

Background: Empirical observations have shown that ozonated autohemotherapy markedly improves the symptoms of chronic limb ischemia (muscular pain at rest, intermittent claudication, etc) in atherosclerotic patients, but mechanisms of action remain unclear.

Aims: Human endothelial cells (HUVECs) are known to release nitrogen monoxide (NO) and we investigated the biological effects of human ozonated serum on HUVECs in culture.

Methods: We assessed the relevance of peroxidation, the release of NO as nitrite and of three classical cytokines.

Results: The treatment of HUVECs with ozonated serum yields a dose dependent increase of thiobarbituric acid reactive substances (TBARS) and of hydrogen peroxide (H_2O_2) and a decrease of protein thiol groups (PTG). Concomitantly, in comparison to either the control or the oxygenated sample, there is a significant and steady increase of nitric oxide (NO) production; this is markedly enhanced by the addition of L-arginine ($20 \mu M$) and inhibited in the presence of the NO inhibitor, L-NAME ($20 mM$). The main mediator of ozone action is H_2O_2 as it has been shown either after its direct measurement or by the addition of 20, 40 and $100 \mu M$. Moreover, during 24 hours incubation we have investigated the production of endothelin 1 (ET-1), E-selectin and Interleukin 8 (IL-8) and it appears that ozonation enhances IL-8, inhibits E-selectin and hardly modifies ET-1 production.

Conclusions: It appears that reinfusion of ozonated blood, by enhancing release of NO, may induce vasodilation in ischemic areas and reduce hypoxia.

Key words: Ozone, Reactive oxygen species, Hydrogen peroxide, Nitric oxide, Endothelial cells, Endothelin-1, E-selectin, Interleukin-8

Studies on the biological effects of ozone: 11. Release of factors from human endothelial cells

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Introduction

Blood vessel tone undergoes a complex regulation by vasodilators (NO, CO, acetylcholine, adenosine, prostacyclin) and vasoconstrictor factors such as superoxide anion, endothelins, thromboxane, angiotensin II and noradrenaline.^{1–3} In vascular disorders, particularly atherosclerosis, the regulation becomes chaotic owing to the interference of oxidants, adhesion molecules, cytokines and growth factors leading to partial vessel occlusion and hypoxia. Chronic limb ischemia is a progressive disease often observed in atherosclerosis and diabetes leading to necrosis of extremities. Conventional therapy is carried out with infusion of prostanoids, vasodilators and antiaggregants but there is vast empirical evidence⁴ that reinfusion of autologous blood briefly exposed to a gas mixture composed of oxygen-ozone (O_2-O_3) is similarly, if not more beneficial.

However, so far double-blind randomized clinical trials have not been performed and, for the time

being, we considered it worthwhile to investigate whether human ozonated plasma elicits any effects on human umbilical vein endothelial cells (HUVECs) in culture that may help to understand the supposed therapeutic effect. The present data show that reactive oxygen species (ROS) and/or lipid oxidation products (LOP) derived from the interaction of ozone with plasma⁵ enhance the production of nitric oxide (NO), interleukin-8 (IL-8) while the release of either E-selectin or endothelin-1 (ET-1) is either inhibited or hardly modified, respectively.

Materials and methods

Materials

Analytical grade H_2O_2 30% was purchased from Fluka GmbH, Switzerland. L-Arginine, the NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) and all the materials not specified below were purchased from Sigma Aldrich srl, Milano, Italy.

Ozone generation and measurements

O₃ was produced from medical grade O₂ using electrical corona arc discharge with a modern O₃ generator (Model Ozonosan PM 100K, Hansler GmbH, Iffezheim, Germany) which allows the gas flow rate and O₃ concentration to be controlled in real time by photometric determination at 253.7 nm as recommended by the Standardization Committee of the International Ozone Association. Tygon polymer tubing and glass syringes were used throughout the reaction procedure to ensure containment of O₃ and consistency in concentration.

Ozone delivery to biological samples

A predetermined volume of O₂/O₃ gas mixture at various O₃ concentrations within the therapeutic range (20–80 µg/ml of gas per ml of serum) was collected with a glass syringe and immediately introduced into a second glass syringe, via a 'Y' connector, containing an identical volume of a pool of human serum (obtained from the Siena Clinical Blood Center). The final gas pressure remained at normal atmospheric pressure. In order to obtain reproducible results it needs to be emphasized that O₃ is a very reactive gas so rapid and precise handling is required. Samples were gently but continuously mixed with the gas for 20 min and afterwards dispensed either into test tubes for analyses or added to cell cultures. Control samples were mixed with an equal volume of O₂. It is worth mentioning that O₂ represents at least 96% of the O₂-O₃ mixture.

Culture of Human Umbilical Vein Endothelial Cells (HUVECs)

Primary endothelial cells were obtained from the neonatal umbilical cord vein according to Jaffe et al.⁶ The main vein of umbilical cords (kindly provided by the nursery of 'Le Scotte' hospital of Siena) was thoroughly washed with PBS and then incubated with 10 ml collagenase (0.2 % dissolved in Medium 199) from *Clostridium histolyticum* (type XI, Sigma) for 20–25 min at 37°C. The endothelial cells layer was removed from the vein using 50 ml of Hanks buffer (Sigma). The cells were sedimented at 250×g for 10 min and washed once with 20 ml of Medium 199 with 20% FCS (Seromed), and resuspended in 5 ml of fresh medium. Cells were grown in Medium 199, pH 7.4 (Gibco BRL, Rockville, MD) supplemented with 20% FCS, 2 mM glutamine, 10 mM Hepes, 100 µg/ml heparin, streptomycin-penicillin and 100 µg/ml of endothelial cell growth factor (ECGF). For subculture, the cells were harvested with 0.01% EDTA-0.1% trypsin.

HUVECs were utilized for experiments at 90–95% (3–3.5×10⁶ cell/well) apparent confluence within

passage 3 and through 6. Passage were performed according to standardized protocols and by diluting the cell population 1:3.

Cell treatment

The cells were pelleted by centrifugation and seeded in 6 well plates pre-treated with 1.5% gelatin (type B from bovine skin). To each well 2 ml of M199 supplemented with 1% FCS, without heparin and ECGF was added for 24 h. The day after cells confluence was around 90–95 % (3–3.5×10⁶ cell/well). The cells were washed once in Hank's balanced salt solution. Ozonated serum (2 ml) was added to the cells but after 20 min incubation the serum was collected for biological determination and the cells were grown as described above, but without phenol red in the medium.

Determinations

Hydrogen peroxide (H₂O₂) was measured in serum before and after addition of oxygen and ozone by the enzymatic method described by Green and Hill.⁷ Protein thiol groups (PTG) were measured in serum according to Hu⁸ using procedure 1 with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) dissolved in absolute methanol. The thiobarbituric acid (TBA) assay was carried out in serum as described by Buege and Aust.⁹ Values are expressed as µM of TBA reactive substances (TBARS) relative to a malondialdehyde standard. By collecting samples at different incubation times (from 2 to 48 h), NO production was assayed by determining the increase in nitrite concentration using the classical Griess reaction.¹⁰ Cytokines such as IL-8, ET-1 and E-selectin present in centrifuged tissue cultured fluids, were kept at –80°C until determinations were carried out by immunoassay using standard kits purchased from R&D Systems (GmbH, Wiesbaden, Germany). Duplicate samples were diluted 1:1 with the appropriate diluent and a 3-cycle automatic washing process was routinely performed.

Statistical analyses

All statistical analyses were performed using one-way analysis of variance (ANOVA) with the Scheffé multi-range test. All values are expressed as Means and Standard Deviation (±SD). *P* values less than either 0.05 or 0.01 were considered significant and are marked with 1 or 2 asterisks, respectively.

Results

Initially we investigated whether ozonated human serum added to HUVECs modifies NO production. Figure 1 shows that the exposure of serum for 1 min to two different concentrations (40 and 80 µg/ml of

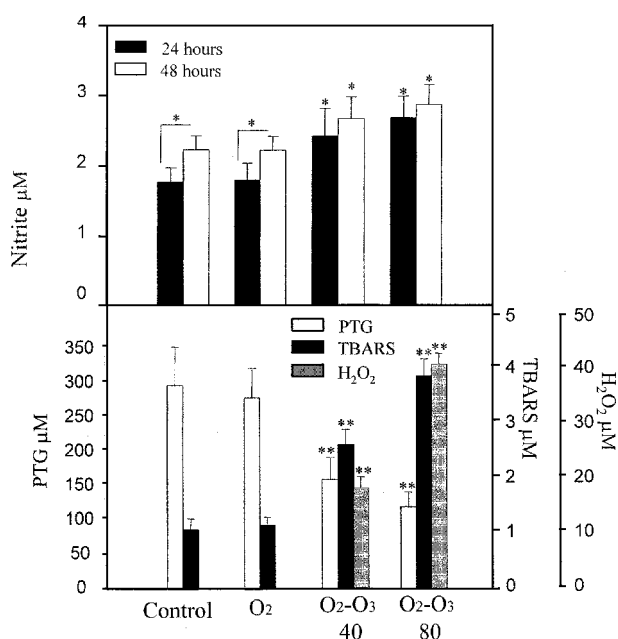


FIG. 1. Effect of different concentrations of ozone on the production of nitrite by HUVECs, 24 and 48 h after addition of ozonated human serum (top panel). Effect of either oxygen or ozone on PTG, TBARS and H₂O₂ levels in the serum before addition to HUVECs. The data are presented as the arithmetic mean ±SD of 6 different experiments.

gas per ml of serum) induces a significant ($P < 0.01$) reduction of PTG levels while TBARS and H₂O₂ levels increase. Both control serum (untreated) or O₂ exposed samples show neither modification of TBARS and PTG values, nor any production of H₂O₂. HUVECs incubated with O₂-O₃ treated serum samples show an increase of NO production measured as nitrite, that becomes significant ($P < 0.05$) after 24 and 48 h and is O₃ dose dependent. Nitrite levels were not modified during the first 6 h of incubation.

Following this we checked if NO release could be influenced by the addition of the substrate L-arginine, or by the inhibitor L-NAME (Fig. 2). L-arginine on its own markedly ($P < 0.05$) increases NO release in both control and O₂-serum treated HUVECs but NO production is significantly higher when cultures are added with ozonated serum samples, particularly at ozone concentration of 80 µg/ml. The presence of L-NAME inhibits NO release in all cases.

Figure 1 shows that ozonation of serum is accompanied by H₂O₂ production confirming Pryor's reaction¹¹ and our previous data.¹² Thus it would be interesting to evaluate if H₂O₂ is able to influence NO release. Figure 3 shows that there is an almost linear increase between NO release and progressively higher amounts (20, 40 and 100 µM) of H₂O₂ added to HUVECs. Moreover addition of L-arginine strongly reinforced NO production while L-NAME inhibited to a great extent, but not entirely, its production.

H₂O₂ is now considered a crucial trigger of NF-κB^{13,14} and it appeared obvious to examine whether

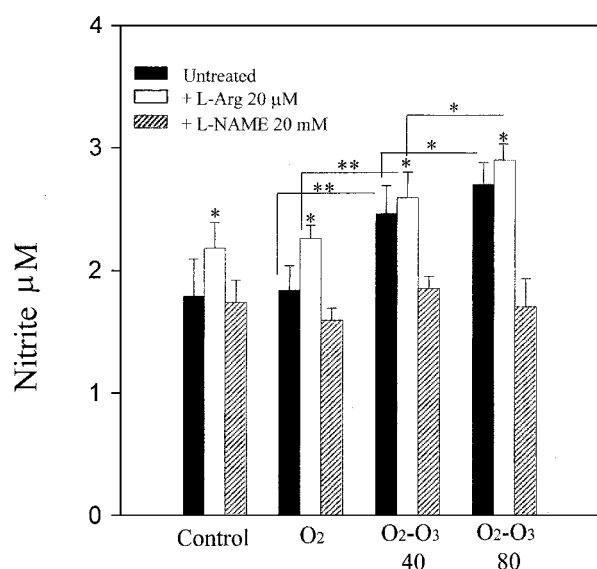


FIG. 2. Production of nitrite by HUVECs, measured after 24 h incubation, after addition of normal human serum either oxygenated or ozonated (at 40 and 80 µg/ml). Effects of addition of L-arginine and L-NAME. The data are presented as the arithmetic mean ±SD of 6 different experiments.

O₃-H₂O₂ induces release of a few typical cytokines such as ET-1 (Fig. 4), E-selectin (Fig. 5) and IL-8 (Fig. 6). Ozone at both concentrations (40 and 80 µg/ml) significantly enhances the release of IL-8 after 4, 6 and 24 hours incubation with and without addition of L-arginine confirming the ability of ozone to induce IL-8 production in blood^{15,16} and respiratory epithelial cells.¹⁷ Release of E-selectin or ET-1 appears either depressed or practically unmodified.

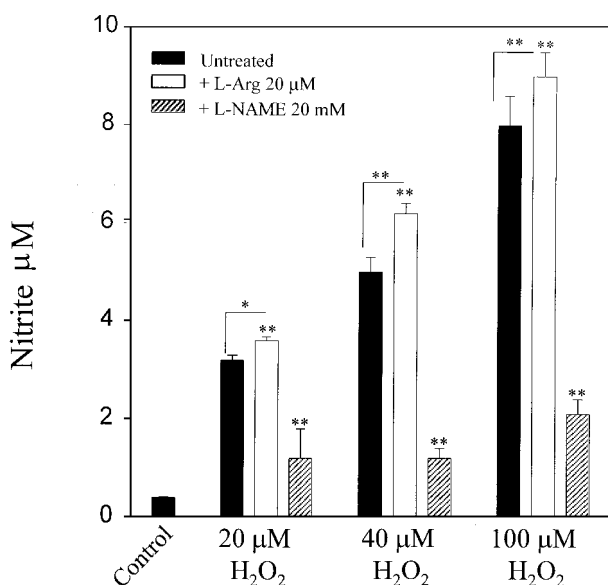


FIG. 3. Production of NO by HUVECs, measured after 24 h incubation, after addition of three concentration of H₂O₂. Effects of the addition of L-arginine and L-NAME. The data are presented as the arithmetic mean ±SD of 6 different experiments.

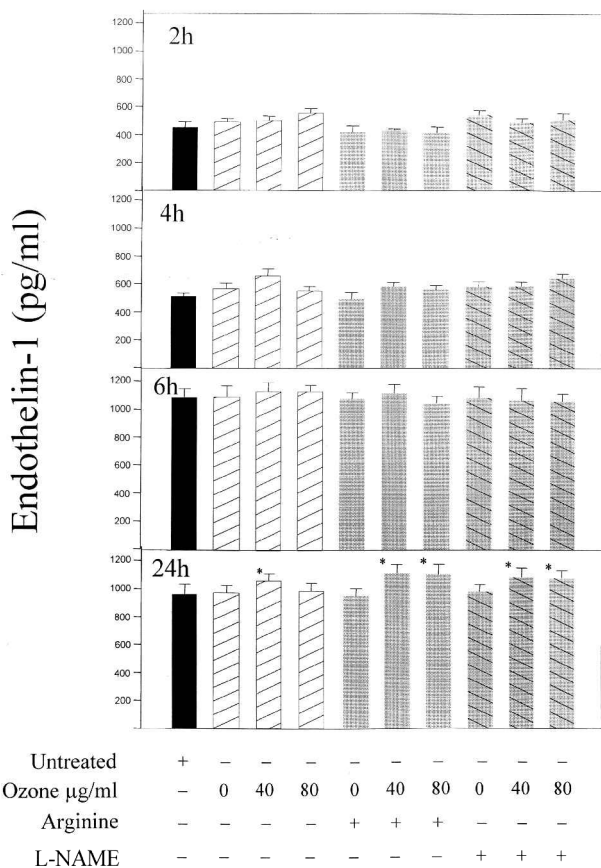


FIG. 4. Kinetic of release of endothelin-1 from HUVECs after addition of human serum either after oxygenation or after ozonation. Effect of the addition of L-arginine and L-NAME. The data are presented as the arithmetic mean \pm SD of 6 different experiments.

Discussion

Vascular disorders caused by atherosclerosis, diabetes and smoking at the level of the brain, heart and limbs represent a formidable social-economical problem affecting several millions of people worldwide. Orthodox medicine uses a variety of approaches such as surgery, anticoagulants, trombotic compounds, vasodilators antioxidants and now even gene therapy¹⁸ for normalizing damaged vessels, reducing restenosis and minimising damage following severe ischemia. During the last decade our laboratory has been involved in clarifying mechanisms of action triggered by ozone when this gas is briefly mixed with human blood.⁴ Like oxygen, ozone dissolves in the plasma and reacts quickly with a number of substrates such as polyunsaturated fatty acids (PUFA), -SH groups present in several compounds and antioxidants.¹¹ An array of generated products namely H₂O₂, malondialdehyde, 4-hydroxynonenal, hydroperoxides and lipoperoxides¹⁹ may act as cellular signals and trigger biological effects. It must be said that these compounds, depending on their concentrations, seem to behave as a double-edged sword.²⁰ For this reason we have

been very careful in defining the biological active dose that, in terms of ozone concentration, ranges between 20 and 80 $\mu\text{g/ml}$ gas per ml of blood without displaying toxic effects neither *in vitro* nor *in vivo*.^{4,21} Empirical observations have shown that ozone enhances delivery of oxygen to hypoxic tissue and we have hypothesized that ozonated plasma during reinfusion may elicit some effects on the vessel wall possibly enhancing vasodilatation. Indeed the present results give credence to this idea by showing an enhanced production of NO, which is an important component of the negative feed-back loop that prevents thrombogenesis. The adherence of platelets to the vessel wall, and their aggregation, is suppressed by nitrovasodilators capable of increasing the intracellular cGMP, with consequent phosphorylation of cGMP-dependent phosphoproteins and suppression of platelet reactivity.²² The possible formation *in vivo* of nitroso compounds, with longer lifetime than NO, may slowly decrease the vasospasm.

Our data showing inhibition of release of E-selectin, especially after 2 and 6 hours, and no increase of ET-1. This is consistent with previous reports²³⁻²⁶ showing a reciprocal regulation of the expression of ET-1 and endothelial cell adhesion molecules in comparison to

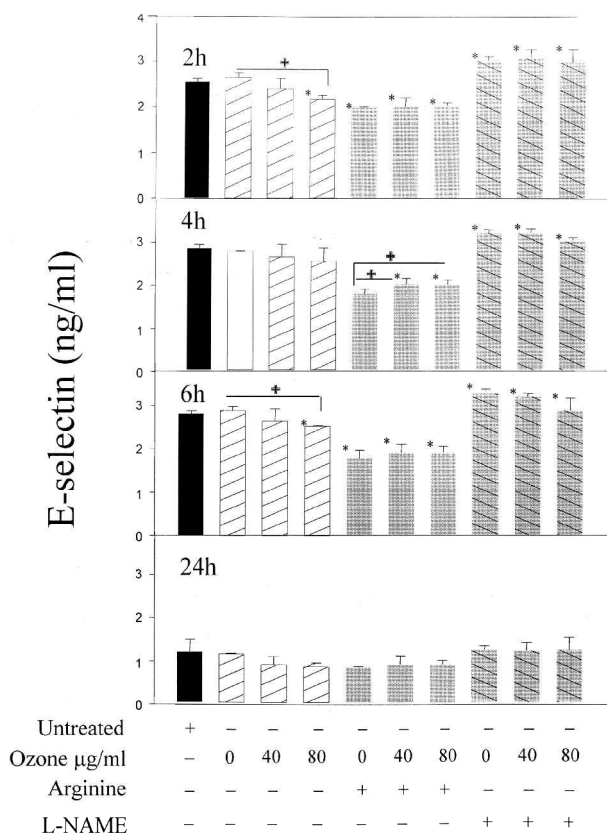


FIG. 5. Kinetics of release of E-selectin from HUVECs after addition of human serum either after oxygenation or after ozonation. Effect of the addition of L-arginine and L-NAME. The data are presented as the arithmetic mean \pm SD of 6 different experiments.

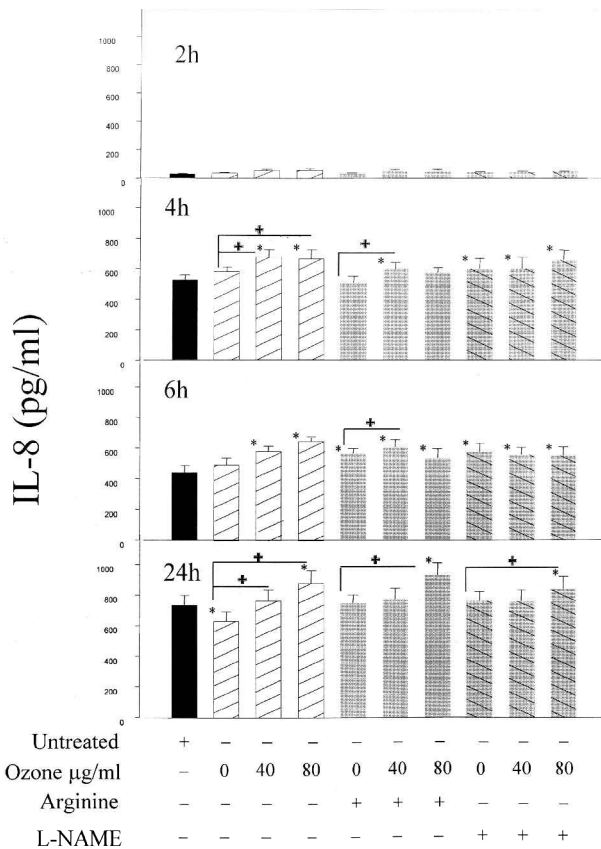


FIG. 6. Kinetics of release of IL-8 from HUVECs after addition of human serum either after oxygenation or after ozonation. Effect of the addition of L-arginine and L-NAME. The data are presented as the arithmetic mean \pm SD of 6 different experiments.

production of NO. At present, we cannot say whether ozonated serum is able to increase gene expression of VEGF,²⁷ that is whether a potent mitogen that by stimulating neoangiogenesis may be helpful in coronary and critical leg ischemia. This is a very important point that unfortunately has not been clarified. However Chua *et al.*²⁸ have clearly shown that VEGF mRNA was expressed in a dose- and time-dependent manner when rat heart endothelial cells were exposed to 0.5–1 mM H₂O₂.

In conclusion we have shown for the first time that human serum briefly exposed to biologically active and atoxic ozone concentration (from 20 to 80 µg/ml per ml of plasma) after a 20 min incubation with HUVECs induces H₂O₂ production (up to 40 µM). This concentration is possibly underestimated because it has been observed¹² that the mean half-life of H₂O₂ in plasma is about 2 min. Addition of H₂O₂ to HUVECs, in turn, as we have now shown, induces a dose-dependent increase of NO further enhanced by L-arginine. It must be emphasized that HUVECs were kept in contact with ozonated serum for only 20 min. This approximately corresponds to the period of time of blood reinfusion. So far we have not addressed the issue of whether NO

production is due to constitutively present NO-synthase (Ca⁺⁺ dependent), typical of the endothelium, or the newly induced enzyme (iNOS, Ca⁺⁺ independent) which seems most likely as at 2, 4 and 6 hours after treatment we could not measure any nitrite increase. However work in progress will clarify this question and elucidate whether ozonated plasma added to HUVECs, simulating the reinfusion of ozonated blood in patients, is able to induce neoangiogenesis.

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