The Ozone Paradox: Ozone Is a Strong Oxidant as Well as a Medical Drug

***Velio Bocci,*[1] *Emma Borrelli,*[2] *Valter Travagli,*[3] *and Iacopo Zanardi*[3]**

[1]Department of Physiology, University of Siena, Siena, Italy
[2]Department of Surgery and Bioengineering, University of Siena, Siena, Italy
[3]Department of Pharmaceutical Chemistry and Technology, University of Siena, Siena, Italy

Published online 3 March 2009 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/med.20150

Abstract: After five decades characterized by empiricism and several pitfalls, some of the basic mechanisms of action of ozone in pulmonary toxicology and in medicine have been clarified. The present knowledge allows to understand the prolonged inhalation of ozone can be very deleterious first for the lungs and successively for the whole organism. On the other hand, a small ozone dose well calibrated against the potent antioxidant capacity of blood can trigger several useful biochemical mechanisms and reactivate the antioxidant system. In detail, firstly ex vivo and second during the infusion of ozonated blood into the donor, the ozone therapy approach involves blood cells and the endothelium, which by transferring the ozone messengers to billions of cells will generate a therapeutic effect. Thus, in spite of a common prejudice, single ozone doses can be therapeutically used in selected human diseases without any toxicity or side effects. Moreover, the versatility and amplitude of beneficial effect of ozone applications have become evident in orthopedics, cutaneous, and mucosal infections as well as in dentistry.
 Med Res Rev, 29, No. 4, 646–682, 2009

Key words: oxidative stress; antioxidants; oxidative preconditioning; ozone; ozonated autohemotherapy

1. INTRODUCTION

A. A Brief Historical Review

Christian Friedrich Schönbein, in 1839, noticed the emergence of a pungent gas with an "electric smell." According to the Greek language, he called it "ozone" and presented a lecture entitled "On the smell at the positive electrode during electrolysis of water" at the Basel Natural Science Society.[1,2] In nature ozone is continuously produced in the stratosphere (at 25–30 km from the Earth surface) by UV radiation (<183 nm) by splitting an atmospheric oxygen molecules into two highly reactive oxygen atoms, in agreement with the Chapman theory. By an endothermic reaction, each of these atoms combines to intact oxygen to form the triatomic ozone.

Correspondence to: Velio Bocci, Department of Physiology, University of Siena, Via Moro 2, 53100 Siena, Italy, E-mail: bocci@unisi.it

It is also produced during the electric discharge of lightning, which catalyzes the formation of ozone from atmospheric oxygen. Ozone has a molecular weight of 48 and it is a bluish gas with a pungent odor and a solubility in water, about ten-fold higher than oxygen (49 mL in 100 mL, 0.02 M, at 0°C), even though an ample variability is present in the literature.[3] While it rapidly dissolves in pure water and obeys Henry's law, in biological water ozone instantly reacts with inorganic and organic molecules dissolved in water generating a variety of free radicals. Ozone as a gas spontaneously decomposes with a half-life of 40 min, at 20°C. This means that ozone is a metastable gas with a temperature-dependent half-life, but it can be stored in liquid form at a temperature below −111.9°C with a specific weight of 1.571 g/mL. Methods for generating ozone are based on UV radiation, corona discharge, and an electrochemical process. Industrial ozone is produced from air but medical ozone must be generated ex tempore only by using medical oxygen because otherwise the simultaneous generation of nitric dioxide (NO_2) will be very toxic.[4] The most recent medical ozone generator can control the electric voltage from 5 kV up to about 14 kV, the space between the electrodes able to modulate a gradual increase in ozone concentration and the flow of pure oxygen usually regulated between 1 and 10 L/min. The final ozone concentration is inversely proportional to the oxygen flow, hence, per unit time, the higher the oxygen flow, the lower the ozone concentration. In the final oxygen–ozone mixture, the maximum ozone concentration can be only 5%.

2. BEHAVIOR OF OZONE

A. Ozone as an Oxidant

Ozone has a cyclical structure assessed by the absorption at 253.7 nm with a distance among oxygen atoms of 1.26 Å and exists in several mesomeric states in dynamic equilibrium[5] (Fig. 1). Among oxidant agents, it is the third strongest ($E^\circ = +2.076$ V), after fluorine and persulphate. Molecular oxygen, by containing two unpaired electrons, is a diradical but it has not the reactivity of ozone and, by a stepwise reduction with four electrons, forms water. On the other hand, ozone having a paired number of electrons in the external orbit is not a radical molecule, but it is far more reactive than oxygen and generates some of the radical oxygen species (ROS) produced by oxygen during mitochondrial respiration. Phagocytes reacting with pathogens[6–8] produce anion superoxide (O_2^-), H_2O_2, and hypochlorous acid (HClO) catalyzed by mieloperoxidase. Wentworth et al.[9,10] have postulated that in atherosclerotic patients human endothelium cells may produce ozone, but their findings remain still doubtful.[11] Moreover, H_2O_2 is produced by almost all cells by the nicotinamide adenine dinucleotide phosphate (NADPH)-oxydase isoenzymes, indicating the relevance of ROS in the normal organism. Interestingly, ozone, in the presence of inorganic and/or organic compounds immediately reacts and generates a great variety of oxidized molecules, disappearing in a matter of seconds.[12]

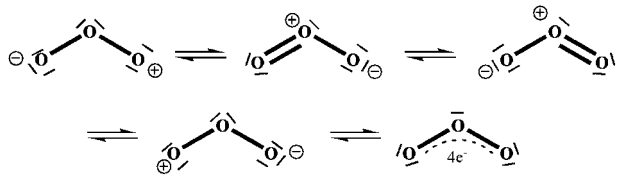


Figure 1. Structure and mesomeric states of ozone.

B. Ozone as UV screen

In the stratospheric layer, ozone has an average concentration of 10 parts per million (ppm) and it has the important role to absorb most of the UV radiations, particularly bands B (from 280 to 320 nm) and C (from 100 to 280 nm), which are mutagenic and can enhance skin carcinogenesis.[13] Unfortunately, during the last decades, short-sighted human activities, by releasing chlorofluorocarbons in the atmosphere, have led to a decreased ozone concentration, particularly in the Antarctic, which will take several decades to be restored.

C. Ozone as an Air Pollutant

On the other hand, the tropospheric amount of ozone ought to be about 1 $\mu g/m^3$ (0.001 ppm), ten times lower than our odor perception threshold for ozone about 20 $\mu g/m^3$ (0.02 ppm). However during the last decades, in large cities, ozone levels in summer time can increase up to dangerous levels ranging from 200 to 900 $\mu g/m^3$. Moreover, additional anthropogenic emissions of NO, NO_2, methane, CO, sulphuric compound, and fine particulates have enhanced the toxicity not only for the respiratory tract but also for the eyes and the skin.

The US Clean Air Act has set an ozone level of 120 $\mu g/m^3$ as an 8 hr mean concentration to protect the health of workers.[14] Evaluation of recent studies[15–18] allows establishing an average environmental ozone concentration of 90 ± 10 $\mu g/m^3$. However, ozone concentration in urban air can exceed 0.8 ppm in high pollution conditions.[19,20] For 8 hr at rest (a tidal volume of about 10 L/min and a retention of inspired ozone of no less than 80%), the ozone dose amounts to 0.70–0.77 mg daily. This is likely the minimal ozone intake because physical activity increases the volume of inhaled air, and, at peak time, the ozone levels can easily augment to 500–900 $\mu g/m^3$, reducing pulmonary functions and markedly enhancing the risk of cardiovascular deaths.[15,17,18]

Ozone levels of 500 $\mu g/m^3$ may not seem too high but one must consider that any single air inhalation implies an ozone dose that immediately reacts with the airway surface fluid and immediately at the epithelial lining fluid (ELF) generates the ROS and lipid oxidation products (LOP) minimally quenched by the scarce antioxidant present in a liquid film of about 0.1 μm.[21] As a consequence, the whole respiratory tract against the continuous inhalation of ozone-contaminated air opposes only the ELF's volume of about 20–40 mL,[22] which is negligible when compared to a plasma volume of about 2700 mL. Thus, throughout the day we must consider, neither simply the ozone concentration nor a single respiratory act, but the ozone cumulative dose that can easily sum up to 1–2 g ozone in 5 months. While ozone vanishes within the ELF,[23] the generated ROS, LOP, and nitrating species[24–28] damage the epithelial lining. The phosphorylation of a protein kinase, by activating the nuclear factor-κB (NF-κB), allows the synthesis and release of a number of cytokines such as TNFα, IL-1, IL-8, IFNγ, and TGFβ1. Moreover, this situation starts a vicious circle because the increased inflow of neutrophils and activated macrophages into the alveolar space worsens and perpetuates the production of more ROS including HClO,[8,26] tachykinins, proteases, alkenals, and F_2-isoprostanes[25,29] able to self-maintain a chronic inflammation. ROS have a very brief half-life and damage mostly the pulmonary microenvironment while alkenals and proinflamatory cytokines are absorbed by the human large expanse (about 70 m^2) of the bronchial–alveolar space. Recent studies[25,30,31] have detected 4-hydroxynonenal (4-HNE), isoprostanes, H_2O_2, and malondialdehyde (MDA) in the bronchoalveolar lavage fluid. The interesting study by Last et al.[32] has clearly shown that mice exposed to 1 ppm for 8 hr during three consecutive nights lose about 14% of their original body weight, decrease their food consumption by 42%, and enter into a cachectic state. Another important aspect of the pulmonary ozone toxicity is its reverberation on the whole organism, especially on the vascular system, heart, liver, brain, and kidneys. The pharmaco-toxicological behavior of both LOP compounds, ceramide signaling, and proinflammatory cytokines is characterized by a continuous absorption from the pulmonary area into the blood and, even

though the half-life of these compounds is brief,[28,33–37] the constant endogenous synthesis insures a constant toxicity explaining the increased morbidity and mortality of population inhaling polluted air for several months of the year.

D. Ozone as a Biological Cytotoxic Agent

Either normal or neoplastic cells in culture are very sensitive to a constant exposure of ozone even if the gas has a very low concentration.[38–40] This observation is correct but it has led to the misleading conclusion that ozone is always cytotoxic. Indeed, we know too well that cells culture studies are mostly performed with air–CO_2 at pH 7.3 but with a pO_2 of 160 mmHg, i.e. more than double of cells in vivo. Even more important is the fact that culture media have a significantly lower level of antioxidants than plasma, particularly of albumin.[41–45] Indeed, the usual fetal calf serum is added at a 5–10% concentration that is equivalent to hardly 50% of the albumin present in the extracellular fluid. Among antioxidants, albumin with its available –SH reducing group is one of the most protective compounds.[46] Moreover, antioxidant components are not dinamically replenished in vitro while cells remain exposed to a constant ozone concentration. Obviously ozone dissolves in the fluid every second, exhausts the scarce antioxidants, and generates toxic compounds that cannot undergo either dilution with extracellular fluid or excretion. This unfavorable situation has been demonstrated when thiobarbituric acid reactive substances (TBARS), incubated in vitro at 37°C and pH 7.3 in human ozonated plasma remain at a constant level for 9 hr.[47] On the other hand TBARS present in ozonated blood declined very rapidly with a half-life of 4.2 ± 1.7 min[48,49] after intravenous infusion in patients with age-related macular degeneration (ARMD) demonstrating the relevance of critical pharmacological properties to be extensively discussed in Section 4A. Moreover, the damaging effect of ozone on saline washed erythrocytes, totally deprived of the plasma protection, has noticeably contributed to consider ozone as a deleterious gas.

3. MAY OZONE BE USED AS A MEDICAL DRUG?

At first sight, the strong oxidizing properties of ozone discard the possibility that this gas may display some therapeutic effects. However, even today some ozonetherapists advance the whimsical idea that ozone, by decomposing in the blood, gifts the body its intrinsic energy accumulated during its synthesis, as shown

$$3O_2 + 68,400 \text{ cal} \rightarrow 2O_3$$

On the 19th century, ozone had been already identified as a potent bactericidal gas and it was used during World War I for treating German soldiers affected by gaseous gangrene due to *Clostridium* anaerobic infections. In two pioneristic studies, Stoker[50,51] reported the first 21 medical cases successfully treated with ozone at the Queen Alexandria Military Hospital. It remains uncertain how a Swiss dentist, E.A. Fisch (1899–1966)[52] had the first idea to use ozone as either a gas or ozonated water in his practice. By a twist of fate, a surgeon, Dr. E Payr (1871–1946) had to be treated for a gangrenous pulpite and remained astonished by the result achieved with local ozone treatment. He enthusiastically extended its application to general surgery and at the 59th Congress of the German Surgical Society (Berlin, 1935) reported "which other disinfectant would be tolerated better than ozone? The positive results in 75% of patients, the simplicity, the hygienic conditions and the safety of the method are some of the many advantages".[53] In 1936, a French physician, Dr. P. Aubourg successfully treated chronic colitis and rectal fistulae by the direct insufflation of oxygen–ozone mixture into the rectum. It seems that Dr. Payr was the first to inject a small volume of the O_2–O_3 gas

mixture directly into the human cubital vein, giving rise to a procedure that in the 90s, adopted by charlatans, became so dangerous to be prohibited. After the invention of the first medical ozone generator by the physicist Joachim Hansler (1908–1981), the physician Hans Wolff (1927–1980) deserves the credit for having developed the ozonated autohemotherapy (O_3-AHT) by insufflating ex vivo the gas into the blood contained in a dispensable ozone-resistant glass bottle. For almost three decades ozone therapy was used in Germany but the lack of scientific and clinical studies arose scepticism and prejudice still common today. Lacking the knowledge of the complexity of biological mechanisms, a distinguished chemist wrote that "ozone is toxic, no matter how you deal with it and should not be used in medicine" (personal communication to V.B.).[54] This negative concept may only be changed by valid scientific and clinical data. It is worthwhile to mention what Timbrell[55] wrote in his book "*The poison paradox; chemicals as friends and foes*." The essential facts are that first it is the dose that makes a chemical toxic, and second and more important, toxicity results from the interaction between chemical and biological defenses. Indeed the subtlety and complexity of biological systems may defy the concept that ozone is always toxic. Interestingly, Paracelsus (1495–1541) did not know biochemistry but guessed that "all things are poison and nothing is without poison, only the dose permits something not to be poisonous."[56]

4. BIOLOGICAL MECHANISMS ELICITED BY OZONE IN HUMAN BLOOD

As it was mentioned, ozone as a gas equilibrates in 5 min in pure water and, in a closed glass bottles, its concentration (about 25% of the ozone concentration in the gas mixture) remains fairly stable for many hours. However, in a physiological environment, it immediately reacts with antioxidants, polyunsaturated fatty acids (PUFA), proteins, carbohydrates and, if in excess, with DNA and RNA.[57,58] Thus, ozone leads to the formation of ROS, LOP, and a variable percentage of oxidized antioxidants.[59,60]

A. Reactions with Plasma Components

Blood is an ideal tissue because it is composed of about 55% plasma and cells, especially erythrocytes, able to cooperate for taming the oxidant properties of ozone. The plasma has a wealth of hydrophilic reductants, such as ascorbic acid (~50 μM), uric acid (~400 μM), and a little amount of reduced glutathione (GSH). These compounds have been measured before and after ozonation.[61–63] Plasma contains albumin (~45 mg/mL) that by virtue of a wealth of –SH groups, is one of the most important antioxidants also because the plasma pool contains about 112 g of albumin.[46] Moreover, the presence of proteins such as transferrin and ceruloplasmin quenches oxidizing reactions by chelating transition metals (mainly Fe^{2+} and Cu^{+}). Presence of traces of these metals must be avoided because either in the presence of hydrogen peroxide, via the Fenton's reaction, or in the presence of anion superoxide (O_2^-) via the Haber–Weiss reaction, they will catalyze the formation of the most reactive hydroxyl radical $^{\cdot}OH$.

$$Fe^{2+} + H_2O_2 \rightleftharpoons Fe^{3+} {}^{\cdot}OH + OH^-$$

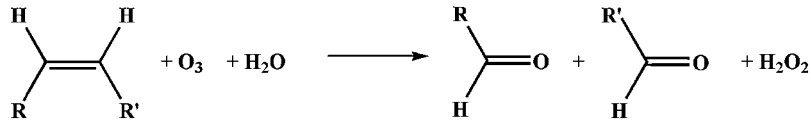
$$O_2^- + H_2O_2 \rightleftharpoons {}^{\cdot}OH + OH^- + O_2$$

Although $^{\cdot}OH$ has a half-life of 1×10^{-9} sec, it reacts with any other molecule and produces another radical. Blood cells contain not only the bulk of GSH (1–5 mM) but also thioredoxin and several lipophilic compounds such as α-tocopherol, retinol, lycopene, ubiquinol, and α-lipoic acid, which are able to cooperatively reduce oxidized compounds, thus restoring the initial antioxidant status. Moreover, blood cells contain a variety of en-

zymes (SOD, catalase, GSPase, GSH-redox system), which cooperate either simultaneously or in a sequential way to restore the redox system. The work performed during the last 18 years in our lab has clarified the most important compounds generated ex vivo during the initial reaction of ozone with some plasma components and how these compounds activate some biochemical pathways in cells revealed by therapeutic effects after the transfusion of ozonated blood in the donor.

The biochemical effects displayed by ozone when it comes in contact with blood components will be briefly reviewed.[47,63] After having performed thousands of treatments, the standard procedure is to add 200 mL of a gas mixture composed of medical oxygen (>95%) with ozone (<5%) to 180 mL of blood after the previous addition of 20 mL of 3.8% sodium citrate at room temperature. The blood–gas volumes are gently mixed in a sterile glass bottle by rotation, avoiding gas bubbling. Within 5 min, about 1.5 mL of O_2 and 2.4 mL of O_3 dissolve in the blood water but their fate is quite different. Oxygen physically diffuses into erythrocytes and fully saturates hemoglobin (Hb_4O_8) but in spite of the pO_2 as high as 450 mmHg, the therapeutic value of oxygenation is irrelevant because the successive infusion of oxygenated–ozonated blood (about 15 mL/min) hardly modify the pO_2 (~40 mmHg) of about 5 L/min of the simultaneous venous blood inflow to the heart. On the contrary, ozone dissolves more readily in plasma water than oxygen, and instantaneously reacts with hydrosoluble antioxidants and with readily available PUFA bound to albumin.

Several years ago, by using a reliable ozone generator able to deliver precise ozone concentrations, the first aim was to define if indeed ozone was always deleterious or if a range of ozone therapeutic concentrations could be determined. The range was determined between 10 μg/mL gas (0.21 μmol/mL) and 80 μg/mL gas (1.68 μmol/mL) per mL of anticoagulated blood, corresponding to total ozone doses comprises between 1 and 8 mg for 100 mL blood, respectively. It was crucial to precisely calibrate the ozone dose (gas volume × ozone concentration) against the individual variable antioxidant capacity of the patient's blood, thereby on one hand avoiding ozone toxicity and, on the other hand, allowing the activation of several biochemical pathways on blood cells. It was proven that during the slow mixing of the blood with the gas phase, all the ozone is consumed in less than 5 min. Several studies[47,51,59,63–65] have clarified that some albumin and uric acid behave as sacrificial molecules whereas several antioxidants after oxidation are rapidly reduced by an efficient recycling system.[66,67] Some ozone reacts with PUFA as follows



leading to the simultaneous formation of 1 mol of H_2O_2 (included among ROS) and 2 mol of LOP.[23,68,69]

The fundamental ROS molecule is H_2O_2, which is not ionized but is an oxidant able to act as an ozone messenger responsible for eliciting several biological and therapeutic effects.[70–75]

As it was mentioned, the old concept that H_2O_2 is always harmful has been widely revised because, in physiological amounts, it acts as a regulator of signal transduction and represents a crucial mediator of host defense and immune responses.[74,76–80] While exposure to oxygen is ineffective, ozone causes the generation of H_2O_2 and of the chemiluminescent reaction in both physiological saline and plasma.[47,81] However, while in saline there is a consistent and prolonged increase in H_2O_2, in the ozonated plasma both chemiluminescence and H_2O_2 increase immediately but decay very rapidly with a half-life of less than 2 min

suggesting that both antioxidants and traces of enzymes rapidly reduce H_2O_2 to water.[47] In ozonated blood the reduction of H_2O_2 is so fast that it has been experimentally impossible to measure it. H_2O_2 is able to easily pass through the cell membrane, but the intracellular concentration increases only 1/10 of the extracellular one.[72,74,78] Its relative stability allows measuring it in plasma; in normotensive subjects its concentration is of 2.5 μM.[70,71] In this case the intracellular concentration of H_2O_2 will be at the most of 0.25 μM, while the maximal intracellular concentration that can be generated for signaling purposes during the ozonation process may reach 0.5–0.7 μM.[47] It appears ubiquitous as it has been detected in urine and in exhaled air.[71] Depending upon its local concentration and cell-type, H_2O_2 can either induce proliferation or cell death.[78,80,82,83] It can regulate vascular tone by causing constrictions of vascular beds or vasodilatation although it remains uncertain if it acts as an endothelium-derived hyperpolarizing factor.[84]

A very enlightening finding was achieved by evaluating the variation of the total antioxidant status (TAS) as measured by the Rice-Evans and Miller's method[85] in plasma after ozonation and 1 min rapid mixing of the liquid–gas phases of either fresh blood or the respective plasma withdrawn from the same ten donor.

Figure 2 shows that, after ozonation of plasma with either a medium or a high ozone concentration (0.84 μmol/mL or 1.68 μmol/mL of gas per mL of plasma, respectively), TAS

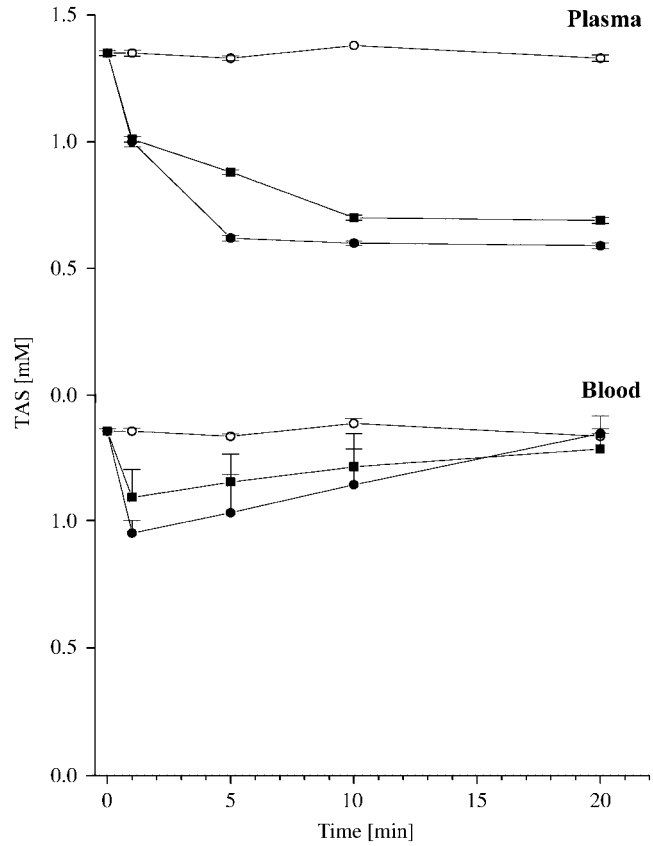


Figure 2. Kinetics of TAS levels in plasma (top) and in blood (bottom) samples from donors ($n = 10$; mean+SD; unpublished results). Plasma and blood samples were exposed for 1 min either O_2 (control,) or O_2–O_3 with ozone concentrations of 40 (■) and 80 (●) μg/mL.

level progressively decreases at first and then remain stable after 20 min.[47] The decrease was ozone-dose dependent and varied between 46 and 63%, respectively. Conversely, TAS levels in blood treated with the same ozone concentrations only decreased from 11 to 33%, respectively, in the first minute after ozonation. Then they recovered and returned to the original value within 20 min, irrespective of the two ozone concentrations, indicating the great capacity of blood to regenerate oxidized antioxidants, namely, dehydroascorbate and GSH disulfide (GSSG). Indeed, Mendiratta et al.[66,67] have found that dehydroascorbate can be recycled back to ascorbic acid within 3 min. Similarly, only about 20% of the intraerythrocytic GSH has been found oxidized to GSSG within 1 min after ozonation, but promptly reduced to normal after 20 min.[86] These data were enlightening and showed that the therapeutic ozonation modifies only temporarily and reversibly the cellular redox homeostasis. There is now full agreement that ascorbic acid, α-tocopherol, GSH, and lipoic acid, after oxidation, undergo an orderly reduction by a well-coordinated sequence of electron donations.[87]

LOP production follows peroxidation of PUFA present in the plasma: they are heterogeneous and can be classified as lipoperoxides (LOO), alkoxyl radicals (LO), lipohydroperoxides (LOOH), F_2-isoprostanes, and alkenals, among which 4-hydroxynonenal (4-HNE), acrolein and MDA. As free radicals and aldehydes are intrinsically deleterious, only precise and appropriate ozone doses must be used in order to generate them in very low concentrations. Figure 3 comparatively shows the modifications of plasma levels of TBARS, hemolysis, TAS, and protein thiols in a typical experiment when 13 human blood samples were exposed to air, O_2, or either 40 or 80 μg/mL ozone concentrations. Plasma TBARS in vitro are far more stable than ROS,[47] but, upon blood reinfusion, they have a brief half-life owing to a marked dilution in body fluids, excretion (via urine and bile), metabolism by glutathione-S-transferases (GST) and aldehyde dehydrogenase (ALDH).

Among the aldehydes, 4-HNE is quantitatively the most important. It is an amphipathic molecule and reacts with a variety of compounds such as albumin, enzymes, GSH, carnosine, and phospholipids.[88,89] There is no receptor for 4-HNE but Poli et al.[89] have reported that, after binding to more than 70 biochemical targets, it exerts some deleterious activity. Luckily, intracellular concentrations of GSH are high enough to frequently prevent or remove 4-HNE from adducts with enzymes. Owing to the unexpected stability of 4-HNE when samples of ozonated human plasma were incubated at 37°C for 9 hr, it was postulated that ozone, for its high solubility in the plasmatic water, steric reasons, and the abundance of albumin molecules prefers to target their bound PUFA. The scheme presented in Figure 4 envisages the events occurring in the plasma phase. It appears reasonable that during the rapid reaction of ozone with albumin PUFA in water, the suddenly generated aldehydes, mainly 4-HNE, will immediately form adducts with contiguous albumin molecules. This hypothesis is now well supported by recent findings,[90–92] which have shown that human albumin, rich in accessible nucleophilic residues, can quench up to 11 different 4-HNE molecules, the first being with Cys34, followed by Lys199 and His146. These important data clarify why ex vivo ozonation of blood does not harm the vascular system during the infusion of ozonated blood. The albumin-4-HNE adducts, not only are rapidly diluted in the blood pool but, being transferred into the extravascular pool, represent only a small aliquot of the whole albumin pool, containing as much as about 310 g protein. On this basis, it would be worthwhile exploring whether either the 4-HNE-modified albumin has an abnormal fate or how the aldehyde is released into other cell compartments, thus becoming able to trigger biochemical mechanisms. 4-HNE is the major product of peroxidation of n-6-PUFA, its concentration in normal plasma varies from 0.07 to 0.15 μM and increases with aging.[93,94] Needless to say that a constant increase in peroxidation as it happens after ischemia-reperfusion, CCl_4 intoxication,

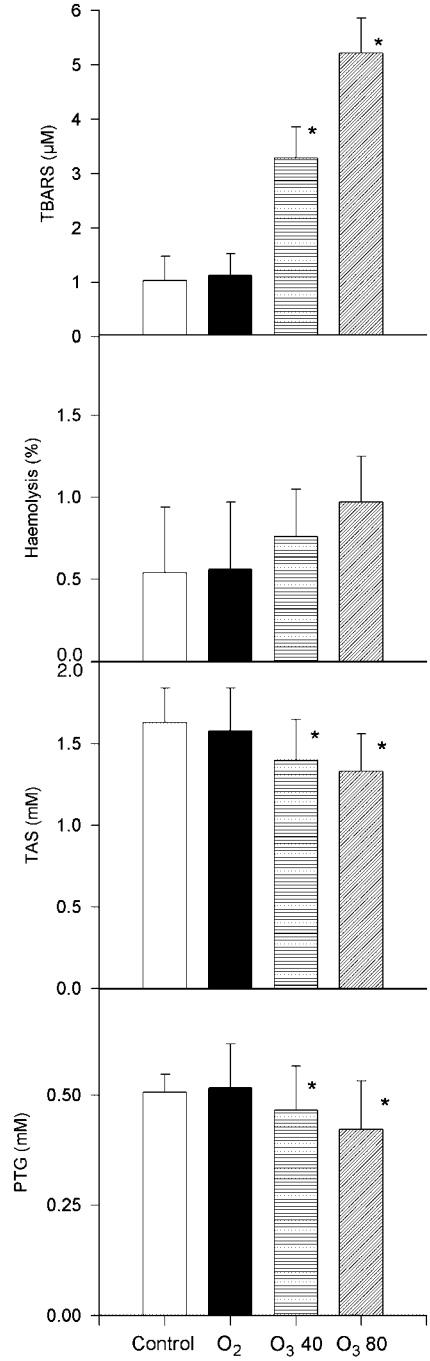


Figure 3. Thirteen human blood samples were exposed to air (control), or O_2, or O_2–O_3 with ozone concentrations of 40 and 80 μg/mL for 1 min. While TBARS, TAS, and PTG levels vary significantly ($p < 0.01$) after ozone exposure, there is a negligible increase in hemolysis. (Bocci V. How does ozone act? *Oxygen–ozone therapy. A critical evaluation*, chap. 13. Figure 40. Kluwer Academic Publishers; 2002. p 114. With kind permission from Springer Science+Business Media, formerly Kluwer Academic Publishers.)

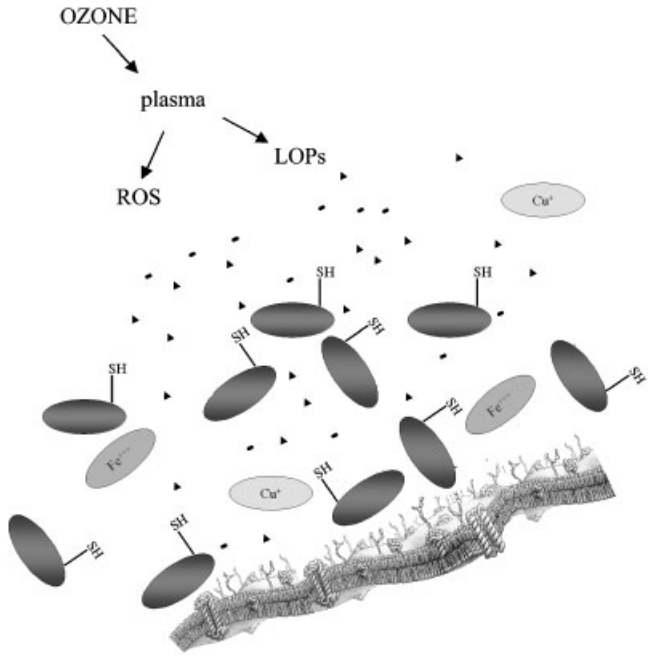


Figure 4. The scheme helps to imagine the multiplicity of substrate reacting with ozone dissolved in plasmatic water. Small circles, triangles, and squares symbolyze hydrosoluble antioxidants present in 100 mL of human blood (uric acid 4.5 mg/dL, ascorbic acid 1.5 mg/dL, glucose 80 mg/dL, etc…). Large albumin molecules (4,000 mg/dL) exposing –SH groups form a cloud over the cell membrane and protect it. Molecules such as transferrin and ceruloplasmin bind Fe^{3+} and Cu^{+} and prevent formation of $OH^{\cdot}$. The exogenous addition of 4–8 mg of ozone to 100 mL of blood is transitory and controlled by antioxidants. In contrast, the endogenous production of ROS is continuous and barely quenched by intracellular antioxidants.

ADP-iron overload, and chronic inflammation typical of some infections disease, diabetes, atherosclerosis, cancer, and degenerative pathologies causes a marked increase in 4-HNE levels, especially in the affected tissues. However, aerobic organisms, for accommodating the toxicity of aldehydic compounds, have simultaneously developed detoxifying systems[37,95–99] and their evaluation is relevant because the infusion of the ozonated blood into the donor patient implies an amount of an albumin-4-HNE adduct.

The following three processes schematically indicated in Figure 5 clarifies why 4-HNE is not a risk:

(1) *Dilution*: The highest concentration of 4-HNE measured after exposing 180 mL of human blood to the highest ozone amount (16 mg) is less than 1 mM in the plasma. During the 20 min intravenous infusion, the aldehyde will be promptly diluted in a total plasma-extracellular fluid volume of about 11 L, causing a transitory increase in the plasma level up to about 0.1 μM.

(2) *Detoxification*: Metabolism of 4-HNE is extremely fast either because small amounts of aldehydes interact with billions of cells endowed with several detoxifying enzymes such as ALDH, aldose reductase, and GST or the formation of an adduct with GSH.[36,37,98–100] Several authors[96,101,102] have determined a metabolic rate so high to conclude that "even with very high lipid peroxidation rates, 4-HNE cannot accumulate in an unlimited way".[89] These data are in agreement with our results in six patients when we could assess a half-life of infused TBARS of 4.2 ± 1.7 min.[48,49] On the contrary when the same preparation in ozonated plasma was incubated (at +37°C, pH 7.3) in acellular medium, TBARS levels hardly declined during the next 9 hr.[47]

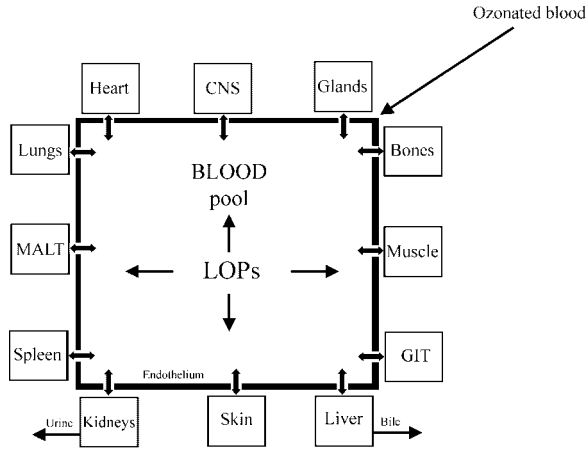


Figure 5. The multivariate biological response of the organism to ozonated blood can be envisaged by considering that ozonated blood cells and the generated LOP interact with a number of organs. Some of these represent real targets (liver in chronic hepatitis, vascular system for vasculopathies), while other organs are probably involved in restoring normal homeostasis. Gastrointestinal tract (GIT); mucosal associated lymphoid tissue (MALT).

(3) *Excretion*: Partially metabolized LOP are eliminated into both bile after hepatic detoxification and urine after renal excretion. In the rat, 4-HNE was detected in the urine as mercapturic acid conjugates.[35,98,103,104]

In normal conditions, owing to the efficiency of these processes, only submicromolar concentrations of LOP can reach organs such as bone marrow, endocrine glands, and even hypothalamic areas deprived of the blood–brain barrier where, via a variety of kinases and even a possible receptor for F_2-isoprostanes, may act as a signaling event of an ongoing acute oxidative stress[105–110] (Fig. 5). As a first conclusion it is clear that the ozonation process either happening in blood ex vivo or in an intramuscular site represents an acute, albeit small, oxidative stress. However, this process is acceptable only if the ozone is precisely calibrated against the antioxidant capacity of either blood or the injected tissue. Moreover, the ozone dose must never lower the antioxidant capacity more than 30% with a process lasting only a few minutes during which ozone reacts and disappears after leaving its messengers. Thus, the process of blood ozonation ex vivo has been characterized by the formation of ROS and LOP mainly acting in two phases. Among ROS, H_2O_2 is the earliest messenger rising and disappearing within 1 min in the plasma, while LOP during drug infusion in the donor reach the vascular systems, act on endothelial cells, and eventually reach parenchymal cells. Their pharmacodynamics minimize their potential toxicity thus making LOP as late and effective messengers.

B. The Effect of Ozone Messengers Onto Blood Cells

There are two questions to be clarified: first, does ozone directly activate the cells? Our methodological approach and experimental results exclude this possibility because when blood is gently mixed ex vivo with O_2–O_3, ozone dissolves rapidly in the water of plasma and there it immediately reacts with antioxidants and PUFA. Blood cell membrane phospholipids surrounded by a cloud of albumin molecules do not come in contact with ozone molecules because the calculated ozone dose is rapidly exausted (Fig. 4). This dangerous interference has been excluded by either a negligible hemolysis, or a change of the hematocrit value, or leakage of K^+ and lactate dehydrogenase, or a change of osmotic fragility, or of electrophoretic mobility, or increased methemoglobin.[47,54,65,111,112] Levels (mg/dL) of fibrinogen, cholesterol, triglycerids, HDL, and LDL in plasma are not modified even using the excessive

ozone concentration of 160 μg/mL per mL of blood.[112] Equally important is the stability of enzymes such as SOD, GSH-Pase, GSH-RD, and G6PDH in the erythrocytes.[112] Moreover, Shinriki et al.[65] after isolating the erythrocytic membranes after blood ozonation within the therapeutic range did neither detect a decrease in αα-tocopherol nor an increase in MDA.

It is unfortunate that in the past other authors[57,68,113–117] have reported that erythrocytes isolated from plasma, after three washings with saline and suspension in protein-free saline, undergo structural changes and intense hemolysis when exposed to ozone. These misleading and unphysiological data have greatly contributed to emphasize the ozone cytotoxicity, which obviously was enhanced by removing plasma antioxidants.[116] Moreover, the critical protective effect of plasma antioxidants has been emphasized in two recent studies.[118,119] These results were particularly evident on saline-washed blood mononuclear cells (BMC) with a marked decrease in mitochondrial functions.[118] Our thinking is well supported by other data[47,120,121] as well as recent results (Fig. 6) obtained after excessive ozonation of samples of normal human blood either collected in heparin or in sodium citrate. Interestingly, heparinized samples were far more susceptible to ozone most likely because of the remaining physiological Ca^{2+} level: in fact, a further addition of 2.5–5 mM Ca^{2+} enhanced the hemolysis up to 40%.

Second, how ozone messengers activate blood cells? Initially, the sudden formation of an H_2O_2 gradient between the ozonated plasma and the intracellular fluid causes the rapid passage of about 10% H_2O_2 into the blood cell cytoplasms and represents the triggering stimulus: depending upon the cell type, different biochemical pathways can be concurrently activated in erythrocytes, leukocytes, and platelets resulting in numerous biological effects. The rapid reduction of H_2O_2 to water is operated by the high concentration of intracellular GSH, CAT, and GSPase but, nonetheless, H_2O_2 must be above the threshold concentration for activating several biochemical pathways as follows.

The mass of erythrocytes mops up the bulk of H_2O_2: GSH is promptly oxidized to GSSG and the cell, extremely sensitive to the reduction of the GSH/GSSG ratio, immediately corrects the unbalance by either extruding GSSG, or reducing it with GSH-Rd at the expenses of ascorbate or of the reduced NADPH, which serves as a crucial electron donor. Next, the oxidized NADP is promptly reduced after the activation of the pentose phosphate

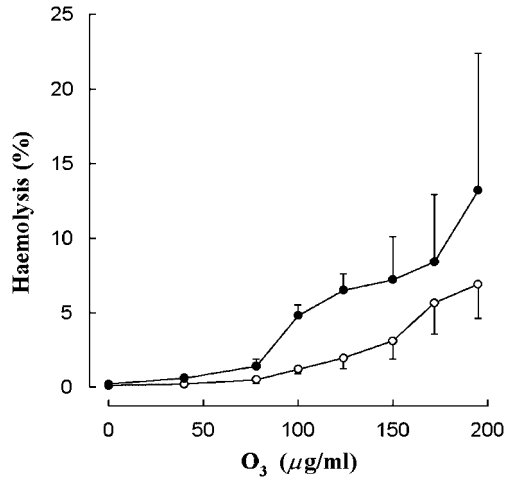


Figure 6. Kinetics of hemolysis in relation to ozone concentration (μg/mL per mL of blood). Blood of five donors was treated with CPD () or with 30 U/mL heparin (●) (mean+SD). (Bocci V. What happens in the intracellular environment after blood ozonation? *Oxygen–ozone therapy. A critical evaluation*, chap. 14. Figure 43. Kluwer Academic Publishers; 2002. p 123. With kind permission from Springer Science+Business Media, formerly Kluwer Academic Publishers).

pathway, of which glucose-6-phosphate dehydrogenase (G6PDH) is the key enzyme. In patients with ARMD, after 13 O_3-AHT, a small increase in ATP formation has been determined but whether this is due to the activation of the pentose cycle or to an increase in phosphofructokinase activity or to both remains to be clarified. The reinfused erythrocytes, for a brief period, enhance the delivery of oxygen into ischemic tissues because of a shift to the right of the oxygen–hemoglobin dissociation curve, due either to a slight decrease in intracellular pH (Bohr effect) or/and an increase in 2,3-diphosphoglycerate (2,3-DPG) levels as shown in Figure 7 (unpublished data). Obviously, an increase in this metabolite has a great significance because it enhances a shift to the right of the oxygenated hemoglobin, hence an increase oxygen delivery to hypoxic tissues. However, Figure 7 shows that the increase has been noted only in three patients where the initial levels were rather low. Thus, this observation needs to be explored in a large number of patients and it will be also necessary to clarify the activation of 2,3-bisphosphoglycerate mutase. Needless to say that one auto-hemotherapeutic treatment has a minimal effect and we need to ozonate at least 3–4 L of blood within a period of 30–60 days.

In another small group of five ARMD's patients after 15–17 O_3-AHT, an increase in some antioxidant enzymes has been determined (Fig. 8). This result has been reported also by other authors[122,123] and it is likely that LOP act as repeated stimuli on the endothelium and bone marrow and cause the adaptation to the ozone stress during erythrogenesis. Whether the enzymatic levels remain sustained for several months during the maintenance therapy need to be evaluated.

Another relevant finding was that in four patients with ARMD, after a cycle of 13 O_3-AHT treatments (in which ca. 3.8 L of blood were ozonated within 7 weeks), isopycnic centrifugation of blood separated old (heavy) and young (light) erythrocytes (RBC), which showed a marked increase in G6PDH in the young erythrocytic fraction generated during the course of ozone therapy (Table I). Whether the enzymatic levels remain sustained with time need to be evaluated. G6PDH activity, expressed as nmol/hr/mg hemoglobin, in total red blood cells was either 357 ± 91 or 406 ± 40, before and after the ozone therapy, respectively. While the enzymatic increase in the

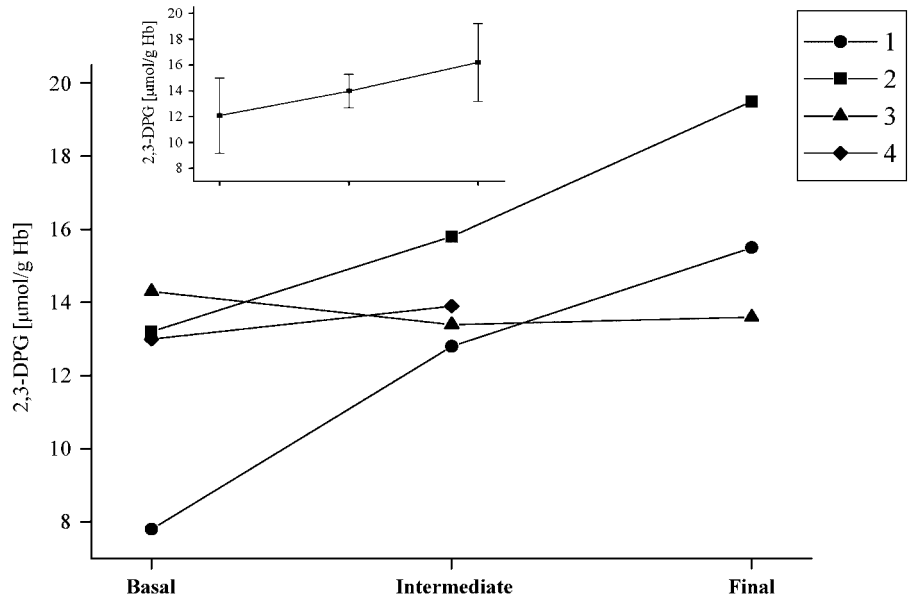


Figure 7. 2,3-DPG level variations in four patients performed before treatment (Basal), after 6–7 treatments (Intermediate), and at the end of treatments (Final). Insert shows the statistical dispersion (mean ± SD) of the data (unpublished results).

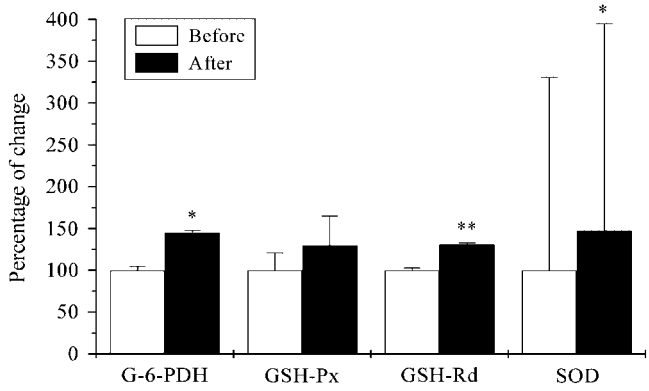


Figure 8. Increase in antioxidant enzymes in ARMD patients after 20–24 O_3-AHTs performed during 7–8 weeks ($n = 10$, mean +SD; unpublished results).

Table I. Evaluation of G6PDH Activity in Total, Young and Old Red Blood Cells (RBC) in Blood Samples from Four Patients With Age-Related Macular Degeneration Before and After an Ozone Therapy Cycle of 13 Treatments (Unpublished Results)

	G6PDH activity[a]		
	Total RBC	Young RBC	Old RBC
Before treatment ($n = 4$)	356.8 ± 90.7	550.3 ± 157.5	310.7 ± 127.3
After treatment ($n = 4$)	406.2 ± 40.4	784.2 ± 181.9	438.8 ± 86.7

[a]G6PDH activity expressed as nmol/hr/mg hemoglobin in whole erythrocyte population and in young and old fractions before and after 13 O_2/O_3 treatments. Results represent mean value $\pm$ SD.

whole erythrocyte population was understandably small, it was found markedly enhanced from 550 ± 157 to 748 ± 182 in very young (light) erythrocytes before and after ozone therapy, respectively. In the so-called old erythrocytes, which practically include the bulk of cells (20–120 days old), G6PDH obviously increased only from 310 ± 127 up to 435 ± 87 nmol/hr/mgHb. It is necessary to mention that the percentage of either young or old erythrocytes remained practically constant throughout the treatments (unpublished data). As a consequence, a patient with chronic limb ischemia (Phase II) undergoing ozone therapy shows a clinical improvement due to the formation of successive cohorts of erythrocytes progressively more capable of delivering oxygen to his ischemic tissues.

Although ozone is one of the most potent disinfectants, it has been shown[124,125] that ozone cannot inactivate bacteria, viruses, and fungi in vivo because, paradoxically, the pathogens are well protected, particularly inside the cells, by the powerful antioxidant system. Thus, the favorable effect of ozone therapy in some infectious diseases has been interpreted as due to ozone acting as a mild enhancer of the immune system, by activating neutrophils and stimulating the synthesis of some cytokines.[64,76,77,79,86,126,127] Once again the crucial messenger is H_2O_2 that after entering into the cytoplasm of BMC, by oxidizing selected cysteines, activates a tyrosine kinase, able to phosphorylate the transcription factor NF-κB. The release of an heterodimer, via effector genes, causes the synthesis of several proteins, among which, the acute-phase reactants, adhesion molecules, and numerous pro-inflammatory cytokines. This process, checked by a phosphatase or inhibited by cytoplasmic antioxidants, is very transitory. The release of several cytokines from ozonated blood upon in

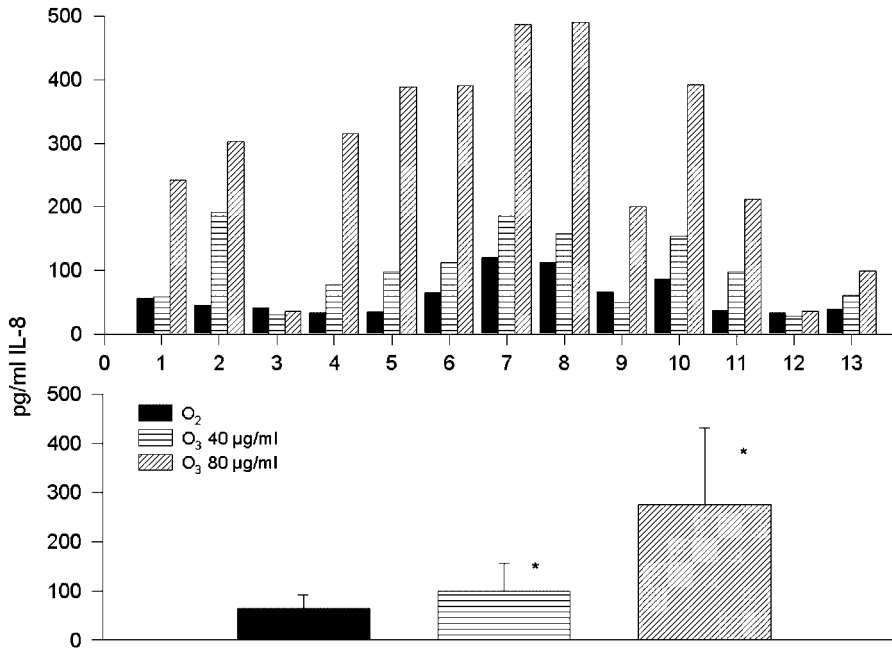


Figure 9. Effect of 1 min exposure of either O_2 or O_3 (40 and 80 µg/mL) on the production of IL-8 after 8 hr incubation of 13 blood samples. Average values are reported in the lower panel after subtraction of control values. *Significant difference ($p < 0.01$) compared with samples treated with O_2. The variable production of IL-8 among donors is noteworthy, particularly the lack of production of donors no. 3 and 12 likely due to a high TAS level. (Bocci V. What happens in the intracellular environment after blood ozonation? *Oxygen–ozone therapy. A critical evaluation*, chap. 14. Figure 53. Kluwer Academic Publishers; 2002. p 134. With kind permission from Springer Science+Business Media, formerly Kluwer Academic Publishers).

vitro incubation has been measured since 1990.[128] Once the ozonated leukocytes return into the circulation, they home in lymphoid microenvironments and successively release cytokines acting in a paracrine fashion on neighboring cells with a possible reactivation of a depressed immune system. This process, described as the physiological cytokine response,[129] is a part of the innate immune system and helps us to survive in a hostile environment. One of our most interesting result has consisted in observing the variable individual production of IL-8 by blood donors in 13 blood ozonated samples.[130] Figure 9 shows that the different release of IL-8 by medium and high ozone concentrations indicates the presence of high, medium, and no responders. The result was interpreted as due to both genetic factors and variable levels of plasma antioxidants.

During ozonation of blood, particularly if it is anticoagulated with heparin, an ozone-dose-dependent increase in activation of platelets has been noted[131,132] with a consequent release of typical growth factors, which will enhance the healing of chronic ulcers in ischemic patients (Fig. 10). Whenever possible, albeit with caution, the use of heparin as an anticoagulant is preferable to sodium citrate because, by not chelating plasmatic Ca^{2+}, reinforces biochemical and electric events.

Finally, during the reinfusion of the ozonated blood into the donor, the vast expanse of the endothelial cells is activated by albumin-LOP resulting in an increased production of NO, plasma *S*-nitrosothiols, and *S*-nitrosohemoglobin.[133–136] Figure 11 shows the in vitro production of nitrite by human vascular endothelial cells after addition of human ozonated serum. Production of NO· was markedly enhanced by the addition of L-arginine (20 µM) and was potentiated by O_3, while it was inhibited in the presence of the NO· inhibitor *N*-ω-nitro-L-arginine-methyl ester (L-NAME). While NO has a half-life of less than 1 sec, protein-

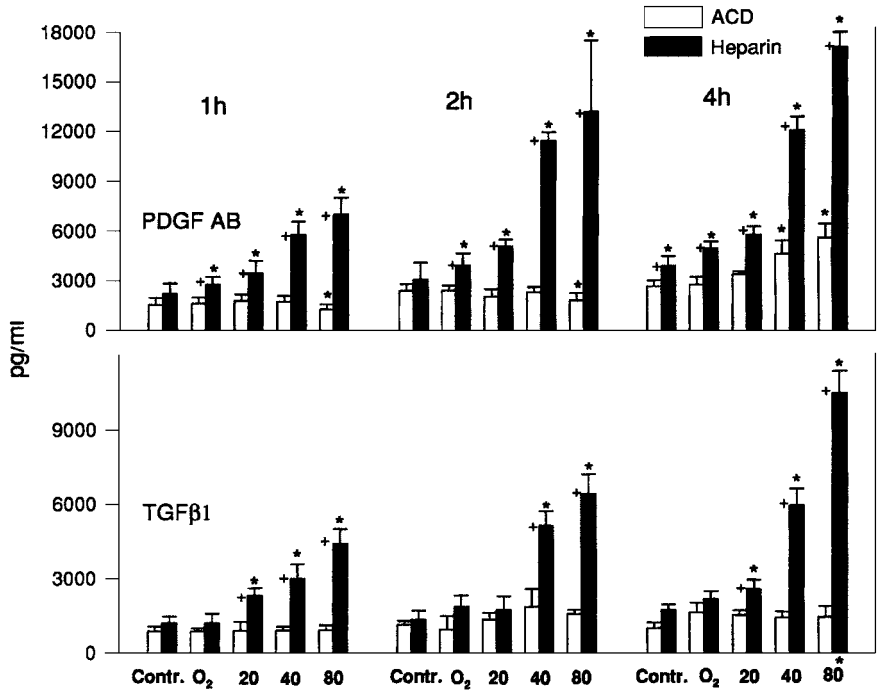


Figure 10. Release of factors from human platelets during 1, 2, and 4 hr incubation. The same PRP samples collected either in heparin or ACD were not exposed (control), or exposed to O_2 alone or to O_2–O_3 at concentrations of 20, 40, and 80 μg/mL for 30 sec before incubation. Statistical significance is indicated by (*) for intergroup analysis and (+) for intragroup analysis. (Bocci V. What happens in the intracellular environment after blood ozonation? *Oxygen–ozone therapy. A critical evaluation*, chap. 14. Figure 65. Kluwer Academic Publishers; 2002. p 158. With kind permission from Springer Science+Business Media, formerly Kluwer Academic Publishers).

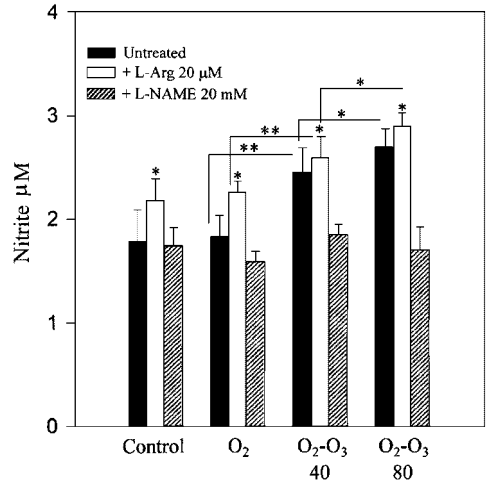


Figure 11. Production of nitrite by HUVECs, measured after 24 hr incubation, after addition of normal human serum either oxygenated or ozonated (at 40 and 80 μg/mL). Effects of addition of L-arginine and L-NAME. The data are presented as the mean+SD of six different experiments. (Bocci V. What happens in the intracellular environment after blood ozonation? *Oxygen–ozone therapy. A critical evaluation*, chap. 14. Figure 68. Kluwer Academic Publishers; 2002. p 165. With kind permission from Springer Science+Business Media, formerly Kluwer Academic Publishers).

bound NO can exert vasodilatation also at distant ischemic vascular sites with relevant therapeutic effect. There is little doubt that the therapeutic advantage observed in many patients with peripheral obstructive arterial disease (POAD) is due to multiple factors such as an increased release of oxygen due to vasodilation by trace amounts of NO and CO, and an increased availability of growth factors from platelets.

All of these data emphasize that submicromolar LOP levels can be stimulatory and beneficial,[137] while it is well established that micromolar levels can be toxic.[89] This conclusion reinforces the concept that optimal ozone concentrations are critical for achieving a therapeutic result: too low concentrations are practically useless (at best elicit a placebo effect), too high may elicit a negative effect (malaise, fatigue), so that they must be just above the threshold level to yield an acute, absolutely transitory oxidative stress capable of triggering biological effects without toxicity. There is no doubt that the process of blood ozonation must be precisely controlled with a calculated ozone dosage: at this condition it is not deleterious and actually capable of eliciting a multitude of useful biological responses and, possibly, reversing a chronic oxidative stress due to ageing, chronic infections, and the several diseases grouped within the metabolic syndrome. Indeed the ozonotherapeutic act has been interpreted as a safe "therapeutic shock" able to restore homeostasis.[138] These aspects are critical and imply two drawbacks: first, if the ozone generator is not well calibrated or periodically checked, it may release erroneous and dangerous ozone amounts and, second, if the ozonetherapist does not fully understand the ozonation process, he may do some mistakes and jeopardize the approach. Other aspects regarding the future of ozone therapy will be evaluated in Section 9.

5. IS OZONE ABLE TO INDUCE AN ADAPTATION TO CHRONIC OXIDATIVE STRESS?

That ozone, one of the most potent oxidizer, may induce an antioxidant response capable of reversing a chronic oxidative stress at first sight seems a paradoxical concept. However, this concept has become common in the animal and vegetal kingdoms.[147–150] Any change of the external or internal environment disturbs cell homeostasis, but if the stress is tolerable, or carefully calibrated in intensity, the cell or the organism can adapt to it and survive. If it is excessive or the cell is already damaged, the cell programmes its own death. Stresses include hyperthermia, hyperoxia, ischemia, hypoglycemia, pH modifications, radiation, very likely mental and hormonal derangement, and chronic infections, which imply an excessive ROS and LOP production. Obviously, ozone has to be included and the phenomenon of ozone tolerance is now well known. The concept of "ischemic preconditioning" for the heart, which after undergoing a brief, nonlethal period of ischemia can become resistant to infarction from a subsequent ischemic insult was pioneered by Murry et al.[151] "Oxidative preconditioning" has been also well demonstrated.[152–157] Therefore, it is of interest that small amounts of ROS and LOP can elicit the upregulation of antioxidant enzymes on the basis of the phenomenon described under the term of "hormesis."[158–162] On the basis of this phenomenon that says "the exposure of an organism to a low level of an agent, harmful at high levels, induces an adaptive and beneficial response,"[159,160,163] it has been postulated that LOP, by acting as long-distance messengers, can transmit to all organs the information of an acute oxidative stress.[54] The bone marrow is particularly relevant because it can upregulate antioxidant enzymes during erythrogenesis and may allow the release of staminal cells for possibly regenerating infarcted organs.

The oxidative preconditioning or, as we prefer, the adaptation to the chronic oxidative stress has been now demonstrated experimentally.[40,45,48] The increased synthesis of enzymes such as SOD, GSPase, GSH-Rd, and CAT has been repeatedly determined in experimental

animals and in patients (reviewed in 57). Iles and Liu[164] have demonstrated the 4-HNE, by inducing the expression of γ-glutamate cysteine ligase, causes an intracellular increase in GSH, which plays a key role in antioxidant defence. Furthermore LOP induce oxidative stress proteins, one of which is heme-oxygenase I (HO-1 or HSP-32) that, after breaking down the heme molecule, delivers very useful compounds such as CO and bilirubin.[165–171] Bilirubin is a significant lipophilic antioxidant and a trace of CO cooperates with NO in regulating vasodilation by activating cyclic GMP. Fe^{2+} is promptly chelated by the upregulated synthesis of ferritin.[172] The induction of HO-1 after an oxidative stress has been described in thousands of papers as one of the most important antioxidant defence and protective enzyme. Both mild ozone inhalation and ozonated plasma induce HSP-70.[170,173] When ozone is judiciously used in small doses, can become a useful drug able to correct an otherwise irreversible state of oxidative stress. There are serious pathologies such as chronic infections, neurodegenerative, and autoimmune diseases in which a vicious imbalance between overproduced oxidants and depleted antioxidant defenses become established and lead to death. How modern medicine correct this imbalance? Several therapeutic approaches among which administration of antioxidants with addition of *N*-acetylcysteine have been often reported[174–176] but they are only partly successful.

The ozone treatment is now envisaged as a transitory and miniaturized oxidative stress resulting in a sort of therapeutic "shock" for the ailing organism. Ozone acting as a prodrug, realizes this shock because generates a number of messengers able to reach all cells in the organism (Fig. 5).

Submicromolar levels of LOP act as key mediators and in still responsive cells may activate a sequence of biochemical mechanisms able to reactivate gene expression leading to a renewed synthesis of HSP and antioxidant enzymes. If the disease has gone too far, cells become anergic and are unable to respond to the treatment. Indeed, we have observed that after intensive chemotherapy, preterminal cancer patients do not improve with ozone therapy. That is also the reason why we always start using low ozone concentrations just above the threshold level to better achieve the ozone tolerance and in-line with the old concept "start low, go slow." Moreover, the stimulation of the endocrine and central nervous systems may help to understand why most of the reactive patients during prolonged ozone therapy report a feeling of euphoria and wellness probably due to an improved metabolism as well as to an enhanced hormonal or neurotransmitters release.

6. WHICH ARE THE ROUTES OF OZONE ADMINISTRATION?

Table II shows that ozone can be administered with great flexibility but it should never be injected intravenously as a gas because of the risk of provoking oxygen embolism, given the fact that the gas mixture contains always no less than 95% oxygen. So far the most advanced and reliable approach has been the O_3-AHT because, on the basis of the patient's body weight, a predetermined volume of blood (200–250 mL) to which has been added either sodium citrate 3.8% (1+9 mL blood) or heparin (20 IU/mL of blood) can be exposed to an equal volume of gas (O_2–O_3) in a stoichiometric fashion, with the ozone concentration precisely determined by using an ozone-resistant, disposable 500 mL glass bottle *under vacuum.*

This simple, inexpensive (all the necessary disposable material costs about 12 US$) procedure has already yielded therapeutic results in vascular diseases superior to those achieved by conventional medicine (discussed in Section 7A). Moreover, the therapeutic modalities, until now restricted to major AHT and to the empirical and imprecise rectal insufflation of gas,[139,177,178] have been extended: they include the quasi-total body exposure

Table II. Routes of Ozone Administration

Parenteral	Topical or locoregional
Intra-arterial (IA)[a]	
Intramuscular (IM)	Nasal[b]
Subcutaneous (SC)	Tubal[b]
Intraperitoneal (Ipe)	Auricular
Intrapleural (IPL)	Oral[b]
Intra-articular (IPL)	Vaginal
(a) Periarticular	Urethral and intrabladder
(b) Myofascial	Rectal
Intradiscal (ID)	Cutaneous
Intraforaminal (IF)	Dental
Intralesional (Iles)[c]	

[a]No longer used for limb ischemia. Hepatic metastasis could be embolized via the hepatic artery.
[b]To be performed during 30–40 sec apnea.
[c]Intratumoral or via a fistula.

to O_2–O_3[140,179] and the extracorporeal blood circulation against O_2–O_3.[141] The latter procedure is rather invasive because blood collected from a vein circulates through an ozone-resistant gas exchanger[180,181] and, with the help of a peristaltic pump, returns to the circulation via a contralateral vein. On the other hand, the partial cutaneous exposure to oxygen–ozone does not need any venous puncture and, owing to the vast expanse of the skin, allows a generalized and beneficial effect. Clearly, today we can select the most suitable method for different pathologies, their stage, and the patient's condition. A discussion on its own is needed for the minor AHT, which basically consists of withdrawing 5 mL of blood to be immediately and vigorously mixed for 1 min with an equal volume of O_2–O_3 at an ozone concentration ranging between 80 and 100 μg/mL of gas per mL of blood already extensively described.[142] The slightly oxidized blood, including the foam, is promptly injected into the gluteus muscle without the need of any anesthetic. As an unspecific immunomodulatory approach, it has been widely used during the last two decades for successfully treating herpetic infections.[143]

The slight hemolysis (~2%) is purposefully required because the heme released in the gluteal muscle will stimulate the synthesis of HO-1.[165,171]

7. *WHICH DISEASES ARE SUITABLY TREATED WITH OZONE THERAPY*

On the basis of the mechanisms of action, ozone therapy can induce the following biological responses: (a) it improves blood circulation and oxygen delivery to ischemic tissue owing to the concerted effect of NO and CO and an increase in intraerythrocytic 2,3-DPG level; (b) by improving oxygen delivery, it enhances the general metabolism; (c) it upregulates the cellular antioxidant enzymes and induces HO-1 and HSP-70; (d) it induces a mild activation of the immune system and enhances the release of growth factors; (e) it has an excellent disinfectant activity when topically used, while this is negligible in the circulation owing to blood antioxidant capacity; (f) it does not procure acute or late side effects;[182] (g) it procures a surprising wellness probably by stimulating the neuro-endocrine system. It does seem that ozone, by acting on many targets, can indirectly help in recovering functional activities gone astray because of a chronic disease and, if this interpretation is correct, ozone therapy acts as a biological response modifier. Although ozone therapy is now used in many countries, it is

mostly used by private physicians and the performance of large clinical trials has been severely hampered by lack of sponsors, disinterest of pharmaceutical as well as health authorities, and prejudice by clinical scientists. However, a number of studies have been performed with the following results:

A. Peripheral Obstructive Arterial Diseases

Even a modest obstruction of limb arteries due to atherosclerosis, diabetes, or Buerger's disease (thromboangiitis obliterans) leads to a progressive reduction of blood flow to the feet. Tissue ischemia and any minor trauma facilitate the formation of an ulcer, which will not heal because oxygen, nutrients, and growth factors indispensable for the repair process are lacking. This pathology is the best suited to be treated with O_3-AHT. According to Fontaine-Leriche classification, patient at either stage II (intermittent claudication and transitory pain), or stage III (continuous pain, cyanosis, and possibly initial ulcers) achieve the best results. Stage IV includes incipient necrosis of toes and unbearable pain leads to surgical amputation that can be avoided with O_3-AHT in about 50% of cases.[183–185] In comparison to pentoxyfilline and prostanoids (the gold standard of orthodox treatment), O_3-AHT has proved more effective and without side effects in ischemic vascular disease. In a small trial, 28 patients were randomized to either receive their own ozonated blood or an IV infusion of prostacyclin.[186] All patients continued conventional treatment with statins, antihypertensive, and antiplatelet aggregation drugs. Ozone therapy proved more effective than prostacyclin in terms of pain reduction and improvement in the quality of life, but no significant difference was seen in vascularization of the lower limbs in either group, most likely due to the short duration of treatment (14 treatments in 7 weeks). More prolonged treatments lead to a satisfactory healing of ulcers.[187] Previous studies[122,188–194] have shown the validity of O_3-AHT in this complex pathology, but it is a mistake to stop therapy too early in these patients because O_3-AHT, as with other conventional drugs, must be continued, albeit less frequently, for life. An improved schedule on a trial in progress consists of two O_3-AHT (225 mL blood plus 25 mL 3.8% sodium citrate solution), given weekly for at least 4 months. Topical therapy performed with ozonated olive oil is extremely useful when initial dry gangrene or ulcers are present. The frequency of O_3-AHT depends upon the stage of the disease and regarding the III and IV stages it can be done every day in the attempt to prevent amputation. How well O_3-AHT works it appears evident by the fact that the nocturnal excruciating pain disappears after the first two to three treatments, indicating the improvement of blood flow in the ischemic tissue and the lack of "stealing" blood away from underperfused muscle.

On January 2008, the Lancet published a double-blind, placebo controlled study (ACCLAIM trial) in 2,426 patients with New York Heart Association (NYHA) functional classes II–IV chronic heart failure (CHF).[195] Beside standard medication, the experimental group during a period of some 24 weeks, underwent about 25 intragluteal injections each patient receiving 10 mL of its own blood heavily oxidized with ozone associated with UV irradiation and heating at 42.5°C. It is unbelivable that 10 mL of blood were oxidized with as many as 75 mg of ozone, a dose that kills all cells and denature plasma proteins. This procedure, which is a sort of minor O_3-AHT,[196] had been invented with the aim to produce immunosuppressive compounds able to counteract the pathophysiological mechanisms responsible for the progression of CHF. Results have been disappointing because no difference in the composite endpoint of death for cardiovascular reasons between the control and the experimental group were noted. A few researchers[197–200] have criticized the approach that had been also a failure in the previous Simpadico trial in patients with chronic limb ischemia.[201] Actually this trial was stopped because of the risk of inducing neoplasia. This

approach has been discussed here because, being based on an irrational concept, may undermine the progress of the real O_3-AHT that utilizes the minimal amount of ozone just sufficient for triggering useful biological activities.

Millions of people suffer from chronic limb, brain, and heart ischemia, which represent the major cause of death worldwide. This has a huge socio-economic impact, particularly in the developing world. If only orthodox medicine will accept O_3-AHT as an adjunct to standard medication, a great leap forward will be noted.

B. Age-Related Macular Degeneration

In the UK alone, some 200,000 patients affected by the "dry" (atrophic) form of ARMD are suitable for treatment with O_3-AHT,[202] but all over the world there are about 30 million people searching for a therapy. Nonetheless, ophthalmologists can only prescribe antioxidants and zinc, which are minimally effective.[203,204] Since 1995, almost 1,000 patients with the dry form of ARMD have been treated with O_3-AHT at our polyclinic and three-quarters have shown an improvement of one to two lines on the visual acuity chart.[144,205]

Usually 15–18 treatments, at an initial ozone concentration of 20 μg/mL of gas per mL blood, slowly upgraded to 60 μg/mL (twice weekly), followed by two monthly session as a maintenance therapy, allows to maintain the improvement. Although uncontrolled, this study emphasizes that O_3-AHT is the only treatment able to dramatically improve the patient's quality of life. In this disease there is progressive degeneration and death of the fovea centralis photoreceptors and of the pigmented retinal epithelium (PRE) as a consequence of several factors, one of which is chronic hypoxia. Although O_3-AHT induces a pleiotropic response, the main advantage is due to an increased delivery of oxygen to the retina, which is the bodily tissue with the highest oxygen consumption. It is worth noting that O_3-AHT is useless, even harmful, in the exudative form of ARMD and in multigenic and progressive disorders (e.g., retinitis pigmentosa and recessive Stargardt's disease).[206] The exudative form, characterized by an aberrant choroidal vascular growth and a vascular hyperpermeability beneath the retina and the PRE, is caused by worsened ischemia, which negatively stimulates the release of the vascular endothelial growth factor. It must be emphasized that O_3-AHT (in the dry form) not only improves visual activity but at least, in part, renders the patient capable of autonomous life.

C. Chronic Infectious Diseases

Ozone is regarded as the best topical disinfectant because bacteria, viruses, fungi, and protozoa, when free in water, are readily oxidized.[207,208] Disappointingly, destruction of free pathogens in plasma by ozone either ex vivo or in vivo is greatly hampered by soluble antioxidants such as albumin, ascorbic acid, and uric acid and they are virtually unassailable when there are intracellular located.[124,125] However, ozone therapy still deserves attention because, by improving metabolism and operating as a mild cytokine inducer,[64] it can have a beneficial influence on infectious diseases. Thus, there remains a place for the application of O_3-AHT as an adjuvant in chronic viral infections (e.g., HIV, HCV, HSV), in combination with highly active anti-retroviral therapy (HAART), pegylated interferon-α plus either lamivudine or ribavirin and the acyclovir.

On the other hand, bacterial septicaemia must be treated with the most suitable antibiotics to prevent toxaemia and multisystem organ dysfunction. Particularly important is the topical application of either (i) ozone as a gas mixture (about 4% ozone and 96% oxygen);[209,210] or (ii) as ozonated water; or (iii) ozonated oils (where ozone is firmly stabilized as a triozonide)[208,211–214] for the treatment of bacterial, viral, and fungal infections, aphthous ulcers, burns, abscesses, and osteomyelitis. Topical therapy is most effective when combined

with O_3-AHT owing to the improved oxygenation of hypoxic tissues. Radiodermatitis[215] and wound healing have been enhanced because ozonated solutions display a cleansing effect, act as a disinfectant, and stimulate tissue reconstruction. A recent review reports that the high rates of diabetes in many parts of the world make foot ulcers a major and increasing public-health problem. Foot ulcers cause substantial morbidity, impair quality of life, engender high treatment costs (about US$17,500–27,987) and are the most important risk factor for lower-extremity amputation.[216] Although the constant use of rectal–colon insufflation of O_2–O_3 is not the optimal approach, it seems to improve the prognosis of diabetes by combining topical therapy with ozonated oil and O_3-AHT.[217] This study needs to be confirmed. Ozonated olive oil is an amazing preparation because combines antibacterial activity with healing properties due to the slow release of oxygen in hypoxic tissues and the stimulation of fibroblasts proliferation.[212,213] Chronic ulcers and/or putrid wounds are one of the most distressing and difficult medical problems with which to deal and are caused by ischemia, diabetes, immunosuppression, and malnutrition. During the past decade the use of ozone derivatives in such cases has proved very beneficial,[143] but so far official medicine has not yet discovered this excellent preparation far more effective than ointments containing often ineffective antibiotics and corticosteroids, which delays healing. With the current increase in health-care costs, O_3-AHT and ozonated oils deserve attention because they reduce hospital assistance and are inexpensive.

D. Pulmonary Diseases

Lung diseases, such as chronic obstructive pulmonary disease (COPD), will soon become the fourth most common cause of death, which, with emphysema and asthma, make significant incapacity. Using corticosteroids, long-acting β_2-agonists, and antibiotics, orthodox medicine has certainly proved helpful,[218] but it cannot change the course of COPD. However, in a series of elderly patients affected by macular degeneration and either emphysema or COPD, a remarkable improvement has been observed by combining ozone therapy[219] (using the schedule adopted for vasculopathies) with the best conventional treatments. It is unfortunate that so far a randomized study evaluating orthodox therapy with or without O_3-AHT has not been performed.

E. The versatility of Ozone Application in Orthopaedics and Dentistry

The application of ozone in low back pain has proved very effective. It can be administered directly (intradiscal),[220–224] or indirectly, via intramuscular administration into the paravertebral muscles. This latter type of administration has been assimilated to a "chemical acupuncture."[145] During the last 6 years, more than 30,000 patients with hernial disc have been treated in Italy with a success rate varying from 62 to 80%. The value of this approach, minimally invasive and without risk, has been already recognized in several countries, from China to Spain and South America. As shown also in another study on pain-related disorders due to sport injury (232 subjects) and inflammatory disorders (770 subjects)[225] it appears that ozone exerts a multiplicity of effects, such as the activation of the anti-nociceptive system, and it has anti-inflammatory action due to lipid peroxidation products, with the consequent inhibition of cyclooxygenase-2 (COX-2).[226,227]

Finally, ozone has proved very useful in dentistry for eliminating infections and blocking primary root carious lesions.[228,229] The interested reader will appreciate the notable book "*Ozone: the revolution in dentistry*."[230] After almost 80 years the intuition of Dr. Fisch could not receive a more enthusiastic appreciation by Prof. Lynch.

8. IS OZONE THERAPY A BAD COPY OF HYPERBARIC OXYGEN THERAPY?

It is often thought that ozone therapy tries to simulate the advantages of the much better known hyperbaric oxygen therapy (HOT)[231–233] and therefore it seems useful to clarify that these two approaches are both theoretically and practically different.

In the former, the drug is represented by ozone and, while we have described its initial reaction and the cascade of active messengers, it has also been pointed out that oxygenation of blood is not its primary intent. Conversely, by breathing almost pure oxygen at 2.6 bar into the hyperbaric chamber, the volume of dissolved oxygen in the plasma increases up to about 5 mL/dL, that is enough to satisfy ischemic tissues even if the absence of fully oxygenated hemoglobin. HOT is only transitorily effective because after 2 hr of therapy, hypoxia resumes in ischemic tissues and therefore the therapeutic effect is temporary. However, HOT has an exclusive role in CO-poisoning, air embolism, decompression sickness, and perhaps clostridial myonecrosis while ozone therapy is far more effective and practical to perform in POAD, heart ischemia, ARMD, diabetic foot, chronic ulcers, and bedsores. Thus, both approaches are relevant but each one has its selected field of application and the difference should be understood for the sake of the patient.[146]

9. CONCLUSIONS

The history of medicine remind us that in the past the application of several important approaches has been delayed owing to prejudice, lack of knowledge, or of sponsors and often by commercial competition. Ozone is inexpensive and therefore ozone therapy does not make an exception in spite of the fact that all chemical, biochemical, physiological, and pharmacological mechanisms elicited by ozone as primum movens are in the realm of orthodox medicine. One wonders if now with the advent of molecular medicine and gene therapy, ozone therapy is obsolete or worthwhile being pursued. Our many treated patients answer for us by saying that it is very beneficial. The compliance is excellent and the patients, as soon as the therapeutic effect declines, ask for a new cycle, showing the benefit and lack of side effects. It has been unfortunate that, in the past, the direct intravenous injection of the gas, now prohibited, the use of primordial ozone generators and misuse of ozone by incompetent quacks has generated serious doubts about its validity. Moreover, pulmonary toxicity due to prolonged inhalation of polluted air and many nonphysiological studies, performed in saline washed erythrocytes unprotected by the potent plasma antioxidants, have generated the dogma that ozone is always toxic and should not be used in medicine. This concept cannot be generalized because it does not take into account the profound difference between the endogenous chronic oxidative stress, due to aging or to a chronic disease, and the calculated, extremely brief, and well-calibrated oxidative stress induced on blood by using a precise and small ozone dose. When the appropriate ozone dose reacts with biomolecules it yields a number of compounds that in spite of their intrinsic toxicity, thanks for their pharmacodynamic, stimulate important biochemical pathways. Indeed, the medical effect depends upon a critical balance between an appropriate small dose of ozone and an almost infinite reacting variables such as the multiplicity of antioxidants, the life-time of ROS and LOP, their in vivo pharmacokinetic, and most important the variability of the biological response depending upon on enzyme reactivity and the stage of the disease.

Since the discovery of NO as a physiological messenger, other gaseous molecules such as CO, H_2S, and H_2,[234–236] in spite of being known as potentially toxic molecules, if used

judiciously are now considered as possible therapeutic agents. Any drug, depending upon its dosage can be either therapeutic or toxic. A striking example is represented by a vital compound such as glucose, its normal concentration in the plasma ranges between 0.7 and 1.0 mg/mL. However, when this concentration falls below 0.4 mg/mL, the consequent hypoglycemic coma can be deadly. On the other hand, if the glucose concentration remains constantly above 1.3 mg/mL, it induces the metabolic syndrome, which is well exemplified by the current diabetic epidemic. Finally, oxygen at 21% concentration in air (and an arterial pO_2 of about 99 mmHg) allows us to live for almost 80 years but it is deadly if we breathe pure oxygen for a few days. Thus, while a further discussion regarding ozone toxicity in medicine appears futile, it is important to examine if, indeed ozone therapy will be able to acquire a right place among the medical armamentarium. In the last decade, ozone therapy has attracted great attention in less-developed countries, while it remains partly prohibited in USA and poorly regarded in other developed countries. What can be done to change this severe outlook? Today we have a comprehensive framework for understanding the biochemical mechanisms and the biological effects of ozone and we have at least in part the capability of recommending ozone therapy in selected diseases either as a first choice or even better in combination with orthodox therapy. Thus, first, we must continue to organize specialized courses for physicians for avoiding conceptual or technical pitfalls. Second, while it is important to continue specific biologic studies, it is imperative to perform controlled and extensive clinical trials to prove beyond any doubt the value of ozone therapy at least in vascular diseases. Unless this is done, there is no future for ozone therapy within official medicine. The stumbling block is represented by lack of sponsors, disinterest of the pharmaceutical industry, and negligence of health authorities. As ozone therapy is a very cheap treatment, especially if it will be performed in all hospitals on a daily basis, it will markedly reduce both medical cost and invalidity. Almost needless to say that ozone therapy, like orthodox medicine, cannot "cure" several human diseases such as ARMD, atherosclerosis, and metabolic diseases. However, the maintenance therapy associated with conventional medication could improve the life of many patients. By considering the huge cost of reliable controlled and randomized clinical trials, unless health authorities give a financial support, ozone therapy will remain in limbo and in the hands of private physicians who can only report anecdotal and yet useless data. Only scientifically well-demonstrated therapeutic advantages will be able to dissipate prejudice and allow oxygen–ozone therapy to become a world wide useful medicinal treatment.

10. ABBREVIATIONS

4-HNE	4-hydroxynonenal
ALDH	aldehyde dehydrogenase
ARMD	age-related macular degeneration
ASF	airway surface fluid
ATP	adenosine triphosphate
BMC	blood mononuclear cells
CAT	catalase
CCl_4	carbon tetrachloride
CGMP	cyclic guanosine monophosphate
CHF	chronic heart failure
CNS	central nervous system
CO	carbon monoxide

COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
DPG	2,3-diphosphoglycerate
ELF	epithelial lining fluid
G6PHD	glucose-6-phosphate dehydrogenase
GSH	glutathione
GSH-Rd	glutathione reductase
GSPase	glutathione peroxidase
GSSG	oxidized gluthathione
GST	glutathione-*S*-transferases
HAART	highly active anti-retroviral therapy
HClO	hypochloric acid
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HO-1	heme oxygenase-1
HOT	hyperbaric oxygen therapy
H_2O_2	hydrogen peroxide
HSP	heat stress proteins
HSV	herpes simplex viruses
HUVEC	human vascular endothelial cells
IFNγ	interferon gamma
IL-1	interleukin-1
IL-8	interleukin-8
LDH	lactate dehydrogenase
L-NAME	*n*-omega-nitro-L-arginine methyl ester
LOP	lipid oxidation products
MA	mercapturic acid
MDA	malondialdehyde
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
NO	nitric oxide
N_2O	nitric dioxide
O_2^-	anion superoxide
$^{\cdot}OH$	hydroxyl radical
O_3-AHT	ozonated autohemotherapy
PDGF	platelet-derived growth factor
POAD	peripheral obstructive arterial disease
ppm	parts per million
PUFA	polyunsaturated fatty acids
RBC	red blood cells
ROS	reactive oxygen species
PRE	pigmented retinal epithelium
SOD	superoxide dismutase
TAS	total antioxidant status
TBARS	thiobarbituric acid reactive substances
TGFβ1	transforming growth factor β1
TNFα	tumor necrosis factor alpha
Trx	thioredoxin
UV	ultraviolet radiation
VEGF	vascular endothelial growth factor

ACKNOWLEDGMENTS

One of us (V.B.) is grateful to the University of Siena for the permission to continue to work in the Department of Physiology as Emeritus Professor of Physiology. We are grateful and thank Mrs. Helen Carter for revising the English manuscript.

REFERENCES

1. Burns DT. Early problems in the analysis and the determination of ozone. Fresenius J Anal Chem 1997;357:178–183.
2. Rubin MB. The history of ozone. The Schönbein period, 1839–1868. Bull Hist Chem 2001;26:40–56.
3. Battino R. Oxygen and ozone. IUPAC solubility data series. Vol. 7. Oxford, UK: Pergamon Press; 1981.
4. Kogelschatz U, Eliasson B, Hirth M. Ozone generation from oxygen and air: Discharge physics and reaction mechanisms. Ozone Sci Eng 1988;10:367–378.
5. Tanaka T, Morino Y. Coriolis interaction and anharmonic potential function of ozone from the microwave spectra in the excited vibrational states. J Mol Spectrosc 1970;33:538–551.
6. Babior BM. Oxygen-dependent microbial killing by phagocytes (first of two parts). N Engl J Med 1978;298:659–668.
7. Babior BM. Oxygen-dependent microbial killing by phagocytes (second of two parts). N Engl J Med 1978;298:721–725.
8. Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic Biol Med 2007;42:153–164.
9. Wentworth Jr P, Wentworth AD, Zhu X, Wilson IA, Janda KD, Eschenmoser A, Lerner RA. Evidence for the production of trioxygen species during antibody-catalyzed chemical modification of antigens. Proc Natl Acad Sci USA 2003;100:1490–1493.
10. Wentworth Jr P, Nieva J, Takeuchi C, Galve R, Wentworth AD, Dilley RB, DeLaria GA, Saven A, Babior BM, Janda KD, Eschenmoser A, Lerner RA. Evidence for ozone formation in human atherosclerotic arteries. Science 2003;302:1053–1056.
11. Smith LL. Oxygen, oxysterols, ouabain, and ozone: A cautionary tale. Free Radic Biol Med 2004;37:318–324.
12. Heng S, Yeung KL, Djafer M, Schrotter J-C. A novel membrane reactor for ozone water treatment. J Membr Sci 2007;289:67–75.
13. Rozema J, Björn LO, Bornman JF, Gaberik A, Häder D-P, Trot T, Germ M, Klisch M, Gröniger A, Sinha RP, Lebert M, He Y-Y, Buffoni-Hall R, de Bakker NVJ, van de Staaij J, Meijkamp BB. The role of UV-B radiation in aquatic and terrestrial ecosystems—an experimental and functional analysis of the evolution of UV-absorbing compounds. J Photochem Photobiol B 2002;66:2–12.
14. US Environmental Protection Agency. National ambient air quality standards for ozone; final rule. Available at: http://www.epa.gov/air/ozonepollution/pdfs/2008_03_finalrule.pdf. Accessed October 13, 2008.
15. Bell ML, McDermott A, Zeger SL, Samet JM, Dominici F. Ozone and short-term mortality in 95 US urban communities, 1987–2000. J Am Med Assoc 2004;292:2372–2378.
16. Mortimer KM, Tager IB, Dockery DW, Neas L, Redline S. The effect of ozone on inner-city children with asthma: Identification of susceptible subgroups. Am J Respir Crit Care Med 2000;162:1838–1845.
17. Ruidavets JB, Cournot M, Cassadou S, Giroux M, Meybeck M, Ferrieres J. Ozone air pollution is associated with acute myocardial infarction. Circulation 2005;111:563–569.

18. Tager IB, Balmes J, Lurmann F, Ngo L, Alcorn S, Kunzli N. Chronic exposure to ambient ozone and lung function in young adults. Epidemiology 2005;16:751–759.
19. Lippman M. Health effects of ozone, a critical review. J Am Air Pollut Control Assoc 1989;39:672–695.
20. Mustafa MG. Biochemical basis of ozone toxicity. Free Radic Biol Med 1990;9:245–265.
21. Bastacky J, Lee CYC, Goerke J, Koushafar H, Yager D, Kenaga L, Speed TP, Chen Y, Clements JA. Alveolar lining layer is thin and continuous: Low-temperature scanning electron microscopy of rat lung. J Appl Physiol 1995;79:1615–1628.
22. Rennard SI, Basset G, Lecossier D, O'Donnell KM, Pinkston P, Martin PG, Crystal RG. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J Appl Physiol 1986;60:532–538.
23. Pryor WA. Mechanisms of radical formation from reactions of ozone with target molecules in the lung. Free Radic Biol Med 1994;17:451–465.
24. Kelly FJ, Mudway I, Krishna MT, Holgate ST. The free radical basis of air pollution: Focus on ozone. Respir Med 1995;89:647–656.
25. Hamilton Jr RF, Eschenbacher WL, Szweda L, Holian A. Potential involvement of 4-hydroxynonenal in the response of human lung cells to ozone. Am J Physiol 1998;274: L8–L16.
26. Spickett CM, Jerlich A, Panasenko OM, Arnhold J, Pitt AR, Stelmaszynska T, Schaur RI. The reactions of hypochlorous acid, the reactive oxygen species produced by myeloperoxidase, with lipids. Acta Biochim Pol 2000;47:889–899.
27. Cho HY, Zhang LY, Kleeberger SR. Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-alpha receptors. Am J Physiol Lung Cell Mol Physiol 2001;280:L537–L546.
28. Goldkorn T, Khan EM. Dual roles of oxidative stress in the lungs. In: Valacchi G, Davis P, editors. Oxidants in biology. The Netherlands: Springer; 2008. pp 231–250.
29. Long NC, Suh J, Morrow JD, Schiestl RH, Murthy GG, Brain JD, Frei B. Ozone causes lipid peroxidation but little antioxidant depletion in exercising and nonexercising hamsters. J Appl Physiol 2001;91:1694–1700.
30. Montuschi P, Nightingale JA, Kharitonov SA, Barnes PJ. Ozone-induced increase in exhaled 8-isoprostane in healthy subjects is resistant to inhaled budesonide. Free Radic Biol Med 2002;33:1403–1408.
31. Corradi M, Alinovi R, Goldoni M, Vettori M, Folesani G, Mozzoni P, Cavazzini S, Bergamaschi E, Rossi L, Mutti A. Biomarkers of oxidative stress after controlled human exposure to ozone. Toxicol Lett 2002;134:219–225.
32. Last JA, Gohil K, Mathrani VC, Kenyon NJ. Systemic responses to inhaled ozone in mice: Cachexia and down-regulation of liver xenobiotic metabolizing genes. Toxicol Appl Pharmacol 2005;208:117–126.
33. Bocci V. Interleukins. Clinical pharmacokinetics and practical implications. Clin Pharmacokinet 1991;21:274–284.
34. Bocci V. Physicochemical and biologic properties of interferons and their potential uses in drug delivery systems. Crit Rev Ther Drug Carrier Syst 1992;9:91–133.
35. Alary J, Geuraud F, Cravedi JP. Fate of 4-hydroxynonenal in vivo: Disposition and metabolic pathways. Mol Aspects Med 2003;24:177–187.
36. Siems W, Grune T. Intracellular metabolism of 4-hydroxynonenal. Mol Aspects Med 2003;24:167–175.
37. Awasthi YC, Ansari GA, Awasthi S. Regulation of 4-hydroxynonenal mediated signaling by glutathione *S*-transferase. Methods Enzymol 2005;401:379–407.
38. Sweet F, Kao MS, Lee SC, Hagar WL, Sweet WE. Ozone selectively inhibits growth of human cancer cells. Science 1980;209:931–933.

39. Tarkington BK, Duvall TR, Last JA. Ozone exposure of cultured cells and tissues. Methods Enzymol 1994;234:257–265.
40. Larini A, Bianchi L, Bocci V. The ozone tolerance: (I) Enhancement of antioxidant enzymes is ozone dose-dependent in Jurkat cells. Free Radic Res 2003;37:1163–1168.
41. Leist M, Raab B, Maurer S, Brigelius-Flohé R. Conventional cell culture media do not adequately supply cells with antioxidants and thus facilitate peroxide-induced genotoxicity. Free Radic Biol Med 1996;21:297–306.
42. Halliwell B. Antioxidants in human health and disease. Annu Rev Nutr 1996;16:33–50.
43. Halliwell B. Antioxidant defence mechanisms: From the beginning to the end (of the beginning). Free Radic Res 1999;31:261–272.
44. Halliwell B. Oxidative stress in cell culture: An under-appreciated problem? FEBS Lett 2003;540:3–6.
45. Larini A, Bianchi L, Bocci V. Effect of 4-hydroxynonenal on antioxidant capacity and apoptosis induction in Jurkat T cells. Free Radic Res 2004;38:509–516.
46. Larini A, Bocci V. Albumin is the most effective antioxidant during human plasma and blood ozonization. Rivista Italiana di Ossigeno–Ozonoterapia 2004;3:15–24.
47. Bocci V, Valacchi G, Corradeschi F, Aldinucci C, Silvestri S, Paccagnini E, Gerli R. Studies on the biological effects of ozone: 7. Generation of reactive oxygen species (ROS) after exposure of human blood to ozone. J Biol Regul Homeost Agents 1998;12:67–75.
48. Bocci V. Does ozone therapy normalize the cellular redox balance? Med Hypotheses 1996;46:150–154.
49. Bocci V. Scientific and medical aspects of ozone therapy. State of the art. Arch Med Res 2006;37:425–435.
50. Stoker G. The surgical uses of ozone. Lancet 1916;188:712.
51. Stoker G. The surgical uses of ozone. Lancet 1917;189:797.
52. Fish E. Apparatus for the production and use of ozone in therapeutics. United States Patent 2,054,367. September 15, 1936.
53. Kühnel K, Seifert V. Erwin Payr and his contributions to neurosurgery. Zentralbl Neurochir 1998;59:27–35.
54. Bocci V. Oxygen–ozone therapy. A critical evaluation. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2002. 440p.
55. Timbrell J. The poison paradox; chemicals as friends and foes. Oxford: Oxford University Press; 2005. 352p.
56. Hunter P. A toxic brew we cannot live without. Micronutrients give insight into the interplay between geochemistry and evolutionary biology. EMBO Rep 2008;9:15–18.
57. Cataldo F, Gentilini L. Chemical kinetics measurements on the reaction between blood and ozone. Int J Biol Macromol 2005;36:61–65.
58. Bocci V, Travagli V. How an ill-conceived methodological approach can condemn the medical use of ozone therapy. Int J Biol Macromol 2005;37:287–288.
59. Van der Vliet A, O'Neil CA, Eiserich JP, Cross CE. Oxidative damage to extracellular fluids by ozone and possible protective effects of thiols. Arch Biochem Biophys 1995;321: 43–50.
60. Bocci V. The question of balance: The interaction between blood and ozone. In: Valacchi G, Davis P, editors. Oxidants in biology. The Netherlands: Springer; 2008. pp 155–165.
61. Cross CE, Reznick AZ, Packer L, Davis PA, Suzuki YJ, Halliwell B. Oxidative damage to human plasma proteins by ozone. Free Radic Res Commun 1992;15:347–352.
62. Mulholland CW, Strain JJ. Total radical-trapping antioxidant potential (TRAP) of plasma: Effects of supplementation. Int J Vitam Nutr Res 1993;63:27–30.
63. Bocci V, Aldinucci C. Biochemical modifications induced in human blood by oxygenation-ozonation. J Biochem Mol Toxicol 2006;20:133–138.

64. Bocci V, Luzzi E, Corradeschi F, Paulesu L, Di Stefano A. Studies on the biological effects of ozone: 3. An attempt to define conditions for optimal induction of cytokines. Lymphokine Cytokine Res 1993;12:121–126.
65. Shinriki N, Suzuki T, Takama K, Fukunaga K, Ohgiya S, Kubota K, Miura T. Susceptibilities of plasma antioxidants and erythrocyte constituents to low levels of ozone. Haematologia 1998;29:229–239.
66. Mendiratta S, Qu ZC, May JM. Erythrocyte ascorbate recycling: Antioxidant effects in blood. Free Radic Biol Med 1998;24:789–797.
67. Mendiratta S, Qu ZC, May JM. Enzyme-dependent ascorbate recycling in human erythrocytes: Role of thioredoxin reductase. Free Radic Biol Med 1998;25:221–228.
68. Uppu RM, Cueto R, Squadrito GL, Pryor WA. What does ozone react with at the air/lung interface? Model studies using human red blood cell membranes. Arch Biochem Biophys 1995;319:257–266.
69. Pryor WA, Squadrito GL, Friedman M. The cascade mechanism to explain ozone toxicity: The role of lipid ozonation products. Free Radical Biol Med 1995;19:935–941.
70. Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. FEBS Lett 2000;486:10–13.
71. Halliwell B, Clement MV, Ramalingam J, Long LH. Hydrogen peroxide: Ubiquitous in cell culture and in vivo? IUBMB Life 2000;50:251–257.
72. Antunes F, Cadenas E. Estimation of H_2O_2 gradients across biomembranes. FEBS Lett 2000;475:121–126.
73. Bocci V, Aldinucci C, Bianchi L. The use of hydrogen peroxide as medical drug. Rivista Italiana di Ossigeno-Ozonoterapia 2005;4:30–39.
74. Stone JR, Yang S. Hydrogen peroxide: A signaling messenger. Antioxid Redox Signal 2006;8: 243–270.
75. Forman HJ. Hydrogen peroxide: The good, the bad and the ugly. In: Valacchi G, Davis P, editors. Oxidants in biology. The Netherlands: Springer; 2008. pp 1–17.
76. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994;12:141–179.
77. Los M, Droge W, Stricker K, Baeuerle PA, Schulze-Osthoff K. Hydrogen peroxide as a potent activator of T lymphocyte functions. Eur J Immunol 1995;25:159–165.
78. Stone JR, Collins T. The role of hydrogen peroxide in endothelial proliferative responses. Endothelium 2002;9:231–238.
79. Grisham MB. Reactive oxygen species in immune responses. Free Radic Biol Med 2004;36: 1479–1480.
80. Ardanaz N, Pagano PJ. Hydrogen peroxide as a paracrine vascular mediator: Regulation and signaling leading to dysfunction. Exp Biol Med 2006;231:237–251.
81. Allen RC, Loose LD. Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. Biochem Biophys Res Commun 1976;69: 245–252.
82. Urschel HC. Cardiovascular effects of hydrogen peroxide: Current status. Dis Chest 1967;51: 180–192.
83. Lacy F, O'Connor DT, Schmid-Schonbein GW. Plasma hydrogen peroxide production in hypertensives and normotensive subjects at genetic risk of hypertension. J Hypertens 1998; 16:291–303.
84. Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I Mukai Y, Hirakawa Y, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. Biochem Biophys Res Commun 2002;290:909–913.
85. Rice-Evans C, Miller NJ. Total antioxidant status in plasma and body fluids. Methods Enzymol 1994;234:279–293.

86. Bocci V, Luzzi E, Corradeschi F, Paulesu L, Rossi R, Cardaioli E, Di Simplicio P. Studies on the biological effects of ozone: 4. Cytokine production and glutathione levels in human erythrocytes. J Biol Regul Homeost Agents 1993;7:133–138.
87. Packer L, Roy S, Sen CK. Alpha-lipoic acid: A metabolic antioxidant and potential redox modulator of transcription. Adv Pharmacol 1997;38:79–101.
88. Petersen DR, Doorn JA. Reactions of 4-hydroxynonenal with proteins and cellular targets. Free Radic Biol Med 2004;37:937–945.
89. Poli G, Schaur RJ, Siems WG, Leonarduzzi G. 4-Hydroxynonenal: A membrane lipid oxidation product of medicinal interest. Med Res Rev 2008;28:569–631.
90. Toyokuni S, Yamada S, Kashima M, Ihara Y, Yamada Y, Tanaka T, Hiai H, Seino Y, Uchida K. Serum 4-hydroxy-2-nonenal-modified albumin is elevated in patients with type 2 diabetes mellitus. Antioxid Redox Signal 2000;2:681–685.
91. Aldini G, Gamberoni L, Orioli M, Beretta G, Regazzoni L, Maffei Facino R, Carini M. Mass spectrometric characterization of covalent modification of human serum albumin by 4-hydroxy-trans-2-nonenal. J Mass Spectrom 2006;41:1149–1161.
92. Aldini G, Vistoli G, Regazzoni L, Gamberoni L, Facino RM, Yamaguchi S, Uchida K, Carini M. Albumin is the main nucleophilic target of human plasma: A protective role against pro-atherogenic electrophilic reactive carbonyl species? Chem Res Toxicol 2008;21:824–835.
93. Selley ML, Bartlett MR, McGuiness JA, Hapel AJ, Ardlie NG, Lacey MJ. Determination of the lipid peroxidation product trans-4-hydroxy-2-nonenal in biological samples by high-performance liquid chromatography and combined capillary column gas chromatography-negative-ion chemical ionisation mass spectrometry. J Chromatogr 1989;488:329–340.
94. Gil L, Siems W, Mazurek B, Gross J, Schroeder P, Voss P, Grune T. Age-associated analysis of oxidative stress parameters in human plasma and erythrocytes. Free Radic Res 2006;40:495–505.
95. Esterbauer H, Zollner H, Lang J. Metabolism of the lipid peroxidation product 4-hydroxynonenal by isolated hepatocytes and by liver cytosolic fractions. Biochem J 1985;228:363–373.
96. Siems W, Zollner H, Esterbauer H. Metabolic pathways of lipid peroxidation product 4-hydroxynonenal in hepatocytes. Quantitative assessment of an antioxidative defense system. Free Radic Biol Med 1990;9:110.
97. Ramana KV, Bhatnagar A, Srivastava S, Yadav UC, Awasthi S, Awasthi YC, Srivastava SK. Mitogenicresponses of vascular smooth muscle cells to lipid peroxidation-derived aldehyde 4-hydroxy-trans-2-nonenal (HNE): Role of aldose reductase-catalyzed reduction of the HNE-glutathione conjugates in regulating cell growth. J Biol Chem 2006;281: 17652–17660.
98. Alary J, Bravais F, Cravedu JP, Debrauwer L, Rao D, Bories G. Mercapturic acid conjugates as urinary end metabolites of the lipid peroxidation product 4-hydroxynonenal in the rat. Chem Res Toxicol 1995;8:34–39.
99. Forman HJ, Dickinson DA, Iles KE. HNE-signaling pathways leading to its elimination. Mol Aspects Med 2003;24:189–194.
100. Forman HJ, Dickinson DA. Introduction to serial reviews on 4-hydroxy-2-nonenal as a signaling molecule. Free Radic Biol Med 2004;37:594–596.
101. Schaur RJ, Zollner H, Esterbauer H. Biological effects of aldehydes with particular attention to hydroxynonenal and malondialdehyde. In: Vigo-Pelfrey C, editor. Membrane lipid peroxidation. Boca Raton, FL: CRC Press; 1991. pp 141–163.
102. Leonarduzzi G, Parola M, Muzio G, Garramone A, Maggiora M, Robino G, Poli G, Dianzani MU, Canuto RA. Hepatocellular metabolism of 4-hydroxy-2,3-nonenal is impaired in conditions of chronic cholestasis. Biochem Biophys Res Comm 1995;214:669–675.
103. Petras T, Siems W, Grune T. 4-Hydroxynonenal is degraded to mercapturic acid conjugate in rat kidney. Free Radic Biol Med 1995;19:685–688.

104. Jardines D, Correa T, Ledea O, Zamora Z, Rosado A, Molerio J. Gas chromatography-mass spectrometry profile of urinary organic acids of Wistar rats orally treated with ozonized unsaturated triglycerides and ozonized sunflower oil. J Chromatogr B Analyt Technol Biomed Life Sci 2003;783:517–525.
105. Dianzani MU. 4-Hydroxynonenal from pathology to physiology. Mol Aspects Med 2003;24: 263–272.
106. Leonarduzzi G, Robbesyn F, Poli G. Signaling kinases modulated by 4-hydroxynonenal. Free Radic Biol Med 2004;37:1694–1702.
107. Yang YS, Sharma R, Sharma A, Awasthi S, Awasthi YC. Lipid peroxidation and cell cycle signaling: 4-hydroxynonenal, a key molecule in stress mediated signaling. Acta Biochim Pol 2003;50:319–336.
108. Dwivedi S, Sharma A, Patrick B, Sharma R, Awasthi YC. Role of 4-hydroxynonenal and its metabolites in signaling. Redox Rep 2007;12:4–10.
109. Kutuk O, Basaga H. Apoptosis signalling by 4-hydroxynonenal: A role for JNK-c-Jun/AP-1 pathway. Redox Rep. 2007;12:30–34.
110. Comporti M, Signorini C, Arezzini B, Vecchio D, Monaco B, Gardi C. F2-isoprostanes are not just markers of oxidative stress. Free Radic Biol Med 2008;44:247–256.
111. Travagli V, Zanardi I, Bocci V. A realistic evaluation of the action of ozone on whole human blood. Int J Biol Macromol 2006;39:317–320.
112. Travagli V, Zanardi I, Silvietti A, Bocci V. A physicochemical investigation on the effects of ozone on blood. Int J Biol Macromol 2007;41:504–511.
113. Goldstein BD, Balchum OJ. Effect of ozone on lipid peroxidation in the red blood cell. Exp Biol Med 1967;126:356–358.
114. Freeman BA, Miller BE, Mudd JB. Reaction of ozone with human erytrocytes. In: Lee SD, Mudd JB, editors. Assessing toxic effects of environmental polluttants. Ann Arbor, MI: Ann Arbor Science Publisher; 1979. pp 151–171.
115. Van der Zee J, van Beek E, Dubbelman TMAR, van StevenickJ. Toxic effects of ozone on murine L929 fibroblasts. Damage to DNA. Biochem J 1987;247:69–72.
116. Fukunaga K, Nakazono N, Suzuki T, Takama K. Mechanism of oxidative damage to fish red blood cells by ozone. IUBMB Life 1999;48:631–634.
117. Górnicki A, Gutsze A. In vitro effects of ozone on human erythrocyte membranes: An EPR study. Acta Biochim Pol 2000;47:963–971.
118. Du Plessis LH, Van der Westhuizen FH, Kotze HF. The effect of blood ozonation on mitochondrial function and apoptosis of peripheral blood mononuclear cells in the presence and absence of antioxidants. Afr J Biotechnol 2007;6:1763–1769.
119. Du Plessis LH, Van der Westhuizen FH, Kotze HF. The protective effect of plasma antioxidants during ozone autohemotherapy. Afr J Biotechnol 2008;7:2472–2477.
120. Galleano M, Puntaruolo S. Role of antioxidants on the erythrocytes resistance to lipid peroxidation after acute iron overload in rats. Biochim Biophys Acta 1995;1271:321–326.
121. Caglayan S, Bayer R. Effects of oxydative stress on erythrocyte deformability and fragility. Proc SPIE 2100 1994;182:183–189.
122. Hernández F, Menéndez S, Wong R. Decrease of blood cholesterol and stimulation of antioxidative response in cardiopathy patients treated with endovenous ozone therapy. Free Radic Biol Med 1995;19:115–119.
123. Hernández Rosales FA, Calunga Fernández JL, Turrent Figueras J, Menéndez Cepero S, Montenegro Perdomo A. Ozone therapy effects on biomarkers and lung function in asthma. Arch Med Res 2005;36:549–554.
124. Bocci V, Venturi G, Catucci M, Valensin PE, Zazzi M. Lack of efficacy of ozone therapy in HIV infection. Clin Microbiol Infect 1998;4:667–669.

125. Burgassi S, Zanardi I, Travagli V, Montomoli M, Bocci V. How much ozone bactericidal activity is compromised by plasma components? J Appl Microbiol, doi:10.1111/j.1365-2672.2008.04141.x.
126. Margalit M, Attias E, Attias D, Elstein D, Zimran A, Matzner Y. Effect of ozone on neutrophil dunction in vitro. Clin Lab Haematol 2001;23:243–247.
127. Larini A, Bocci V. Effects of ozone on isolated peripheral blood mononuclear cells. Toxicol In Vitro 2005;19:55–61.
128. Bocci V, Paulesu L. Studies on the biological effects of ozone 1. Induction of interferon on human gamma leucocytes. Haematologica 1990;75:510–515.
129. Bocci V. Roles of interferon produced in physiological conditions. A speculative review. Immunology 1988;64:1–9.
130. Bocci V, Valacchi G, Corradeschi F, Fanetti G. Studies on the biological effects of ozone: 8. Effects on the total antioxidant status and on interleukin-8 production. Mediators Inflamm 1998;7:313–317.
131. Bocci V, Valacchi G, Rossi R, Giustarini D, Paccagnini E, Pucci AM, Di Simplicio P. Studies on the biological effects of ozone: 9. Effects of ozone on human platelets. Platelets 1999;10:110–116.
132. Valacchi G, Bocci V. Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets. Mediators Inflamm 1999;8:205–209.
133. Valacchi G, Bocci V. Studies on the biological effects of ozone: 11. Release of factors from human endothelial cells. Mediators Inflamm 2000;9:271–276.
134. Stamler JS. *S*-nitrosothiols in the blood: Roles, amounts, and methods of analysis. Circ Res 2004;94:414–417.
135. Gladwin MT, Schechter AN. NO contest: Nitrite versus *S*-nitroso-hemoglobin. Circ Res 2004;94:851–855.
136. Foresti R, Bains S, Sulc F, Farmer PJ, Green CJ, Motterlini R. The interaction of nitric oxide with distinct hemoglobins differentially amplifies endothelial heme uptake and heme oxygenase-1 expression. J Pharmacol Exp Ther 2006;317:1125–1133.
137. Dianzani MU. 4-Hydroxynonenal and cell signalling. Free Radic Res 1998;28:553–560.
138. Bocci V. Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005; 295p.
139. Bocci V. Rectal insufflation of oxygen–ozone. Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005. pp 49–56.
140. Bocci V. Quasi-total body exposure to oxygen–ozone. Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005. pp 56–65.
141. Bocci V. Extracorporeal blood circulation against oxygen–ozone. Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005. pp 66–73.
142. Bocci V. Minor ozone autohemotherapy. Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005. pp 42–44.
143. Bocci V. Infection diseases (bacterial, viral, fungal, parasitic). Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005. pp 100–122.
144. Bocci V. Retinal degenerative disorders. Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005. pp 132–144.
145. Bocci V. The paradoxical effect of ozone in orthopaedic diseases. The problem of back-ache. Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005. pp 198–208.
146. Bocci V. The dilemma between hyperbaric oxygen therapy (HOT) and ozonetherapy. Ozone. A new medical drug. Dordrecht, The Netherlands: Springer; 2005. pp 227–230.
147. Kangasjärvi J, Talvinen J, Utriainen M, Karjalainen R. Plant defence system induced by ozone. Plant Cell Environ 1994;17:783–794.
148. Sharma YK, León J, Raskin I, Davis KR. Ozone-induced responses in *Arabidopsos thaliana*: The role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. Proc Natl Acad Sci USA 1996;93:5099–5104.
149. Desikan R, Neill SJ, Hencock JT. Hydrogen peroxide-induced gene expression in *Arabidopsis thaliana*. Free Radic Biol Med 2000;28:773–778.

150. Ranieri A, Petacco F, Castagna A, Soldatini GF. Redox state and peroxidase system in sunflower plants exposed to ozone. Plant Sci 2001;159:159–167.
151. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124–1136.
152. Kume M, Yamamoto Y, Saad S, Gomi T, Kimoto S, Shimabukuro T, Yagi T, Nakagami M, Takada Y, Morimoto T, Yamaoka Y. Ischemic preconditioning of the liver in rats: Implications of heat shock protein induction to increase tolerance of ischemia-reperfusion injury. J Lab Clin Med 1996;28:251–258.
153. León OS, Menéndez S, Merino N, Castillo R, Sam S, Pérez L, Cruz E, Bocci V. Ozone oxidative preconditioning: A protection against cellular damage by free radicals. Mediators Inflamm 1998;7:289–294.
154. Sun JS, Lu FJ, Huang WC, Hou SM, Tsuang YH, Hang YS. Antioxidant status following acute ischemic limb injury: A rabbit model. Free Radic Res 1999;31:9–21.
155. Barber E, Menéndez S, León OS, Barber MO, Merino N, Calunga JL, Cruz E, Bocci V. Prevention of renal injury after induction of ozone tolerance in rats submitted to warm ischaemia. Mediators Inflamm 1999;8:37–41.
156. Yamamoto H, Yamamoto Y, Yamagami K, Kume M, Kimoto S, Toyokuni S, Uchida K, Fukumoto M, Yamaoka Y. Heat-shock preconditioning reduces oxidative protein denaturation and ameliorates liver injury by carbon tetrachloride in rats. Res Exp Med (Berl) 2000;199:309–318.
157. Peralta C, Xaus C, Bartrons R, Leon OS, Gelpi E, Roselló-Catafau J. Effect of ozone treatment on reactive oxygen species and adenosine production during hepatic ischemia-reperfusion. Free Radic Res 2000;33:595–605.
158. Goldman M. Cancer risk of low-level exposure. Science 1996;271:1821–1822.
159. Wolff S. Aspects of the adaptive response to very low doses of radiation and other agents. Mutat Res 1996;358:135–142.
160. Calabrese EJ. Paradigm lost, paradigm found: The re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. Environ Pollut 2005;138:379–411.
161. Calabrese EJ. Hormesis and medicine. Br J Clin Pharmacol 2008;66:594–617.
162. Stark M. Hormesis, adaptation, and the sandpile model. Crit Rev Toxicol 2008;38:641–644.
163. Olivieri G, Bodycote J, Wolff S. Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. Science. 1984;223:594–597.
164. Iles KE, Liu RM. Mechanisms of glutamate cysteine ligase (GCL) induction by 4-hydroxynonenal. Free Radic Biol Med 2005;38:547–556.
165. Maines MD. The heme oxygenase system: A regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997;37:517–554.
166. Bach FH. Heme oxygenase-1 as a protective gene. Wien Klin Wochenschr 2002;114:1–3.
167. Baranano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: A major physiologic cytoprotectant. Proc Natl Acad Sci USA 2002;99:16093–16098.
168. Zuckerbraun BS, Billiar TR. Heme oxygenase-1: A cellular Hercules. Hepatology 2003;37:742–744.
169. Iles KE, Dickinson DA, Wigley AF, Welty NE, Blank V, Forman HJ. HNE increases HO-1 through activation of the ERK pathway in pulmonary epithelial cells. Free Radic Biol Med 2005;39:355–364.
170. Bocci V, Aldinucci C, Mosci F, Carraro F, Valacchi G. Ozonation of human blood induces a remarkable upregulation of heme oxygenase-1 and heat stress protein-70. Mediators Inflamm 2007;2007:26785.
171. Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev 2008;60:79–127.
172. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. Ferritin: A cytoprotective antioxidant strategem of endothelium. J Biol Chem 1992;267:18148–18153.

173. Su WY, Gordon T. In vivo exposure to ozone produces an increase in a 72-kDa heat shock protein in guinea pigs. J Appl Physiol 1997;83:707–711.
174. Polidori MC, Mecocci P, Levine M, Frei B. Short-term and long-term vitamin C supplementation in humans dose-dependently increases the resistance of plasma to ex vivo lipid peroxidation. Arch Biochem Biophys 2004;423:109–115.
175. Victor VM, McCreath KJ, Rocha M. Recent progress in pharmacological research of antioxidants in pathological conditions: Cardiovascular health. Recent Patents Anti Infect Drug Discov 2006;1:17–31.
176. Roberts 2nd LJ, Oates JA, Linton MF, Fazio S, Meador BP, Gross MD, Shyr Y, Morrow JD. The relationship between dose of vitamin E and suppression of oxidative stress in humans. Free Radic Biol Med 2007;43:1388–1393.
177. Bocci V, Borrelli E, Corradeschi F, Valacchi G. Systemic effects after colorectal insufflation of oxygen/ozone in rabbit. Int J Med Biol Environ 2000;28:109–113.
178. Eliakim R, Karmeli F, Rachmilewitz D, Cohen P, Zimran A. Ozone enema: A model of microscopic colitis in rats. Dig Dis Sci 2001;46:2515–2520.
179. Bocci V, Borrelli E, Valacchi G, Luzzi E. Quasi-total-body exposure to an oxygen–ozone mixture in a sauna cabin. Eur J Appl Physiol Occup Physiol 1999;80:549–554.
180. Bocci V, Di Paolo N. Oxygenation–ozonation of blood during extracorporeal circulation (EBOO). Part III: A new medical approach. Ozone Sci Eng 2004;26:195–205.
181. Bocci V, Zanardi I, Travagli V, Di Paolo N. Oxygenation–ozonation of blood during extracorporeal circulation: In vitro efficiency of a new gas exchange device. Artif Organs 2007;31:743–748.
182. Jacobs MT. Untersuchung uber zwishenfalle und typische komplikationen in der ozon-sauerstoff-therapie. OzoNachrichten 1982;5:1–5.
183. Rokitanski O, Rokitanski A, Steriner J, Trubel W, Viebahn R, Washüttl J. Die ozontherapie bei peripheren, arteriellen durchblutungs-störungen; klinik, biochemische und blutgasanalytische untersuchungen. In: Wasser IOA, editor. Berlin: Ozon-Weltkongress; 1981. pp 53–75.
184. Rokitanski O. Klinik und biochemie der ozontherapie. Hospitalis 1982;52:643–647.
185. Matassi R, D'Angelo F, Bisetti P, Colombo R, Vaghi M. Terapia con ozono per via parenterale nelle arteropatie obliteranti periferiche: Meccanismo biochimico e risultati clinici. Il Giornale di Chirurgia 1987;VIII:109–111.
186. Di Paolo N, Bocci V, Salvo DP, Palasciano G, Biagioli M, Meini S, Galli F, Ciari I, Maccari F, Cappelletti F, Di Paolo M, Gaggiotti E. Extracorporeal blood oxygenation and ozonation (EBOO): A controlled trial in patients with peripheral artery disease. Int J Artif Organs 2005;28:1039–1050.
187. De Monte A, van der Zee H, Bocci V. Major ozonated autohemotherapy in chronic limb ischemia with ulcerations. J Altern Complement Med 2005;11:363–367.
188. Giunta R, Coppola A, Luongo C, Sammartino A, Guastafierro S, Grassia A, Giunta L, Mascolo L, Tirelli A, Coppola L. Ozonized autohemotransfusion improves hemorheological parameters and oxygen delivery to tissues in patients with peripheral occlusive arterial disease. Ann Hematol 2001;80:745–748.
189. Tylicki L, Nieweglowski T, Biedunkiewicz B, Burakowski S, Rutkowski B. Beneficial clinical effects of ozonated autohemotherapy in chronically dialysed patients with atherosclerotic ischemia of the lower limbs—pilot study. Int J Artif Organs 2001;24:79–82.
190. Tylicki L, Nieweglowski T, Biedunkiewicz B, Chamienia A, Debska-Slizien A, Aleksandrowicz E, Lysiak-Szydlowska W, Rutkowski B. The influence of ozonated autohemotherapy on oxidative stress in hemodialyzed patients with atherosclerotic ischemia of lower limbs. Int J Artif Organs 2003;26:297–303.
191. Clavo B, Pérez JL, López L, Suárez G, Lloret M, Rodríguez V, Macías D, Santana M, Morera J, Fiuza D, Robaina F, Günderoth M. Effect of ozone therapy on muscle oxygenation. J Altern Complement Med 2003;9:251–256.

192. Clavo B, Catalá L, Pérez JL, Rodríguez V, Robaina F. Ozone therapy on cerebral blood flow: A preliminary report. Evid Based Complement Altern Med 2004;1:315–319.
193. Tylicki L, Biedunkiewicz B, Nieweglowski T, Chamienia A, Slizien AD, Luty J, Lysiak-Szydlowska W, Rutkowski B. Ozonated autohemotherapy in patients on maintenance hemodialysis: Influence on lipid profile and endothelium. Artif Organs 2004;28:234–247.
194. Biedunkiewicz B, Tylicki L, Nieweglowski T, Burakowski S, Rutkowski B. Clinical efficacy of ozonated autohemotherapy in hemodialyzed patients with intermittent claudication: An oxygen-controlled study. Int J Artif Organs 2004;27:29–34.
195. Torre-Amione G, Anker SD, Bourge RC, Colucci WS, Greenberg BH, Hildebrandt P, Keren A, Motro M, Moyé LA, Otterstad JE, Pratt CM, Ponikowski P, Rouleau JL, Sestier F, Winkelmann BR, Young JB. Advanced chronic heart failure clinical assessment of immune modulation therapy investigators. Results of a non-specific immunomodulation therapy in chronic heart failure (ACCLAIM trial): A placebo-controlled randomised trial. Lancet 2008;371:228–236.
196. Cooke ED, Pockley AG, Tucker AT, Kirby JDT, Bolton AE. Treatment of severe Raydaud's syndrome by injection of autologous blood pretreated by heating, ozonation and exposure to ultraviolet light (H-O-U) therapy. Int Angiol 1997;16:250–254.
197. Sliwa K, Ansari AA. Immunosuppression as therapy for congestive heart failure. Lancet 2008;371:184–186.
198. Fildes JE, Shaw SM, Yonan N, Williams SG. Non-specific immunomodulation in chronic heart failure. Lancet 2008;37:2083.
199. Bocci V. Non-specific immunomodulation in chronic heart failure. Lancet 2008;37:2083.
200. Bocci V. The failure of the ACCLAIM trial is due to an irrational technology. Int J Cardiol 2008, doi:10.1016/j.ijcard.2008.10.001.
201. Olin JW. A multicenter, randomized, double-blind, placebo-controlled study of immune modulation therapy in patients with symptomatic peripheral arterial disease: The SIMPADICO trial. American College of Cardiology 55th Annual Scientific Sessions, Atlanta, GA, March 11–14, 2006. Late-breaking clinical trials I.
202. Owen CG, Fletcher AE, Donoghue M, Rudnicka AR. How big is the burden of visual loss caused by age related macular degeneration in the United Kingdom? Br J Ophthalmol 2003;87:312–317.
203. Krinsky NI, Landrum JT, Bone RA. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. Annu Rev Nutr 2003;23:171–201.
204. Coleman H, Chew E. Nutritional supplementation in age-related macular degeneration. Curr Opin Ophthalmol 2007;18:220–223.
205. Bocci V. Die senile makulopathie und verwandte erkrankungen. In: Viebahn-Hänsler R, Knoch HG, editors. Ozon-Handbuch. Vol. 5.1. Landsberg, Germany: Ecomed Verlag; 2001. pp 1–26.
206. Berson EL, Remulla JF, Rosner B, Sandberg MA, Weigel-DiFranco C. Evaluation of patients with retinitis pigmentosa receiving electric stimulation, ozonated blood, and ocular surgery in Cuba. Arch Ophthalmol 1996;114:560–563.
207. Carpendale MT, Freeberg JK. Ozone inactivates HIV at noncytotoxic concentrations. Antiviral Res 1991;16:281–292.
208. Sechi LA, Lezcano I, Nunez N, Espim M, Duprè I, Pinna A, Molicotti P, Fadda G, Zanetti S. Antibacterial activity of ozonized sunflower oil (Oleozon). J Appl Microbiol 2001;90: 279–284.
209. Church L. Ionozone therapy for skin lesions in elderly patients. Physiotherapy 1980;66:50–51.
210. Turcić J, Hancević J, Antoljak T, Zic R, Alfirević I. Effects of ozone on how well split-thickness skin grafts according to Thiersch take in war wounds. Results of prospective study. Langenbecks Arch Chir 1995;380:144–148.
211. Matsumoto A, Sakurai S, Shinriki N, Suzuki S, Miura T. Therapeutic effects of ozonized olive oil in the treatment of intractable fistula and wound after surgical operation. Proceedings of the 15th

Ozone World Congress, Vol. 1, London, UK. London, UK: Speedprint MacMedia; 2001. pp 77–84.
212. Valacchi G, Fortino V, Bocci V. The dual action of ozone on the skin. Br J Dermatol 2005;153:1096–1100.
213. He QC, Tavakkol A, Wietecha K, Begum-Gafur R, Ansari SA, Polefka T. Effects of environmentally realistic levels of ozone on stratum corneum function. Int J Cosmet Sci 2006;28:349–357.
214. Zanardi I, Travagli V, Gabbrielli A, Chiasserini L, Bocci V. Physico-chemical characterization of sesame oil derivatives. Lipids 2008;43:877–886.
215. Jordan L, Beaver K, Foy S. Ozone treatment for radiotherapy skin reactions: Is there an evidence base for practice? Eur J Oncol Nurs 2002;6:220–227.
216. Cavanagh PR, Lipsky BA, Bradbury AW, Botek G. Treatment for diabetic foot ulcers. Lancet 2005;366:1725–1735.
217. Martínez-Sánchez G, Al-Dalain SM, Menéndez S, Re L, Giuliani A, Candelario-Jalil E, Alvarez H, Fernández-Montequín JI, León OS. Therapeutic efficacy of ozone in patients with diabetic foot. Eur J Pharmacol 2005;523:151–161.
218. Barnes PJ. Scientific rationale for inhaled combination therapy with long-acting beta2-agonists and corticosteroids. Eur Respir J 2002;19:182–191.
219. Bocci V. May oxygen-ozonetherapy improve the prognosis of BPCO? Giorn It Mal Tor 2007;61:434–446.
220. Andreula CF, Simonetti L, De Santis F, Agati R, Ricci R, Leonardi M. Minimally invasive oxygen–ozone therapy for lumbar disk herniation. AJNR Am J Neuroradiol 2003:996–1000.
221. Gallucci M, Limbucci N, Zugaro L, Barile A, Stavroulis E, Ricci A, Galzio R, Masciocchi C. Sciatica: Treatment with intradiscal and intraforaminal injections of steroid and oxygen–ozone versus steroid only. Radiology 2007;242:907–913.
222. Oder B, Loewe M, Reisegger M, Lang W, Ilias W, Thurnher SA. CT-guided ozone/steroid therapy for the treatment of degenerative spinal disease-effect of age, gender, disc pathology and multi-segmental changes. Neuroradiology 2008;50:777–785.
223. Muto M, Ambrosanio G, Guarnieri G, Capobianco E, Piccolo G, Annunziata G, Rotondo A. Low back pain and sciatica: Treatment with intradiscal-intraforaminal O(2)–O(3) injection. Our experience. Radiol Med 2008;113:695–706.
224. Wu ZQ, Wei LZ, Li J, Wanga Y, Ni DH, Yang P, Zhang Y. Percutaneous treatment of non-contained lumbar disc herniation by injection of oxygen–ozone combined with collagenase. Eur J Radiol 2008, in press. DOI: 10.1016/j.ejrad.2008.07.029.
225. Re L, Martínez-Sánchez G, Malcangi G, Mercanti A, Labate V. Ozone therapy: A clinical study on the pain management. Int J Ozone Therapy 2008;7:37–44.
226. Bochkov VN, Leitinger N. Anti-inflammatory properties of lipid oxidation products. J Mol Med 2003;81:613–626.
227. Tamoto K, Yamazaki A, Nochi H, Miura T. Ozonides of olive oil and methyl oleate inhibit the expression of cyclooxygenase 2 through the suppression of kB/NFkB pathway in lipopolysaccharide-stimulated macrophage-like THP1 cells. Proceedings of the 17th World Ozone Association Congress, Strasbourg, France, 2005. p 37.
228. Baysan A, Whiley RA, Lynch E. Antimicrobial effect of a novel ozone-generating device on micro-organisms associated with primary root carious lesions in vitro. Caries Res 2000;34:498–501.
229. Azarpazhooh A, Limeback H. The application of ozone in dentistry: A systematic review of literature. J Dent 2008;36:104–116.
230. Lynch E. The revolution in dentistry. Copenhagen: Quintessence Publisher; 2004; 300p.
231. Weaver LK, Hopkins RO, Chan KJ, Churchill S, Elliott CG, Clemmer TP, Orme Jr JF, Thomas FO, Morris AH. Hyperbaric oxygen for acute carbon monoxide poisoning. N Engl J Med 2002;347:1057–1067.

232. Cianci P. Advances in the treatment of the diabetic foot: Is there a role for adjunctive hyperbaric oxygen therapy? Wound Repair Regen 2004;12:2–10.
233. Gill AL, Bell CN. Hyperbaric oxygen: Its uses, mechanisms of action and outcomes. QJM 2004;97:385–395.
234. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 2007;13:688–694.
235. Wood KC, Gladwin MT. The hydrogen highway to reperfusion therapy. Nat Med 2007;13: 673–674.
236. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R. H_2S as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. Science 2008;322:587–590.

Velio Bocci, M.D., Ph.D., *in Physiology. He is an Emeritus Professor of Physiology at the University of Siena, Italy. His main research fields are plasma proteins characterization and labelling; metabolism and pharmacokinetics of interferons and cytokines; biological and medical evaluation of oxygen–ozone therapy.*

Emma Borrelli, M.D., Ph.D., *in Cardiovascular Pathophysiology. She is the Director of the Laboratory of Cardiovascular pathology in the Department of Surgery and Bioengineering of the University of Siena, Italy. Her research interest deals with cardiovascular pathology and the application of ozone therapy.*

Valter Travagli *is an Associate Professor of Pharmaceutical Technology at the University of Siena, Italy. His main research fields are the interactions of biological macromolecules with degrading agents and the inner Quality concept for drugs, based on manufacturing processes.*

Iacopo Zanardi, Ph.D., *in Pharmaceutical Sciences, Department of Pharmaceutical Chemistry and Technology, University of Siena, Italy. His main research fields are the chemico-physical evaluation of biological macromolecules and ozone therapy.*