	3.6±0.4	NS
Baseline mean flow (mL/m)	93±9	92±10	NS
Flow-mediated dilation (%)	11.8±2.4	10.5±0.4	NS
Hyperemic to baseline mean flow ratio (%)	580±46	571±49	NS
Peripheral arterial disease patients			
Baseline brachial diameter (mm)	4.6±0.5	4.6±0.7	NS
Baseline flow (mL/m)	99±13	103±14	NS
Flow-mediated dilation (%)	6.3±6.1	5.6±5.5	NS
Hyperemic to baseline mean flow ratio (%)	433±61	438±72	NS

Results presented as mean ± SD. ANOVA for repeated measures followed by two-tailed paired t test was used to assess differences before and after the ozonized autohemotransfusion. NS Nonsignificant

TABLE 3
Heart rate and arterial pressure* before and after ozonized autohemotransfusion

	Before			After		
	Baseline	Peak flow	P	Baseline	Peak flow	P
Healthy subjects						
Heart rate, beats/min	76±2	75±4	NS	75±4	75±3	NS
SBP, mmHg	139±4	138±6	NS	138±5	139±6	NS
DBP, mmHg	77±3	76±5	NS	76±4	76±5	NS
Peripheral arterial disease patients						
Heart rate, beats/min	73±5	72±6	NS	76±2.4	77±5	NS
SBP, mmHg	142±8	142±6	NS	141±9	142±8	NS
DBP, mmHg	75±6	75±6	NS	76±4	76±6	NS

*Measured at baseline and at peak increase in brachial artery flow (15 s to 60 s after cuff deflation). Results presented as mean ± SD. ANOVA for repeated measures followed by two-tailed paired t test was used to assess differences between values at baseline and at peak flow. DBP Diastolic blood pressure; SBP Systolic blood pressure; NS Nonsignificant

changes in heart rate or blood pressure (before and after cuff inflation) between the groups throughout the test.

After ozonized autohemotransfusion, MDA plasma levels (Figure 1) significantly increased, in both healthy subjects and in PAD patients from 0.07 ± 0.01 µmol/L to 0.71 ± 0.15 µmol/L, $P<0.001$; and from 0.19 ± 0.06 µmol/L to 2.22 ± 0.47 µmol/L, $P<0.001$, respectively. Measures of vascular function did not show any significant change compared with the respective baseline (before ozonized autohemotransfusion) (Table 2). Heart rate and blood pressure values did not change significantly throughout the test (Table 3).

DISCUSSION

The main result of the study is that the ozonized autohemotransfusion (reinfusion of autologous blood after ozonization in a glass box) does not significantly influence (neither impairs nor improves) the flow-mediated dilation of large arteries.

Based on a number of small clinical studies (1-5) that showed the beneficial effects of such therapy (longer pain-free intervals between *claudicatio intermittens* episodes, a decreased amputation

ratio), ozonized autohemotransfusion has been used as a complementary therapeutic modality in patients with critical ischemia of lower limbs. The most probable mechanism of these effects is the hemostatic-hemorheological one (6,8). A shift of the O₂ saturation curve to the right, with an improvement in the release of O₂ from hemoglobin into the tissues, has also been demonstrated (7).

However, because of its potent oxidizing capability on a variety of biological molecules (20-22), ozone could interfere with the normal activity of the L-arginine-nitric oxide pathway – the endothelium-localized metabolic pathway for arterial vasodilation (11). Results of large controlled clinical trials are absent in the scientific literature, but the hypothetical risk that acute vascular events, most importantly acute coronary events, could be triggered by the therapeutic use of ozonized autohemotransfusion demands attention.

There is no doubt that to achieve biological effects, ozone must induce acute oxidative stress (27). The balance between the potency of the natural antioxidant defences and the oxidizing effects of ozone is important for determining its toxic or therapeutic effect in tissues (28-31). Many clinical and experimental observations indicate that respiratory ozone toxicity occurs because of the minimal availability of neutralizing and antioxidant compounds in the enormous expanse of the bronchoalveolar space. With so few of these compounds, ozone and its derivatives are free to trigger an inflammatory reaction, documented by the release of reactive oxygen species, proteinases and proinflammatory cytokines (32-34).

The significant increase in the plasma levels of MDA observed in the present study, after ozonized autohemotransfusion, demonstrates that oxidative stress significantly increases with blood ozonation. In our previous experimental *in vitro* study (35), we observed that MDA plasma levels significantly increased and uric acid (a free radical scavenger) plasma levels significantly decreased after blood exposure to the O₂-O₃ mixture, with a positive dose-effect correlation. In any case, the results of the present study did not show any harmful effects of ozone on endothelium-dependent vascular dilatory mechanisms, which supports the clinical experiments indicating that ozonized autohemotransfusion is a substantial therapeutic instrument.

This apparent discrepancy between the beneficial effects reported in the present study and the generally reported toxic

effects of ozone might be explained by the following two considerations.

First, human blood has very high antioxidant defences (36), linked to a number of plasmatic (transferrin, ceruloplasmin, hemopexin, haptoglobin, albumin, uric acid), intracellular (superoxides, dismutases, catalases, glutathione peroxidases, ferritin) and intraextracellular (alpha-tocopherol) molecules. In the ozonized autohemotransfusion procedure, the autologous blood is slowly reinfused into the vascular system after it was thoroughly mixed in a glass box with the O₂-O₃ mixture. A very large spectrum of biochemical, morphological and functional changes has been documented (7,13,21,22,35) after extracorporeal exposure of whole blood, plasma or red blood cells to ozone, as a result of the confrontation between the natural antioxidant defences and the oxidizing effects of ozone. These changes include a decrease in formation of lipid hydroperoxides; a decrease in serum uric acid, coagulable plasmatic fibrinogen, platelet aggregability, whole blood viscosity and hemoglobin-oxygen affinity; an increase in plasmatic lactic dehydrogenase, free hemoglobin, malonyldialdehyde, K⁺ leakage and red blood cell filterability; and inhibition of membrane-associated Na⁺/K⁺ ATPase, glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase enzymes. Because of differences in ozone concentrations and experimental protocols employed, blood ozonation showed very different effects in different studies; these results together demonstrate rapid, highly multiform interactions between blood and ozone that are likely able to extinguish within a few minutes, or to limit the oxidative insult on the blood contained in the glass box.

Second, nitric oxide – possibly the main target for ozone-dependent interference with arterial vasodilation – is produced in endothelial cells, and diffuses rapidly and isotropically through the tissues in the smooth muscle of blood vessel walls. It binds to soluble guanylate cyclase, which activates and initiates the responses to ischemia in the blood vessels within seconds; it also enters red blood cells where it is rapidly destroyed by reacting with oxyhemoglobin (18). Because nitric oxide is not substantially transported via the blood vessels, it is not likely that the oxidizing activity in ozonized autohemotransfusion is mediated via nitric oxide.

For the time being, this discussion remains speculative. At the time of writing we can only say that ozonized autohemotransfusion does not modify endothelium-dependent ischemia-induced vascular reactivity.

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