

Extracorporeal blood oxygenation and ozonation: clinical and biological implications of ozone therapy

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Some lines of evidence have suggested that the challenge to antioxidants and biomolecules provoked by pro-oxidants such as ozone may be used to generate a controlled stress response of possible therapeutic relevance in some immune dysfunctions and chronic, degenerative conditions. Immune and endothelial cells have been proposed to be elective targets of the positive molecular effects of ozone and its derived species formed during blood ozonation. On the bases of these underlying principles and against often prejudicial scepticism and concerns about its toxicity, ozone has been used in autohemotherapy (AHT) for four decades with encouraging results. However, clinical application and validation of AHT have been so far largely insufficient. Latterly, a new and more effective therapeutic approach to ozone therapy has been established, namely extracorporeal blood oxygenation and ozonation (EBOO). This technique, first tested *in vitro* and then *in vivo* in sheep and humans (more than 1200 treatments performed in 82 patients), is performed with a high-efficiency apparatus that makes it possible to treat with a mixture of oxygen–ozone (0.5–1 µg/ml oxygen) in 1 h of extracorporeal circulation up to 4800 ml of heparinized blood without technical or clinical problems, whereas only 250 ml of blood can be treated with ozone by AHT. The EBOO technique can be easily adapted for use in hemodialysis also. The standard therapeutic cycle lasts for 7 weeks in which 14 treatment sessions of 1 h are performed. After a session of EBOO, the interaction of ozone with blood components results in 4–5-fold increased levels of thiobarbituric acid reactants and a proportional decrease in plasma protein thiols without any appreciable erythrocyte haemolysis. On the basis of preliminary *in vitro* evidence, these simple laboratory parameters may represent a useful complement in the routine monitoring of biological compliance to the treatment. The clinical experience gained so far confirms the great therapeutic potential of EBOO in patients with severe peripheral arterial disease, coronary disease, cholesterol embolism, severe dyslipidemia, Madelung disease, and sudden deafness of vascular origin. Extensive investigation on oxidative stress biomarkers and clinical trials are under way to validate this new technique further.

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Theoretical approach to ozone therapy

Ozone is erroneously regarded as exclusively toxic for the human body. Although it may cause severe damage if inhaled,^{1–3} it can have a range of therapeutic uses (reviewed by Bocci¹). In particular, this gas has topical therapeutic activity in various skin diseases.⁴ In the early

1990s, the bactericidal properties of ozone were demonstrated and are now used to sterilise food and water.⁵

In 1974, Wolff⁶ described a method in which a small quantity of blood was exposed to ozone in sealed glass bottles and then re-infused into the patient, stimulating interesting therapeutic responses. So far, ozone has been used in therapy in an empirical way. Positive and often surprising experiences have been reported^{1,7-10} with renewed interest on this topic in the medical literature. However, the absence of codified procedures, specific rationale, scientific rigour or practical knowledge represents insurmountable limits to the validation of this therapy.

The main therapeutic use of ozone is that already recorded and described by Wolff, and known as ozone autohemotherapy (AHT).^{1,2,6} Recent studies to clarify therapeutic mechanisms showed that the contact between ozone and blood produces effects that can be useful in medicine.

Exposure of human blood to a mixture of oxygen and ozone is not toxic, providing exposure times and concentrations are appropriate.¹¹⁻¹⁴

Unlike the respiratory system, the components of human blood are in a dynamic state and they can neutralise the oxidising power of ozone by means of specific defence systems. Like other gases (*i.e.* O₂, CO₂), ozone must be dissolved in water in order to interact with organic substrates. In blood, it dissolves in plasma and instantly decomposes in a cascade of reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), hydroxyl radical (HO[•]) and hypochlorous acid (HClO).^{1,15-17} ROS are normally produced during several processes of physiological relevance such as cell respiration by mitochondria, the respiratory burst that accompanies phagocytosis and xenobiotic detoxification, being also important cell messengers and gene transcription regulators.^{16,17} Some of these species are highly reactive compounds with a short half-life, therefore, representing potentially toxic compounds that may affect different biochemical targets leading to the formation of reactive by-products and molecular lesions. Repair or elimination by metabolic processing and/or excretion are key responses of living organisms to the ROS- (and thus ozone) dependent damage to biomolecules.

Since ROS may be toxic, aerobic organisms have developed several lines of cellular and extracellular defence such as antioxidant systems and sacrificial biomolecules. These include non-enzymatic defences such as uric acid, ascorbic acid, protein (mainly albumin) and non-protein thiols, vitamin E and bilirubin, and enzymes such as superoxide dismutase (SOD) and catalase (CAT), and the glutathione (GSH) system with the enzymes glutathione peroxidase (GSH-Px), glutathione transferase (GST) and glutathione reductase (GSH-R), the latter reducing oxidized glutathione (GSSG) to GSH with reducing equivalents provided by the

hexose monophosphate shunt through the coenzyme NADPH.^{16,18,19}

Most of the ozone dose that comes into contact with blood is partly reduced by water-soluble antioxidants and partly transformed into ROS including lipid peroxides and other bioactive molecules, which are also checked by the antioxidant and scavenger systems before they can damage blood cells. A first pharmacological effect of ozone is due to the slight excess of ROS acting as chemical messengers for membrane receptors and various biological functions,^{20,21} whereas bioactive molecules such as some lipid peroxides may act on practically all cells after re-infusion of ozonized blood as done in AHT.

The oxidation chemistry of ozone is known to produce H₂O₂ that enters cells where it has various effects. In red blood cells (RBCs), it shifts the hemoglobin dissociation curve to the right and facilitates release of oxygen,^{22,23} while in leukocytes and endothelial cells it can stimulate the production of interleukins, interferons, growth factors and nitric oxide.^{24,25} In platelets, it favours the release of growth factors;^{21,26} in other cell types, such as macrophages²⁷ and respiratory epithelial cells,²⁸ H₂O₂ stimulates cell activation, cytokine secretion and long-term efficiency of antioxidant systems in adaptation to its pro-oxidant action.

These cellular responses derive from a transient and acute event of oxidative stress which can be appropriately tuned when appropriate exposure times and ozone doses are used. Ozone, therefore, may act like a drug within a defined therapeutic window. In other words, ozone is not toxic if administered within its therapeutic range, and is ineffective if the dose is too low,¹ with its derived species totally quenched by antioxidants.

Monitoring the time and concentration dependence of the interaction of ozone with biomolecules in the blood provides a key tool to delivering an appropriate therapy and to check the compliance of the patient (see below). For instance, one approach is to select an array of biomarkers such as cell messengers or specific biological effects triggered by ROS reactivity, also measuring parameters that could demonstrate the compliance of the antioxidant systems. The capacity of the latter should not be irreversibly surpassed by the pro-oxidant challenge provided.²⁹

As a further aspect of the therapeutic action of ozone, there is its capacity to regulate the cell antioxidant network positively.^{30,31} This aspect is of key relevance in all those conditions in which an imbalance between production and neutralisation of ROS may develop, resulting in localized or systemic events of oxidative stress. These events may turn into a self-feeding cycle in which oxidative stress is sustained by micro- and macro-inflammatory reactions that lead to cell and tissue degeneration and necrosis. This scenario features the pathogenetic

role of oxidative stress in several chronic, degenerative disease states such as chronic viral infections, atherosclerosis, tumour growth, neurodegenerative diseases and accelerated aging.³¹ Under such conditions, the administration of exogenous antioxidants has been widely proposed as a strategy to slow down this adverse cycle thus preventing degenerative events. Unfortunately, this kind of approach has shown many limitations and often provided only weak results giving rise to controversial opinions or scepticism about its clinical relevance.

Another strategy may be to stimulate adaptation responses that would increase the resistance of cells against conditions of mild-to-severe oxidative stress. Ozone administered at therapeutic and progressively increasing doses was proposed to stimulate and then to re-inforce the intracellular antioxidant system.³² This adaptation response may also occur through the induction of silent or rarely expressed genes which encode the production of shock proteins such as heat shock proteins (HSPs), glucose-regulated proteins (GRPs) and oxidative shock proteins (OSPs).^{1,16,33}

Again, chronic inflammatory conditions are accompanied by a partial or sometimes large resetting of the immune system to a pro-inflammatory and pro-oxidant state. This response has gross implications also in the integrity of vascular components and may represent a sensitive therapeutic target of chronic, degenerative conditions. On the basis of these theoretical foundations, reported therapeutic applications for ozone therapy were the activation of the immune system in infectious diseases³⁴ and cancer,^{35,36} and an improved oxygen utilisation and release of growth factors that can reduce the extent of ischemic lesions in vascular diseases.³⁷

The body of *in vitro* and preliminary clinical evidence supporting the therapeutic uses of blood ozonation has been obtained thanks to the experience gained with the aforementioned model of AHT.^{1,2} In the last few decades, this technique (still regarded as alternative medicine) has become increasingly used throughout the world. However, its clinical application has remained limited due to some unconvincing results and the difficulty of demonstrating a sure mechanism of action *in vivo*.²

These aspects have been critically and extensively re-examined by our group. The most convincing explanation for these limits of AHT is that only small quantities of autologous blood (200–300 ml/week) were treated *in vitro* and then re-infused to the patient. This may considerably constrain the therapeutic potential of biological effects deriving from the blood–ozone interaction and their systemic relevance once the treated blood is re-infused. Therefore, research work has endeavoured to increase the blood volumes that are treated, and also to improve the ozonation technique. Efforts in this direction lead to the development of a completely new therapeutic approach based on the exposure of blood to a

mixture of oxygen–ozone within an extracorporeal circulation system; the technique was named extracorporeal blood oxygenation and ozonation (EBOO). This original approach opens a new era of ozone therapy, but much further development work is required.

In this review paper, we describe the basic principles and biological implications of ozone therapy. At the same time, we present a review of the literature that has marked the key steps in the development of this therapy to its latest (but surely not final) manifestation – EBOO.

HISTORY OF EBOO DEVELOPMENT

Development of suitable gas exchange devices and preliminary set up of the technique

In the last 13 years, an intensive collaboration between different academic institutions and industry partners made it possible to achieve some key steps in the establishment of EBOO. The first and necessary one was the development of an oxygen–ozone exchanger (with a view to a disposable device) that could resist corrosion by ozone and enable efficient gaseous exchange with large quantities of blood in an extracorporeal circulation.

The first gas exchange device (GED) was designed for capillary blood flow on a plane surface measuring 250 cm² in direct contact with the gas. The exchanger was sealed so that gas did not escape. The blood circulated at 40 ml/min and a mixture of oxygen and ozone (at an ozone concentration of 3–20 µg/ml of oxygen) flowed through the exchanger. *In vitro* experiments showed that under these conditions there is poor ozonation and oxygenation of treated blood.^{1,2}

The solution of increasing exchange area, as in extracorporeal oxygenation during heart surgery, met apparently insurmountable problems: all known materials used for constructing commercial oxygenators were attacked by ozone and this enhanced blood clotting by triggering platelet adhesion to modified oxygenator surfaces. Moreover, it was impossible to develop a mathematical model that could help in interpreting the performance of these blood–gas exchange prototypes, the diffusion and permeability coefficients of ozone being unknown. Again, the solubility coefficient of ozone in water is 49 cm³/dl at 0°C, but it had never been determined in blood.

The only alternative to resolving these initial drawbacks was to build a GED with ozone-resistant materials and undertake an empirical approach to evaluation of its performance. The critical component of this new generation of GED was the membrane, which had to be impermeable to liquids, permeable to ozone and oxygen and resistant to corrosion by ozone. Thanks to a collaboration with the Materials Science Institute of Pisa

University, Italy, it was established that the only suitable materials for such a membrane were polypropylene or polyethylene. A hollow-fiber structure was chosen and suitable materials for potting and housing of the fibers in the cartridge were also identified.

In the early 1990s, extruded polypropylene membranes were the first to be tested because they were commercially available, even though in a limited amount, from some Japanese companies. Therefore, the first GEDs were built like capillary oxygenators in which gas flowed inside the fibers and blood outside them.² This meant that there were only two inlet and two outlet ports allowing the gas and blood to flow in opposite directions throughout the GED. *In vitro* testing performed either with a glucose solution or blood, showed good gas flow at various concentrations without any apparent corrosion of the materials.³⁸ The greatest problem was platelet adhesion, which greatly reduced exchange efficiency within minutes.

Several years went by before a GED with heparin coating on the blood surface was produced. That step finally opened the way for the next generation of experiments to validate the technique.

Experiments with animals

As a required step in testing this unexplored therapy, *in vivo* experiments were carried out using an animal model. Sheep

were chosen because of the need for a large animal to practise extracorporeal circulation. Practically, it provides characteristics of suitable vascular access, sufficient blood volume, and so forth. These studies carried out in collaboration with experienced veterinarians demonstrated several fundamental points.^{2,38-40} First, EBOO was possible in animals without any technical problem. Second, but of key importance, was the evidence that EBOO is completely atoxic over a wide time period and dose range: ozonation for more than 60 min with a blood flow of 100 ml/min and exposure to an oxygen/ozone mixture with ozone concentrations of 20–60 µg/ml did not have clinical side-effects in any case, either during treatment or afterwards. Only slight hemolysis (measured as an increase in the levels of serum lactate dehydrogenase activity) was observed with ozone concentrations above 20 µg/ml blood. Biological effects were already detectable in sheep, as they were *in vitro* (see elsewhere), at doses of 1–2 µg/ml blood.

Specific tests³⁸ showed no release of plastic substances indicating that corrosion of GED materials by ozone did not occur during extracorporeal circulation, even at high ozone doses. Since then, it has become relatively easy to obtain GEDs built with suitable materials and a more recent generation of membranes has further improved the efficacy and biocompatibility of these devices. Polypropylene membranes coated on the blood side with a film of phospholipids have been produced,

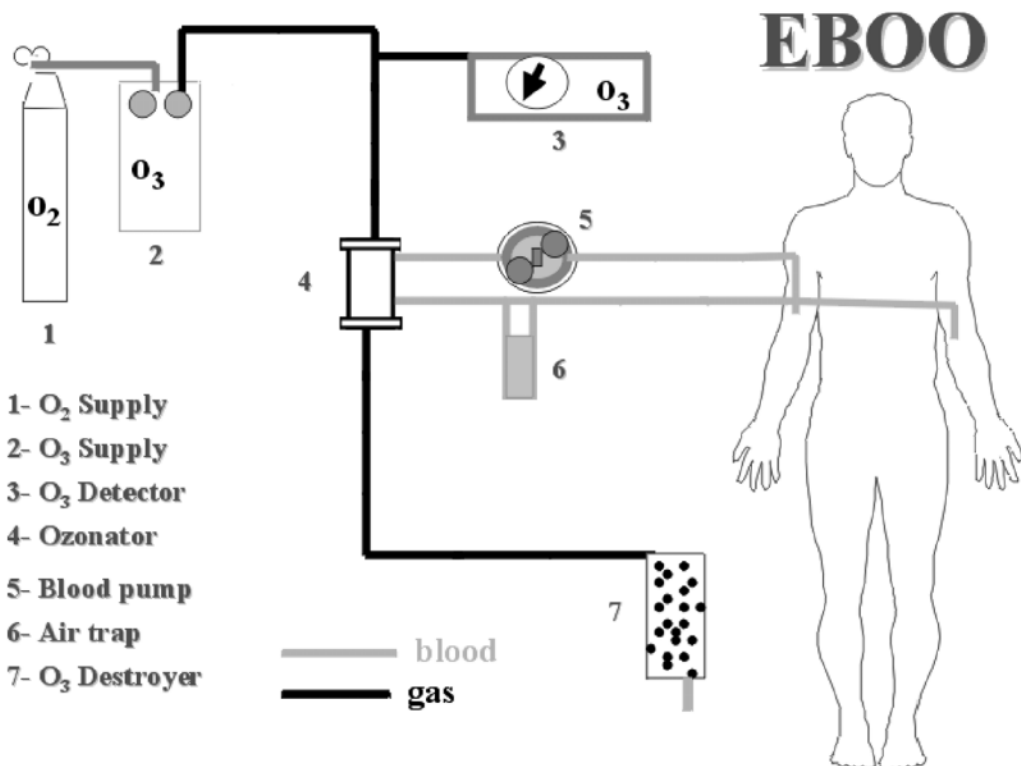


Fig. 1. Schematic representation of EBOO apparatus.

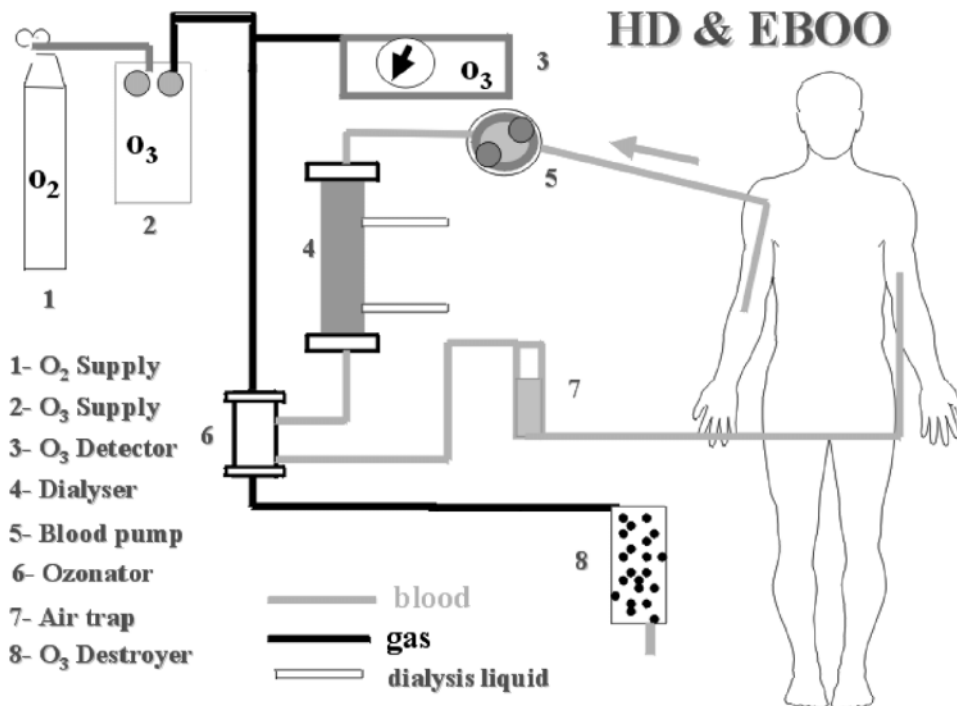


Fig. 2. Schematic representation of EBOO performed during hemodialysis

which are more effective than heparin-coated membranes in inhibiting ozone-induced platelet aggregation.² High-efficiency, highly biocompatible GEDs completely avoiding platelet adhesion are now available (DIDECO-BELLCO, Mirandola, Modena, Italy) and have been extensively tested in EBOO by our group.

Hence, experiments with the sheep model demonstrated that successful ozonation of large quantities of blood is possible and applicable to humans.

EBOO technique for human therapy

One of the present authors volunteered for the first four treatments in the early 1090s. Since then, more than 1200 treatments have been performed in 82 patients with various pathologies. Therapy normally consists of cycles of 14 sessions over a period of 7 weeks.

The EBOO apparatus (Fig. 1) currently in use consists of a blood pump with various alarms and pressure monitors, for extracorporeal circulation (BELLCO, Mirandola, Modena Italy). The blood circuit of the extracorporeal circulation is connected by arterial and venous lines to the GED (surface area, 0.6 m²), and blood flow is maintained at a rate of 75–80 ml/min. Ozone is produced by an Ozonline International generator (Medica, Bologna) with an O₃ output of 1–10 µg/ml of oxygen at a pressure of 0.2 bar. A specific photometer (Ozonosan 590, Iffezheim, Germany) controls the quantity of ozone supplied. The gas flows through the GED

and then into a decomposer (Catalyzer Sonder Zubehor, Ozonosan, Iffezheim, Germany) that destroys the ozone by means of palladium salts heated to about 60°C, so that none escapes into the atmosphere. For environmental safety, the ozone therapy room is fitted with ozone detectors (Ozon Sensor, Mod. C. 307 X) and aspirators incorporating ozone decomposers (Ozonline Air Nov Mini/578, Medica, Bologna, Italy) which come into operation automatically in the case of accidental release of gas. The blood line contains an air trap with automatic level detector. On the basis of *in vitro* tests, and *in vivo* studies with sheep and then with humans, we chose to use ozone (mixed with 95–98% oxygen) at final concentrations of 0.5–2 µg/ml of oxygen.

Ozone therapy is done, after overnight fasting, via the cubital veins of both arms. Using the latest GED, one heparin injection (5000 IU) at the start of treatment has been found sufficient to avoid clotting over the 1-h span of an EBOO session. Once the extracorporeal circuit is stable, the ozone/oxygen mixture is allowed into the gas compartment and treatment begins. Maximum oxygenation and ozonation were obtained with blood flows in the range 75–85 ml/min (*i.e.* 4.5–4.8 l of blood treated in 1 h). Using ozone doses of only 0.5–1 µg/ml of oxygen, blood samples obtained at the outlet of the GED showed a 5–6-fold increase in pO₂ without any significant change in general arterial pO₂,¹⁶ and a 4–5-fold increase in concentrations of thiobarbituric acid reactants (TBARs) and a similar decrease in protein thiol groups (PTGs).^{38–40} (See also the next section for further information on these parameters).

Table 1. Some biomarkers used to characterize the response to the *in vitro* exposure of human blood components or cell cultures to oxygen–ozone mixtures

Parameter	Biological sample/experimental setting	Effect	Type of response
TAS	Plasma ^{a,c} Whole blood ^{a,c}	↓ ↔ (compensation by blood cells)	Time and concentration dependent Time and concentration dependent
PTGs	Plasma ^{a,c}	↓	Time and concentration dependent
TBARs	Plasma ^{a,c}	↑	Time and concentration dependent
Haemolysis	Plasma (as free hemoglobin) ^{a,c}	↔ ^{doses ≤ c} ↑ ^{doses ≥ d}	Concentration dependent
MDA	Plasma ^d RBC ^d	↑ ↔	Concentration dependent /
α-Tocopherol	Plasma ^d RBC ^d	↓ ↔	Concentration dependent /
PDGF and TGF-1β	Platelet ^{a,c}	↑	/
TNF-α	Whole blood (a, b)	↑	Concentration dependent
INF-g	Whole blood (a, b)		Concentration dependent
IL-2	Whole blood (a, b)		/
IL-8	Whole blood (a, b)		Concentration dependent
NO ₂ (iNOS activity)	HUVECs ^{*a,c}	↑	Concentration dependent

*Exposed to human serum treated with the doses of O₃ a and c and assessed for the formation of TBARs and H₂O₂ and the consumption of PTG.

O₃ doses used in these *in vitro* experiments were: ^a40 μg/ml; ^b70 μg/ml; ^c80 μg/ml; ^d100 μg/ml.

Experiments were performed over an incubation time of 1 min except in those cases in which time-dependence was evaluated (as specified in the column on the right).

Abbreviations: TAS, total antioxidant status; PTG, protein thiol groups; TBARs, thiobarbituric acid reactants; MDA, malonyldialdehyde, HUVECs, human umbilical vein endothelial cells.

Data were from references reported in the text.

These ozone concentrations did not cause any acute RBC hemolysis or troubles related to increased platelet activation and clotting. No significant changes in routine blood chemistry parameters were found after treatment for up to 60 days after the end of cycle of 14 sessions.

Extracorporeal circulation was successful in 85% of cases with the cubital veins; when these were not accessible, the jugular veins were used. No technical problems were experienced. It is advisable to monitor blood pressure at the inlet and outlet so as to detect any resistance in GED or the return vein. No side effects were reported during or between sessions. Patients did not report any particular sensation during treatment. After treatment, they reported a feeling of well-being and euphoria that lasted for several hours.

We are currently examining the possibility of adapting EBOO to the treatment of patients during hemodialysis (Fig. 2).

The first series of treatments has shown an interesting therapeutic potential in patients with severe peripheral

arterial disease, coronary disease, cholesterol embolism, severe dyslipidemia, Madelung disease, and sudden deafness of vascular origin.^{1,13,38,40} Improvements continued to manifest up to 2 months after the end of the treatment cycle, which is in line with the mechanism of action of ozone.⁴¹ In our experience, there have been only two drop-outs. The first was due to infection of the permanent subclavian catheter site and the second due to deterioration of the cubital veins.

Control studies to verify these encouraging preliminary results are currently under way, supervised by the Siena University Ethical Committee.

Biomarkers for monitoring ozone therapy

A large body of information available on the molecular actions of ozone when reacting with blood constituents and presented in this section was obtained thanks to pioneering studies performed by Bocci and his co-workers

(reviewed in Bocci¹). They extensively investigated *in vitro* models resembling the principle of ozone therapy administered by the AHT technique (see above). The findings of these experiments are summarized in Table 1 and have been used to identify laboratory indices useful in monitoring the biological compliance to blood ozonation during EBOO.

In practice, it is technically impossible to measure ozone directly in the blood because of its very brief half-life making it impossible to characterize ozone pharmacokinetics directly. Therefore, surrogate markers useful in the evaluation of the biological compliance to blood ozonation are identified in the indices of biomolecule damage and modifications of plasma antioxidants.

The kinetics of total antioxidant status (TAS) in plasma and whole blood of normal donors exposed for 1 min to either O₂ or O₂/O₃ with ozone concentrations of 40 and 80 µg/ml and then evaluated over a time of 20 min, demonstrated a time- and concentration-dependent decrease of TAS in both plasma and whole blood samples.¹ However, in whole blood samples, after an initial drop, a major compensation for this O₃-induced *in vitro* effect was found. This finding suggests the key role of blood cells (mainly erythrocytes) in the restoration/buffering of blood TAS. Other experiments carried out with the same design confirmed a concentration-dependent decrease of TAS and a decrease of PTG in plasma. At the same time, the levels of TBARs increased significantly while a negligible increase in red-cell lysis was observed. Hemolysis appears stepwise when the ozone dose increases over an identified therapeutic window (upper limit 100 µg/ml).⁴² These doses of O₃ in heparinized blood were also preliminarily observed to increase Ca²⁺ influx in red blood cells and to activate phospholipase C (Bocci *et al.*, unpublished observation; reported in Bocci¹). At the same time, early evidence suggested positive changes in microrheology of ozonized blood.⁴³ This interpretation does not fit with that of other experiments⁴⁴ and thus needs further careful examination to be confirmed.

As further evidence supporting the role of blood cells in compensating for the pro-oxidant effect of ozone on blood components, other *in vitro* studies have shown that the exposure of blood to O₃ at a concentration of 100 µg/ml can lead to the formation of TBARs and consumption of α-tocopherol in plasma, but not in the erythrocyte membranes.⁴⁵ Other studies have clearly shown that when erythrocytes are protected by plasma antioxidants, membrane lipoperoxidation is efficiently prevented (reviewed in Bocci¹).

Under the same experimental conditions, O₃ stimulates cytokine secretion in peripheral blood mononuclear leukocytes. Again, the incubation of human umbilical vein endothelial cells (HUVECs) in the presence of ozonized plasma or H₂O₂ (a product of O₃ decomposition in solution)^{16,17} stimulates the release of the vasodilating agent

NO and the production of the chemokine IL-8 without affecting the expression of E-selectin and endothelin 1.⁴⁶

Platelet activation with consequent release of the growth factors PDGF and TGFβ1 has also been suggested as an important effect that may provide a specific therapeutic potential to blood ozonation techniques, particularly in patients with chronic limb ischemia.²¹

These data (Table 1) seem to confirm that the generation of ROS (mainly H₂O₂) by the exposure of blood components (mainly unsaturated lipids) to O₃ transiently modifies the antioxidant status and may lead to a stress stimulus on blood cells. Circulating erythrocytes may play a role in the compensation of the pro-oxidant challenge by O₃, while leukocytes, endothelial cells and platelets are transiently activated and produce cytokines and possibly other paracrine signals such as nitric oxide, adhesion molecules, growth factors and prostaglandins. This stress response is hypothesized to represent the stimulus to molecular events that explain the therapeutic role of ozone.

The available literature on ozone therapy clearly demonstrates some major limits in the selection of parameters that would describe accurately events of oxidative stress or biological responses underlying the therapeutic role of blood ozonation. There are several reasons for this. For instance, in the case of oxidative stress, the parameters now considered the gold standards in monitoring this biochemical condition remain difficult to adapt to routine clinical applications and only a few laboratories are equipped with the required instrumentation and have the appropriate knowledge to measure them. As a consequence, selected parameters adopted to monitor the biochemical compliance to ozone therapy have been:

1. **The total antioxidant status (TAS).** Among the different methods available to measure this parameter,^{16,29} we used that based on the evaluation of the inhibitory effect of antioxidants contained in biological fluids (usually plasma or whole blood) on the ROS-mediated generation of a cationic radical from the water-soluble compound 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ATBS). The ATBS⁺ is a fairly stable radical absorbing at 600 nm and then easily measurable by spectrophotometric analysis.
2. **The assay of protein thiol groups (PTGs).** Protein thiols (or sulphhydryl groups) are one of the first lines of defence against oxidants in plasma. PTGs can be detected after reaction with the Ellman reagent as a coloured product measured spectrophotometrically at 412 nm.⁴⁷ Ozone causes a decrease of PTGs in plasma.
3. **The assay of TBARs.** As described above, ozone in plasma reacts with unsaturated fatty acids to produce

a wide range of carbonyls, which include the hallmark malonyldialdehyde (MDA) as one of the most abundant by-products. The method routinely used to measure TBARs is that described by Buege and Aust,⁴⁸ and consists of a colorimetric determination based on reaction of these free carbonyls in the plasma with the reagent thiobarbituric acid (TBA). Although less specific than other assay procedures such as the analysis of 8-iso-PGF_{2α},^{49,50} this determination can be considered an approximation of the degree of peroxidation in the treated blood.

The selection of these parameters was based mainly on the fact that they are easily measured and suitable for routine use but, at the same time, are sufficiently reliable and informative.

Therefore, based on the early evidence of increased formation of TBARs and decrease of PTGs during EBOO,³⁸⁻⁴⁰ there is sufficient indication of the presence of oxidative stress occurring as a consequence of the interaction between ozone and blood constituents. Research work is in progress to characterize better molecular changes due to this interaction both in oxidative stress and biological/adaptive responses. Parameters of definite interest are hallmarks of plasma protein damage (such as protein carbonyls, 3'-nitro-tyrosine and di-tyrosine, and the glycoxidation end-products pentosidine and carboxymethyl-lysine),⁵¹ lipid oxidation (mainly by the assay of urinary or plasma 8-iso-PGF_{2α})^{49,50} and DNA oxidation of circulating leukocytes and solid tissues (by means of the assay of blood or urinary 8-OH-dG).⁵²

Of particular interest in the cellular responses to blood ozonation is the stimulatory effect on leukocytes and epithelial cells with production of cytokines, adhesion molecules and pro-inflammatory eicosanoids.^{12,24-28} In particular, the interferon- γ (INF- γ)-producing effect of blood ozonation is a response with a great therapeutic potential in immunodeficiency and chronic, degenerative conditions such as chronic liver disease and rheumatoid arthritis.

DISCUSSION AND CONCLUSIONS

Ozone is incorrectly regarded as toxic to the human body.^{11,27,28} Although it may cause severe damage and even death if inhaled, blood ozonation via AHT has been increasingly used in the past decades and found useful in various diseases. It is reported to activate the immune system in infectious diseases,^{3,4,18} to improve the utilization of oxygen and stimulate release of growth factors and other mediators that may re-activate the immune system and reduce ischemia in vascular disease.^{3,19,21,46} It also activates the immune system in cancer patients and was reported to kill cancer cells.^{32,34,36} However, a major

limitation to the therapeutic application of AHT is the limited amount of blood treated with this technique.

These limits have been surpassed and a new prospective in ozone therapy has been introduced thanks to the establishment of an extracorporeal technique named EBOO. Successful ozonation of large quantities of blood was demonstrated to be possible and totally non-toxic in sheep. In this animal model, a DL₅₀ for ozone administration cannot be established even using doses 100-fold higher than the doses normally used in humans with AHT. The technique of EBOO in human patients is simple for hospital staff familiar with extracorporeal circulation.² EBOO was perfectly tolerated and showed promising therapeutic effects in various atheromatous and immune disease states. Pro-oxidant effects of blood ozonation can be monitored during clinical practice by simple blood tests such as the assay of plasma TBAR and PTG assays.^{47,48,53-55} Even though of limited scientific value, these tests are necessary to monitor therapy compliance. In other words, they can provide an indication for the adjustment of the dose of ozone administered and represent a partial proof of the biological outcome of the therapy. Other and more comprehensive biomarkers and clinical evaluations need careful attention to verify clinical outcomes and the therapeutic relevance of blood ozonation during the treatment of different disease states with EBOO.

The clinical relevance of this technique is still debated mainly due to lack of knowledge, unsubstantiated prejudices and scarce interest by the industries. As a consequence, extensive clinical evaluation is missing and clinicians are still subject to poor information and familiarity with the use of ozone in clinical practice.⁵⁶⁻⁵⁸ Clinical studies are under way to confirm the preliminary results obtained with EBOO. Further studies on the biological mechanisms by which ozone can provide its curative effect are also required to understand better the potential of this evolutionary step in ozone therapy.

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