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**R. Giunta · A. Coppola · C. Luongo · A. Sammartino
S. Guastafierro · A. Grassia · L. Giunta · L. Mascolo
A. Tirelli · L. Coppola**

Ozonized autohemotransfusion improves hemorheological parameters and oxygen delivery to tissues in patients with peripheral occlusive arterial disease

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Abstract Twenty-seven subjects suffering from peripheral occlusive arterial disease (POAD, clinical stage II–III according to Fontaine) were enrolled in this study to evaluate the effect of oxygen-ozone therapy upon hemorheological parameters and hemoglobin-oxygen affinity in patients with POAD. All patients underwent a major ozonized autohemotransfusion consisting of the slow reinfusion of 100 ml of autologous blood, previously exposed to a O_2-O_3 mixture in a glass box for 10 min. Whole blood viscosity, erythrocyte filterability, hematocrit, and fibrinogen levels were assessed at the basal time and 30 min after the reinfusion of ozonized blood. At the same time p50 standard (p50std) values (an indicator of hemoglobin-oxygen affinity) and plasma values of malonyl dialdehyde (MDA, an indicator of lipid peroxidation) were evaluated. At the baseline, patients had significantly higher ($p<0.05$–$p<0.001$) whole blood viscosity, MDA, and p50std values and significantly lower blood filterability ($p<0.01$) as compared with 20 matched healthy volunteers (controls). Thirty minutes after the end of a major autohemotransfusion, whole blood viscosity significantly decreased ($p<0.01$). This was accompanied by a significant fall in plasma fibrinogen level ($p<0.01$) with no change in hematocrit. Blood filterability, MDA plasma level, and p50std values increased significantly at the same time ($p<0.01$–$p<0.005$). The 2,3–DPG value did not change significantly. No significant changes occurred when the same patients received a non-ozonized autohemotransfusion (control test). In conclusion, ozonized autohemotransfusion may be useful to improve both the poor rheological properties of the blood and the oxygen delivery to tissues in patients suffering from POAD.

Keywords Ozonized autohemotransfusion · Hemorheology · Peripheral occlusive arterial disease

R. Giunta · A. Coppola · A. Grassia · L. Giunta · L. Coppola
Department of Geriatrics and Metabolic Diseases,
Second University of Naples, Piazza L. Miraglia, 2,
80138 Naples, Italy

C. Luongo · A. Sammartino · L. Mascolo
Hyperbaric Medicine Service, Second University of Naples,
Piazza L. Miraglia, 2, 80138 Naples, Italy

S. Guastafierro (✉) · A. Tirelli
Department of Hematology, Second University of Naples,
Largo Madonna delle Grazie, 2, 80138 Naples, Italy
e-mail: ematologia@libero.it
Fax: +039-81-5665587

Introduction

Data from large epidemiological investigations indicate that hemostatic and rheological factors play a major role in the pathogenesis of atherosclerosis and its complications (myocardial infarction, stroke, and peripheral ischemia) [1, 2, 3, 4, 5, 6]. Amelioration of resting pain, intermittent claudication, and ischemic-dependent metabolic disorders of the leg have been reported after oxygen-ozone therapy, which has validated its empirical use in the treatment of peripheral vascular disease [7, 8]. In previous studies we demonstrated that oxygen-ozone treatment increases erythrocyte deformability and reduces both plasma and whole blood viscosity [9, 10]. These findings support the view that ozone-induced clinical improvement of chronic arterial insufficiency might be dependent on its influence on the blood rheological properties. It is well-known that optimal tissue oxygenation depends on three main factors: blood flow, hemoglobin concentration, and affinity of hemoglobin for oxygen [11]. The adjustment of hemoglobin affinity for oxygen is important to maintain optimal tissue oxygenation in many pathological circumstances in which blood flow is reduced (mainly in peripheral arterial disease).

The aim of this study was to evaluate the effects of ozonized autotransfusion on both hemorheological parameters and hemoglobin-oxygen affinity in patients suffering from peripheral arterial disease.

Materials and methods

Population

Informed consent was obtained, after a clear explanation of the potential hazards of the experimentation, from 27 consecutive patients (15 males and 12 females, mean age: 66±11 years) with clinical evidence of peripheral arterial disease (stage II–III according to Fontaine: intermittent claudication, resting pain). A classic physical and noninvasive testing schedule confirmed the diagnosis. A group of 20 matched healthy volunteers was enrolled as controls.

The most important exclusion criteria for the study were (1) diabetes, (2) anemia, (3) polycythemia, (4) hemoglobinopathies, (5) chronic pulmonary disease, and (6) actual smokers of more than ten cigarettes daily. The patients were not taking thrombolytic or anticoagulant drugs; compounds known to interfere with hemorheological parameters were not permitted in the 7 days before testing.

Ozonized autotransfusion and control test

On the day of the test, in the morning after an overnight fast, two Teflon catheters were inserted into a large antecubital vein in both arms of each patient. One was immediately inserted and kept patent by a slow saline (150 mmol NaCl) infusion to allow free-flowing blood sampling, and the other was inserted after 15 min and used for autohemotransfusion consisting of the slow reinfusion of 100 ml of autologous blood previously exposed to a O_2-O_3 mixture in a glass box. Ozone was made up with a machine (Multiossigen Medical 93, Multi Tech, Milan, Italy); the required quantity (3.6 mg of total ozone) was withdrawn with a syringe and added to the blood in the glass box. The blood was gently mixed for 10 min and then slowly reinfused (5 ml/min).

Before and 30 min after the reinfusion of ozonized blood, venous blood samples were withdrawn for the determination of both hemorheological parameters (whole blood viscosity, erythrocyte deformability, fibrinogen, and hematocrit) and hemoglobin-oxygen affinity.

A control test was performed on a different day and in random order for each patient to exclude the possible influence of simple blood manipulation by evaluating the same parameters before and after a non-ozonized autohemotransfusion.

Methodology

Whole blood viscosity was evaluated at 37°C at two different rates of shear (225 s^{-1} and 45 s^{-1}), in a Wells–Brookfield micro-cone/plate digital viscometer (Model LVT 0.8°cone, Brookfield Engineering Laboratories Inc., Stoughton, Mass., USA). The calibration of the cone and cup was frequently performed using two Brookfield silicon fluid standards. Erythrocyte deformability was measured with a filtration technique according to the method of Reid et al. [12]. Blood was filtered through a Nucleopore polycarbonate sieve containing cylindrical channels with a diameter of 5 μm under a pressure of 20 cm of water. Results are expressed as the volume of red blood cells filtered in 1 min according to the formula: red blood cell velocity (VRBC) (ml/min)=60×*Ht*/*t*, where *t* indicates the time occurring for the filtration of 1 ml of blood and *Ht* is the hematocrit value. Fibrinogen was determined by a clotting method [13] and hematocrit by standard procedures. The hemoglobin-oxygen affinity was assessed using the p50 standard value (p50std). This value is defined as oxygen tension (mmHg) at 50% oxygen saturation, at pH 7.4, at 37°C, and at PCO_2 40±2 mmHg. It indicates the position of the oxygen-hemoglobin saturation curve and is calculated according to the formula [14]:

$$p50std = anti\log\left[\log PO_2 - \frac{\log SO_2}{2.7} - 0.4 \times (7.4 - pH)\right]$$

The plasma values of both 2,3-diphosphoglycerate (2,3-DPG), an important regulator of oxygen unloading, and of malonyl dialdehyde (MDA), an indicator of lipid peroxidation, were evaluated in all patients before and 30 min after ozonized autohemotransfusion. The 2,3-DPG values were assessed on a protein-free supernatant (3 ml of 8% trichloroacetic acid plus 1 ml of heparinized blood) using a quantitative enzymatic method involving Sigma Diagnostic reagents (Sigma, St. Louis Mo. USA). MDA levels were measured as thiobarbituric acid reactant according to Warawdekar and Saslaw [15].

Statistical analysis

All data are presented as mean±standard deviation (SD). A preliminary analysis of variance (ANOVA) was used to assess differences (before and after treatment) within and between the groups (peripheral arterial occlusive patients and normal subjects). When a significant *p* value was found, the differences between baseline and post-ozone measures were assessed by a paired, two-tailed *t*-test.

Results

Table 1 shows the clinical and laboratory characteristics of the subjects investigated. At baseline, patients with peripheral arterial disease had significantly higher ($p<0.05$–$p<0.001$) whole blood viscosity, MDA, and

Table 1. Clinical and laboratory characteristics of the subjects investigated. Values are mean±SD. *BMI* body mass index, *MDA* malonyl dialdehyde, *2,3-DPG* 2-3diphosphoglycerate

	Peripheral arterial occlusive disease	Normal subjects
n	27	20
Age (years)	66±11	65±10
Sex (M/F)	15/12	11/9
BMI (kg/m^2)	26±0.9	26±2.7
Blood viscosity (mPa.s)		
225 s^{-1}	7±1.3	5.3±0.8**
45 s^{-1}	15±3	7.3±0.6**
Blood filterability VRBC (ml/min)	0.58±0.1	0.98±0.1**
Fibrinogen (g/l)	4.1±0.8	3.4±0.6**
Hematocrit (%)	40±2	40±1.3
MDA (μg/dl)	6±1.3	1.2±0.3***
p50std (mmHg)	28±0.9	27±0.8*
2,3-DPG (mmol/l)	2.5±0.2	2.3±0.6

*$p<0.05$; **$p<0.01$; ***$p<0.001$

Table 2 Effect of ozone treatment on hemorheological parameters, hemoglobin-oxygen affinity, and MDA plasma level. Data are mean±SD. *MDA* malonyl dialdehyde, *2,3-DPG* 2-3diphosphoglycerate

	Before	After
Blood viscosity (mPa.s)		
225 s^{-1}	7±1.3	6.1±1.1*
45 s^{-1}	15±3	9.8±2.1*
Blood filterability VRBC (ml/min)	0.58±0.1	0.9±0.1**
Fibrinogen (g/l)	4.1±0.8	3.5±0.8*
Hematocrit (%)	40±2	40±2.4
MDA (µg/dl)	6±1.3	28±5.4**
p50std (mmHg)	28±0.9	32±1.1**
2,3-DPG (mmol/l)	2.5±0.2	2.5±0.3

*p<0.05; **p<0.01

Table 3 Effect of simple non-ozonized autohemotransfusion on hemorheological parameters, hemoglobin-oxygen affinity, and MDA plasma level. Values are mean ± SD. *MDA* malonyl dialdehyde, *2,3-DPG* 2-3diphosphoglycerate

	Before	After
Blood viscosity (mPa.s)		
225 s^{-1}	7.2±1.4	7.0±1.1
45 s^{-1}	14±4.1	15±3.9
Blood filterability VRBC (ml/min)	0.56±0.1	0.6±0.2
Fibrinogen (g/l)	4.0±1.4	4.0±1.1
Hematocrit (%)	40±2	40±2
MDA (µg/dl)	5.9±1.4	8.3±2.7
p50std (mmHg)	27.9±1.2	28.2±1.3
2,3-DPG (mmol/l)	2.6±0.3	2.6±0.4

p50std values whereas they had significantly lower blood filterability (p<0.01) than normal subjects. The effects of ozonized autohemotransfusion therapy are shown in Table 2. Thirty minutes after the end of a major autohemotransfusion, whole blood viscosity at both rates of shear (225 s^{-1} and 45 s^{-1}) significantly decreased (p<0.01). This was accompanied by a significant fall in plasma fibrinogen level (p<0.01) with no change in hematocrit. Blood filterability, MDA plasma level, and p50std values significantly increased (p<0.01–p<0.005). The 2,3-DPG value did not change significantly.

No significant changes (Table 3) occurred when the same patients received a non-ozonized autohemotransfusion (control test).

Discussion

Data from the present study confirm that ozone favorably influences hemorheological parameters in patients suffering from peripheral occlusive arterial disease in agreement with results from our previous studies on the in vitro effects of an oxygen-ozone mixture [10]. In fact, both whole blood viscosity and plasma fibrinogen levels (increased at baseline) and the erythrocyte filterability (decreased at baseline) tend to normalize 30 min after ozonized autohemotransfusion.

Furthermore, our results allow the hypothesis that ozonized-autotransfusion increases the oxygen delivery to tissues. This is supported by the significant increase of the p50std value, which indicates a shift towards the right in the oxygen-hemoglobin dissociation curve.

The mechanism by which oxygen-ozone therapy exerts its effects is unknown. The absence of significant changes in control tests (non-ozonized autohemotransfusion) excludes that the results are due to blood manipulation. This study shows that plasma concentration of MDA significantly increases after ozonized autotransfusion. Increased oxidation stress and lipid peroxidation (evaluated by malonyl dialdehyde release) can, at least in part, account for the effects of ozone both on hemorheological parameters and on hemoglobin-oxygen affinity.

We hypothesize that a very selective in vivo cellular lysis of aged and more rigid erythrocytes occurs during O_2-O_3 therapy. Therefore, whole blood viscosity and erythrocyte filterability may improve even without a significant decrease of the hematocrit value. This hypothesis is supported by results from our previous in vitro study with an oxygen-ozone mixture at different ozone final concentrations on washed blood cells [16]. When the ozone final concentration changes from 6.5 to 78 µg/ml, the increase of the lipoperoxidation indicator (MDA) plasma level significantly correlates with the increase of both free hemoglobin in the supernatant and lactic dehydrogenase (LDH) level (indicators of blood cell lysis).

On the other hand, ozone-induced lipid peroxidation of red blood cell membrane may alter intracellular pH and shift the oxygen-hemoglobin dissociation curve towards the right (Bohr effect), interfering with ionic transmembrane pumps. In any case, 2,3-DPG concentration, the other primary regulatory mechanism of oxygen delivery to tissues, does not significantly change after acute ozone therapy.

In conclusion, this study demonstrates that ozone significantly improves hemorheological parameters and increases oxygen unloading of hemoglobin in patients with peripheral occlusive arterial disease. The patients, when interviewed, related positive clinical effects (amelioration of resting pain and improvement in exercise capacity) after ozonized autohemotransfusion. These results encourage the use of ozonized autohemotransfusion in this field. However, controlled clinical studies are required to confirm clinical efficacy of ozonized autotransfusion in these patients.

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