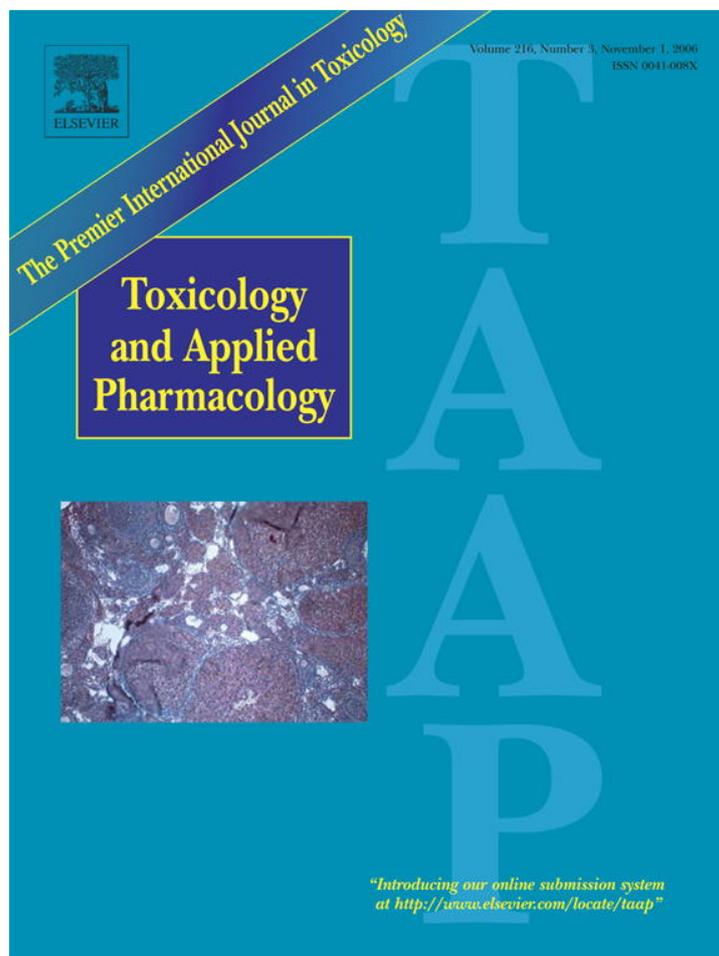


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Short Communication

Is it true that ozone is always toxic? The end of a dogma

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Abstract

There are a number of good experimental studies showing that exposure by inhalation to prolonged tropospheric ozone damages the respiratory system and extrapulmonary organs. The skin, if extensively exposed, may also contribute to the damage. The undoubted strong reactivity of ozone has contributed to establish the dogma that ozone is always toxic and its medical application must be proscribed. Although it is less known, judiciously practiced ozonotherapy is becoming very useful either on its own or applied in combination with orthodox medicine in a broad range of pathologies. The opponents of ozonotherapy base their judgment on the ozone chemistry, and physicians, without any knowledge of the problem, are often skeptical. During the last 15 years, a clear understanding of the action of ozone in biology and medicine has been gained, allowing today to argue if it is true that ozone is always toxic. The fundamental points that are discussed in this paper are: the topography, anatomical and biochemical characteristics of the organs daily exposed to ozone versus the potent antioxidant capacity of blood exposed to a small and precisely calculated dose of ozone only for a few minutes. It is becoming clear how the respiratory system undergoing a chronic oxidative stress can release slowly, but steadily, a huge amount of toxic compounds able to enter the circulation and cause serious damage. The aim of this paper is to objectively evaluate this controversial issue.

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Keywords: Ozone; Lungs; Antioxidants; Acute oxidative stress; 4-Hydroxynonenal; Ozonotherapy

Introduction

Ozone has become a famous gas because in the stratosphere it blocks an excessive ultraviolet irradiation of the earth, while, in the troposphere, associated to several other pollutants, it damages lung functions and can lead to severe ailments. There are quite a few remarkable studies (Lippman, 1989; Devlin et al., 1991; Aris et al., 1993; Kelly et al., 1995; Krishna et al., 1998; Broeckaert et al., 1999; Bhalla and Gupta, 2000; Cho et al., 2001; Long et al., 2001; Bell et al., 2004; Savov et al., 2004; Tager et al., 2005) showing that prolonged inhalation of ozone damages the respiratory system and extrapulmonary organs (Soulage et al., 2004; Ruidavets et al., 2005). “Epidemiology” has recently reported a series of meta-analysis and evaluations of geographic and seasonal ozone relative risk providing striking evidence of the relationship between ozone and mortality (Bell et al., 2005; Ito et al., 2005; Levy et al., 2005; Bates, 2005; Goodman, 2005). It is not surprising that the release of noxious compounds such as substance P, NO (Fakhrzadeh et al., 2002), IL-1 β , IL-8 and TNF α

has been amply demonstrated (Wang et al., 2002; Janic et al., 2005). Reports by Gohil et al. (2003) and Last et al. (2005) are particularly instructive because they have further shown that mice, exposed to 1.00 ppm ozone breathing for 8 h for three consecutive nights, upregulate the synthesis of a few pulmonary proteins including the just mentioned pro-inflammatory cytokines and, concomitantly, down-regulate a number of hepatic enzymes related to fatty acids and carbohydrate metabolism including suppression of the cytochrome P450 superfamily consistent with a systemic cachexic response.

In order to understand the problem of the multiform toxicity induced by ozone, it appears useful to discuss firstly the origin and nature of the toxic compounds, secondly, their noxious activity in the lungs and, thirdly, their distribution and fate in body fluids and organs.

Origin, distribution and fate of toxic compounds released by the pulmonary system during and after ozone exposure

At the airspace level, the alveolar cells are constantly overlaid by a film composed of water, salts and a myriad of biomolecules such as a profusion of surfactant phospholipids

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and small amounts of proteins, lipophilic and hydrophilic antioxidants. Any inspired gas, depending upon its relative concentration and pressure, must first dissolve into the aqueous layer before reaching the alveolar microcirculation and the erythrocytes. This process implies a physical transport regulated by a pressure gradient and a diffusion process. On the other hand, it is known that ozone, in contact with biological water, does not follow Henry's law and, although its solubility is ten-fold higher than oxygen, it is not transferred into the alveolar capillaries because it reacts immediately with the biomolecules present in the epithelial lining fluid (ELF).

As it was hypothesized (Pryor, 1994; Pryor et al., 1995), ozone does not penetrate the cells but oxidizes available antioxidants and reacts instantaneously with surfactant's polyunsaturated fatty acids (PUFA) present at the air–ELF interface to form reactive oxygen species (ROS), such as hydrogen peroxide and a mixture of heterogenous LOPs including lipoperoxyl radicals, hydroperoxides, malonyldialdehyde, isoprostanes, the ozonide radical, O_3^- (Ballinger et al., 2005) and alkenals, particularly 4-hydroxy-2,3-*trans*-nonenal, HNE (Mustafa, 1990; Esterbauer et al., 1991; Hamilton et al., 1998; Long et al., 2001; Schaur, 2003; Iles et al., 2005). As cholesterol is a component of the ELF and because its double bond is readily attacked by ozone, it can give rise to biologically active oxysterols (Pulfer et al., 2005; Sathishkumar et al., 2005) of which 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al (CSeco) has been implicated in pulmonary toxicity, Alzheimer's disease and atherosclerosis.

In Table 1, the antioxidant capacity present in the human ELF indicates only average values and, although different portions of the respiratory tract may have different antioxidant levels, these are always irrelevant in comparison to the amount

of antioxidants that, in blood, easily tame the ozone reactivity. First of all, by considering the expanse of the alveolar surface (1 m²/kg body weight) in a 70 kg human, it can be calculated that the normal volume of ELF ranges between 17 and 20 ml, whereas 5 l of blood include about 2.7 l plasma. Moreover, the erythrocyte mass, amounting to about 2.3 kg, has an enormous antioxidant capacity due to hydro-lipophilic antioxidants and enzymes able to reduce any antioxidant in a few minutes (Mendiratta et al., 1998a; 1998b). Erythrocytes, via glucose-6-phosphate dehydrogenase activity in the pentose cycle, can continuously supply NADPH-reducing equivalents. The amount of plasma albumin acting as a "sacrificial compound" against oxidants is impressive (99.9% higher than in ELF), and only free GSH appears higher in ELF than in plasma. However, erythrocytes have a GSH content of about 2.2 mM (almost 700-fold higher than plasma) and therefore they contain a huge reserve (Di Simplicio et al., 1998). In the course of evolution, aerobic organisms have developed a sophisticated antioxidant system against oxygen at the air–tissue barrier and, although about 2% of the inhaled oxygen generates $^{\cdot}O_2$, this is normally neutralized at an alveolar pO_2 pressure of 100 mm Hg. It is useful, however, to bear in mind that rats inhaling pure oxygen (alveolar pressure at about 700 mm Hg) die within 60–66 h (Crapo, 1986). Ozone is far more reactive than oxygen, and breathing air containing 10.0 ppm ozone causes death within 4 h in rats (Mehlman and Borek, 1987; Mustafa, 1990).

In order to understand the effects of a daily 8-hour ozone exposure (April–October), we need to know the average environmental ozone levels that vary considerably for many reasons. The US Clean Air Act has set an ozone level of 0.06 ppm (120 $\mu\text{g}/\text{m}^3$) as an 8-h mean concentration to protect the health of workers (U.S. Environmental Protection Agency, 2005). Evaluation of recent studies (Mortimer et al., 2000; Ruidavets et al., 2005; Bell et al., 2004; Tager et al., 2005) allows establishing an average environmental ozone concentration of 90 ± 10 ppb. However, ozone concentration in urban air can exceed 800 ppb in high pollution conditions (Mustafa, 1990). For 8 h 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 or 21.0–23.1 mg monthly. 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 200–300 ppb, reducing pulmonary functions and enhancing the risk of cardiovascular death (Ruidavets et al., 2005; Bell et al., 2004, 2005; Tager et al., 2005; Ito et al., 2005). Moreover, the toxicity is certainly augmented by the presence of NO_2 , CO, SO_2 and particles (PM_{10}). On this basis, it appears clear how the ozone generated ROS and LOPs at the ELF level, after being only partly quenched by the scarce antioxidants, will act as cell signals able to activate nuclear factor-kappa B (NF- κ B), NO synthase and some protein kinases, thus enhancing the synthesis and release of $TNF\alpha$, IL-1, IL-8, $IFN\gamma$ and $TGF\beta 1$ and the possible formation of nitrating species. With an increasing inflow into the alveolar space of neutrophils and activated macrophages, a vicious circle will start, perpetuating the production of an excess of ROS including also hypochlorous acid (Spickett et al., 2000),

Table 1

A comparison between the composition of ELF and blood of a normal 70 kg human showing the great difference in antioxidant capacity of these two fluids

ELF	Blood
Volume: 17–20 ml	Plasma volume: ~2.7 l Erythrocytes: ~2.3 kg
Total proteins: ~7 mg/ml (total: ~130 mg)	Total plasma proteins: ~75 mg/ml (total: ~202.5 g)
Albumin: ~3.5 mg/ml (total: ~63 mg)	Albumin: ~45 mg/ml (total: ~121.5 g)
Transferrin: ~0.3 mg/ml	2–4 mg/ml
Ceruloplasmin: ~25 $\mu\text{g}/\text{ml}$	140–400 $\mu\text{g}/\text{ml}$
Lactoferrin: ~0.5 $\mu\text{g}/\text{ml}$?
GSH: 300–400 μM	In plasma: ~3 μM In erythrocytes: ~2.2 mM
Vitamin E: ~2 $\mu\text{g}/\text{ml}$	10–20 $\mu\text{g}/\text{ml}$
Vitamin C: ~3.5 $\mu\text{g}/\text{ml}$	~9 $\mu\text{g}/\text{ml}$
Uric acid: ~0.05 mg/ml	0.04–0.07 mg/ml
Glucose: ~0.4 mg/ml	0.7–1.0 mg/ml
Total Bilirubin: ?	~1.0 mg/dl
Na: ~82; Cl: ~84; K: ~29 mM	Na: ~139; Cl: ~103; K: ~4 mM
pH 6.9	pH 7.4

Data reported by Schultze and Heremans (1966); Davis and Pacht (1997); Lubman et al., 1997; Di Simplicio et al. (1998); Hawgood (1997); Repine and Heffner (1997); Widdicombe, 1997; Whitsett (1997).

LOPs, isoprostanes, tachykinins, cytokines and proteases, which will self-maintain the inflammation after ozone exposure.

Although the present studies have shown the complexity of the induced pathology caused by a variety of toxic agents, we do not have enough information regarding their amount, turnover and rates of absorption into the general circulation via lymphatics and capillaries. However, measurements of the peroxidation markers level in experimental animals before and after ozone exposure have been reported: Hamilton et al. (1998) detected HNE adducts in the bronchoalveolar lavage fluid (BAF) of human subjects exposed to 0.4 ppm ozone for 1 h with exercise and Long et al. (2001) have demonstrated the presence of F2-isoprostane in the bronchoalveolar lavage fluid of hamsters exposed to 3.0 ppm (but not to 0.12 ppm) ozone for 6 h. Montuschi et al. (2002) found that pretreatment with budesonide did not affect the increase in exhaled 8-isoprostane in healthy volunteers exposed to inhale air containing ozone (400 ppb) for 2 h. Corradi et al. (2002) measured H₂O₂, MDA and 8-isoprostane in plasma and exhaled breath condensate (EBC), while 8-hydroxy-2'-deoxyguanosine (8-OHdG) and deoxyguanosine were assessed in peripheral lymphocytes. Healthy volunteers were exposed to 0.1 ppm of O₃ for only 2 h and yet a subgroup of "susceptible" subjects showed a significant increase of H₂O₂ in EBC and of 8-isoprostane and 8-OHdG in blood immediately after the ozone exposure to indicate that the pulmonary inflammation rapidly reverberated in the general circulation. This is to be expected as the ozone stress lasts several hours, and the production of ROS, LOPs and cytokines continues after ozone exposure.

ROS have a very brief half-life and are most likely acting only on the pulmonary microenvironment, while toxic LOPs, particularly HNE and pro-inflammatory cytokines, can be continuously absorbed. Regarding their amount, I can only speculate that, by considering the very large expanse of the bronchial-alveolar space, it must be a huge one. In Last et al.'s study (2005), mice were exposed to 1 ppm for 8 h during three consecutive nights and unsurprisingly they lost 14% of their original body weight with a 42% decrease in total food consumption. The maximum work site concentration (WSC) corresponds to 0.1 ppm (0.2 µg/l) over a breathing period of 1 h, and therefore those mice breathed a more than ten-fold higher ozone dose. But it is not the static value of 1 ppm that counts because we must consider that, during summer, there is a continuous flow of ozone entering the respiratory space and also the very fact that ozone dissolves in the ELF and reacts immediately; thus, every second, more ozone reacts so that in a 6-month period the cumulative dose (likely up to 150–300 mg ozone) becomes really deleterious. In cell culture studies (Tarkington et al., 1994), where the medium contains a lower level of antioxidants than plasma (Leist et al., 1996; Halliwell, 2003; Larini et al., 2004), cell death, occurring within a few hours, is due to the successive doses of ozone that, although small, continuously dissolve, exhaust the scarce antioxidants and produce toxic compounds.

The next problem has pharmacotoxicological relevance and concerns the distribution and fate of the absorbed cytokines and LOPs. TNF α , IL-1, IL-8, IFN γ and TGF β 1 can easily

reach their respective receptors in any organ and, in spite of a half-life of a few hours (Bocci et al., 1987, Bocci, 1981; 1987; 1991; 1992), the prolonged, endogenous synthesis insures a saturation of the available binding sites. Given the toxicity of aldehydic lipid peroxidation compounds, it is important to know their metabolism and fate: Alary et al. (2003) have reported that about 70% of [3H]HNE was excreted in urine within 2 days after its intravenous (IV) administration in rats. Siems and Grune (2003), after investigating the metabolism of HNE in several mammalian cells and organs, demonstrated that HNE, at a concentration of 100 µM, was degraded within 3 min of incubation at 37 °C, while it took only 10–30 s to restore the physiological level of about 0.2 µM. We have measured the kinetic of disappearance from mildly ozonated blood of thiobarbituric acid reactive substances (TBARS), including MDA and HNE, in six patients with age-related macular degeneration (ARMD), and their half-life was equivalent to 4.2±1.7 min (Bocci, 1996a; 1996b; 2006). On the other hand, when the same samples were incubated *in vitro* (at +37 °C and pH 7.3), LOPs levels hardly declined during the next 9 h, indicating their stability in an acellular medium and suggesting the relevance of cellular catabolism (Bocci et al., 1998a). As far as the cholesteryl ester hydroperoxide is concerned, Yamamoto (2000) has emphasized the role of the enzymatic degradation and hepatic uptake. On the whole, it appears that mammals have developed an efficient detoxification machinery to metabolize HNE and minimize its toxicity: Awasthi et al. (2005), not only have indicated six enzymes, glutathione S-transferases, aldo-ketoreductases, aldose reductase, aldehyde dehydrogenases Cyp₄₅₀ 4A and β -oxidation enzymes, important in the metabolism of HNE, but they and other authors (Li et al., 2000; Yang et al., 2003; Iles et al., 2005; Foucaud et al., 2006; Zhang et al., 2006) have emphasized that HNE stress-preconditioned cells can develop a significant adaptive response by upregulating the synthesis of γ -glutamate cysteine ligase, γ -glutamyltransferase, γ -glutamyl transpeptidase, HSP-70, heme oxygenase-1 and a variety of antioxidant enzymes. There is now ample consensus on the importance of the induction of cell tolerance to low levels of HNE (Takahashi et al., 1997, Cheng et al., 2001; Yang et al., 2003).

At this point, it seems useful to point out that mammalian organisms, for controlling HNE toxicity due to oxidative stress and maintaining it at physiological plasma level of 0.3–0–7 µM (Strohmaier et al., 1995; Dianzani, 2003; Forman et al., 2003; Forman and Dickinson, 2004; Barrera et al., 2004), enact the following processes:

- a) *Dilution*, a simple calculation indicates that a bolus injection of a dose of 500 µMHNE in 10 ml of plasma, once diluted in a plasma-extracellular fluid volume of 12 l of a normal human, irrespective of any other process, yields a concentration of as low as 0.04 µM.
- b) *Detoxification*, due to the direct inactivation of HNE with GSH and ascorbate or to the interaction with billions of cells endowed with detoxifying enzymes (Forman and Dickinson, 2004; Awasthi et al., 2005).

- c) *Excretion*, into bile and urine after hepatic detoxification (Yamamoto, 2000) and renal excretion (Alary et al., 2003; Jardines et al., 2003) and
- d) *Cell internalization*, this is a crucial and interesting point because the consequent biological effects can be either negative or positive. There is no doubt that chronically inflamed lungs, by maintaining a steady and high levels of LOPs and pro-inflammatory cytokines in the circulation for hours or days, will cause cell degeneration and a cachetic state as shown by Last et al.'s (2005) work. Several months exposure to ozone or to a prolonged oxidative stress due to a chronic disease (atherosclerosis, diabetes, inflammation) can possibly raise HNE plasma levels up to 5–20 μM and, in spite of continuous detoxification, they can exert pathological effects as those observed *in vitro* studies performed with endothelial cells (Herbst et al., 1999), leukemic cells (Zhang et al., 2001), lens epithelial cells (Choudhary et al., 2002), Jurkat T cells (Larini et al., 2004) and when testing CSeco in cardiomyoblasts (Sathishkumar et al., 2005). Interestingly, tolerance to ozone or HNE is far more easily achieved by small and repeated oxidative stresses than after a continuous and heavy oxidation (Awasthi et al., 2005; Iles et al., 2005).

On the other hand, a normal endogenous HNE level (0.1–0.7 μM) appears to act as a defensive agent against itself and other toxic compounds (Dianzani, 1998; 2003; Barrera et al., 2004). Thus, the biological behavior of HNE is an enlightening example of how the physiological serum level of a potentially toxic aldehyde produced by the normal peroxidation (Strohmer et al., 1995) can activate a number of useful signaling pathways (Siems and Grune, 2003).

Finally, it is worthwhile to mention that the vast cutaneous surface, possibly exposed for hours to ozone and UV radiation, can contribute to the overall toxicity: several studies performed by exposing hairless mice to ozone have shown not only depletion of the skin antioxidants but the induction of a remarkable oxidative stress (Thiele et al., 1997; Podda et al., 1998; Cotovio et al., 2001; Valacchi et al., 2000; 2002; 2003; 2004). As a consequence, humans, living in hot countries and during summer, become particularly susceptible to ozone and UV irradiation. On the contrary, a quasi-total (excluding the neck and the head) exposure of human volunteers to a very low ozone concentration in a sauna cabin for 20 min results in a very transient increase of LOPs in the peripheral circulation that exerts therapeutic effects in chronic limb ischemia's patients (Bocci et al., 1999b; Bocci, 2005) interpreted as due to an induction of antioxidant enzymes and HO-1.

In conclusion, although ozone is not the only culprit for adverse health effects, it significantly contributes to exacerbate respiratory illnesses and enhances mortality in about 40% of the total US population (Bell et al., 2004; 2005; Ruidavets et al., 2005). The problem is linked to the abnormal ozone concentration of thropospheric ozone and the chronic production of noxious compounds that damage the lungs and other vital organs. The overall toxicity, due to the constant aggressiveness of ozone on lungs and partly on the exposed skin, associated

with the relative efficiency of the detoxifying system progressively overwhelmed by the perennial stress, favors pathological effects such as inflammation and cell degeneration particularly on lungs, liver (fibrosis), heart, kidneys and brain (Poli and Schaur, 2000; Chiarpotto et al., 2005). The drawing presented in Fig. 1 aims to visualize how vital organs continuously undergo a kind of a “toxic rain”. Obviously, this knowledge has popularized the idea of ozone toxicity but, in the next section, it will be clarified that the generalization of this concept is incorrect.

Ozone is not always toxic and can be used as a real drug

In our studies (Bocci et al., 1993a, 1993b; 1998a, 1998b; 1999a), exposing human blood to a gas mixture composed of medical oxygen and ozone (~96 and ~4%, respectively), both gases present in the phase overlying a superficial layer of about 10 μ of blood, at first dissolve in the water of plasma. The gas solubilization goes on continuously when the blood is gently rotated in a glass bottle. Oxygen equilibrates with the extracellular and intraerythrocytic water before becoming bound to hemoglobin until it is fully oxygenated as shown by the rapid increase of the $p\text{O}_2$ from about 40 up to 400 mm Hg. On the contrary, ozone, more soluble than oxygen, readily dissolves in water and reacts instantaneously with several substrates, oxidizing ascorbic acid, urate, free cysteine, GSH

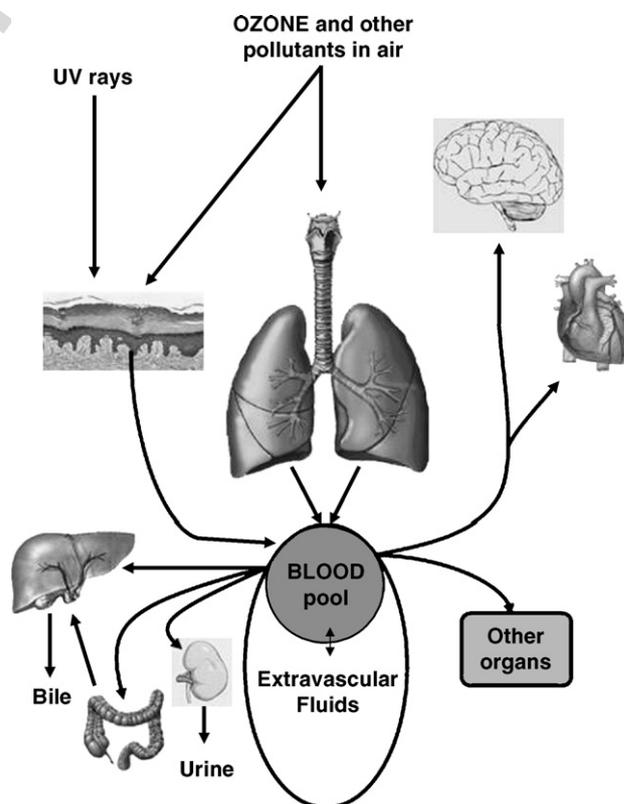


Fig. 1. A chronic exposure of the pulmonary system and skin to ozone and UV radiation causes the formation of a great number of toxic compounds, which, in spite of the natural antioxidant system, flow continuously into the blood and reach vital organs complicating the pulmonary damage.

molecules and albumin thiol groups. Ozone doses, within the therapeutic range (10–80 $\mu\text{g/ml}$ of gas per ml of blood), are partly neutralized by well-known sacrificial reactions: Cross et al. (1992a) investigated the oxidative action of ozone on plasma proteins but did not detect any electrophoretic modification of lipoproteins. Albumin-SH groups undergo oxidation and in fact albumin is considered the main sacrificial molecule and surely prevents lipoprotein damage. The small amount of oxidized albumin is rapidly removed from the circulation and does not affect the plasma level. We have provided evidence that oxygen–ozone behaves similarly when this gas mixture comes in contact with a moist human skin (Bocci et al., 1999b) and the rabbit colon–rectal mucosa (Bocci et al., 2000): ozone dissolves immediately in the water overlaying the epithelium and reacts with sebum, mucoproteins, feces and any other biomolecules present in the liquid film generating hydrogen peroxide (H_2O_2), possibly other ROS and lipid ozonation products (LOPs). Only LOPs are absorbed via lymphatics and venous capillaries and reach first the liver and then enter into the general circulation where these have been measured.

During the last 15 years, we have evaluated the biochemical reactions occurring when human blood is exposed for a few minutes to oxygen and ozone. After the instantaneous reactions of the dissolved ozone with biomolecules (antioxidants and PUFA), the newly formed hydrogen peroxide (H_2O_2) and a heterogeneous number of lipid oxidation products (LOPs) represent the chemical mediators of the totally extinct ozone (Bocci and Paulesu, 1990; Paulesu et al., 1991; Bocci et al., 1993a, 1993b; 1998a, 1998b; 1999a). Although the reaction of ozone with either blood or ELF is somewhat similar, there are profound differences in regard to the quantity and composition of components and antioxidants (Table 1).

The behavior and pharmacodynamic of H_2O_2 have been ascertained: the initial formation of a gradient between plasma and intracellular water allows its entrance into the erythrocytes and lymphocytes but its concentration remains around a few micromoles because it is quickly reduced to H_2O by free GSH, catalase and GSH-Px (Stone and Collins, 2002; Bocci et al., 1998a, 1998b; Valacchi and Bocci, 1999; 2000; Bocci and Aldinucci, 2005). Its half-life is of about 1 s and yet its intracellular concentration is critical because, in order to activate some biochemical pathways (formation of GSSG

with consequent activation of the pentose cycle in the red cell and activation of a tyrosine kinase in lymphocytes), it must reach a critical threshold that, nonetheless, is not cytotoxic. The concept of threshold is physiologically important and means that an ozone dose below 10 $\mu\text{g/ml}$ of gas per ml of blood, in most cases, is biologically ineffective because the ozone dose is totally neutralized by the plasma antioxidants. In other words, the concept of a threshold helps to understand that a too low ozone dose may be ineffective (placebo effect) while a dose higher than the therapeutic one can be toxic. It is almost needless to add that saline-washed erythrocytes suspended in saline, even if exposed to very low ozone concentrations, undergo conspicuous hemolysis, an artificial result (Goldstein and Balchum, 1967) that has favored the concept of ozone toxicity.

Provided that the ozone dose is within a well defined, experimentally determined range (10–80 $\mu\text{g/ml}$ or 0.21–1.68 mM per ml of blood), there is only a transitory decrease (no more than 25%) of the potent antioxidant capacity of plasma (Rice-Evans and Miller, 1994), fully reconstituted within 20 min owing to the efficiency of the redox system (Mendiratta et al., 1998a, 1998b). There is neither damage to erythrocytes: hemolysis is negligible (from 0.4 up to 1.2%) and methemoglobin remains normal (Bocci et al., 1998b; Shinriki et al., 1998; Bocci et al. 2005; Bocci, 2006; Travagli et al., in press) nor to other blood cells. It must be added that ozonated erythrocytes show an improved glycolysis with an increase of ATP and 2,3-DPG levels, which are able to shift the dissociation curve of HbO_2 to the right, confirming the observation of an improved delivery of oxygen in peripheral obstructive arterial disease (Rokitansky, 1982; Mattassi et al., 1987; Viebahn, 1999). Extensive data have been reported in reviews (Bocci, 1999, 2004, 2006) and two books (Bocci, 2002, 2005). It is now clear that a “physiological” ozone dose (most frequently ranges between 10 and 40 $\mu\text{g/ml}$ or 0.21 and 0.84 mM per ml of blood) triggers an acute and precisely calculated oxidative stress able to activate several biological processes summarized in Fig. 2.

What happens during the rapid reinfusion of the hyperoxygenated-ozonated blood into the donor? The hyperoxygenation of blood ($p\text{O}_2 \sim 400$ mm Hg) is irrelevant because, during the 15-min infusion period, it mixes with about 75 l venous blood so that the final venous $p\text{O}_2$ relative pressure is hardly

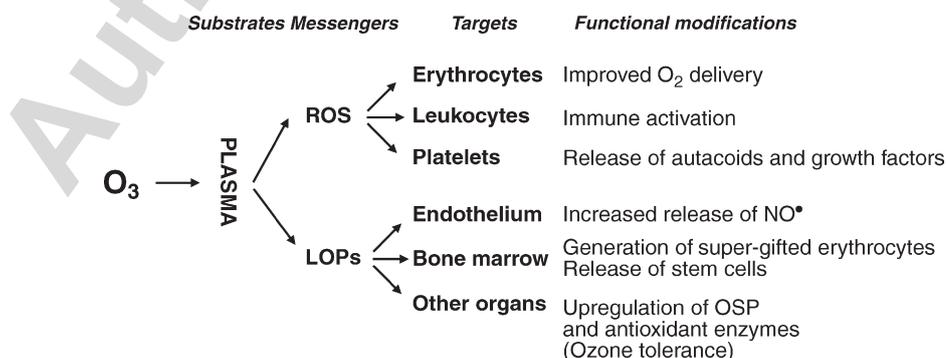


Fig. 2. A summary of the main biological effects elicited during exposure of human blood to oxygen–ozone, ex vivo, and after its reinfusion in the donor. The term of super-gifted erythrocytes is attributed to cohorts of erythrocytes that, after repeated treatments, show an increase of antioxidant enzymes.

modified. LOPs (mainly HNE), as already mentioned, disappear from the circulation within a few minutes, and yet they can exert stimulatory effects throughout the body without toxicity because their concentration, at a submicromolar level, is transitory. This is a crucial consideration to keep in mind and emphasizes how a small and precise ozone dose can act as a biological response modifier. At variance with the high and fairly constant LOPs levels generated by lungs exposed to ozone, HNE can act as useful and not injurious signals (Dianzani, 1998; 2003; Parola et al., 1999; Barrera et al., 2004) and can be regarded as a physiological messenger informing the organism of a minimal oxidative stress that is the critical stimulus for inducing the adaptive response.

What then is the difference between a chronic exposure to ozone and a transitory, precisely calculated ozone stress to a small volume of blood *ex vivo*? The atoxicity of blood ozonation is explained by the use of small and well-calibrated doses of ozone that are tamed by the antioxidant system and the short span (only a few minutes) of ozone exposure. In other words, the ozonation of blood implies that most of the ozone dose is consumed by the antioxidants and only a small percentage elicits biological effects. Blood, in comparison to the lungs, is a much more resistant “tissue,” by virtue of a redundancy of plasmatic and intracellular antioxidants (Halliwell, 1994, 1996, 1999) able to check a bland pulse of ozone. Moreover, it is amazing how quickly a partial depletion of antioxidants returns to normal, thanks to the recycling of dehydroascorbate, GSSG, alpha-tocopheryl radical and lipoate to the reduced counterparts (Packer et al., 1997; Mendiratta et al., 1998a, b; Halliwell, 1999).

Another important biological effect is the amply demonstrated induction of adaptation to oxidative stress (Bocci, 1996a; Leon et al., 1998; Barber et al., 1999; Jalil et al., 2001; Candelario-Jalil et al., 2001; Larini et al., 2003, 2004), a phenomenon described also as “ozone tolerance” or “oxidative preconditioning”. This interesting process is universally present from bacteria to fungi to plants and mammals (extensively discussed in Bocci, 2002, pages 233–37). Goldman (1996) used the term “hormesis” to indicate “the beneficial effect of a low level exposure to an agent that is harmful at high levels” (see also Calabrese, 2005). The repetition of small ozonated autohemotherapies in patients upregulates the synthesis of several antioxidant enzymes (SOD, GSH-Px, GSH-Rd, GSH-Tr and G6PD) and of heme oxygenase-1 (HO-1), which is one of the most protective enzymes catalyzing the release of bilirubin and CO from heme (Pannen et al., 1998; Snyder and Baranano, 2001; Duckers et al., 2001; Zuckerbraun and Billiar, 2003; Iles et al., 2005; Bocci, 2006). I fully agree with one of the referees’s comments that a trace of hemolysis (0.4–0.8%), unavoidable when blood is ozonated in a glass bottle, is useful because it acts as an inducer of HO-1. Thus, a small, acute stress on blood *ex vivo* is quite different from the prolonged, endogenous, oxidative stress due to thropospheric ozone because the former paradoxically upregulates the antioxidant defenses and the latter induces a progressive inflammation and degeneration.

The so-called “major ozonated autohemotherapy” was invented in Germany by Wolff (1974) and until now millions

of treatments have been performed in patients all over the world without any acute or chronic toxicity (Jacobs, 1982). However, a few deaths have been caused by malpractice performed by quacks, who, at the height of HIV infections, injected the gas, intravenously provoking pulmonary oxygen embolism. These unfortunate episodes caused a justifiable outcry and greatly helped to condemn ozonotherapy. Briefly, the correct method consists in collecting 100–200 ml of blood (plus an anticoagulant) in an ozone-resistant glass bottle, adding an equivalent gas volume containing ozone at a precise concentration, gently mixing for 5 min and returning the oxygenated–ozonated blood to the donor during the next 15 min, obviously without the gas. In this way, some of the chemical messengers generated by ozone *ex vivo* diffuse into all the organs and elicit a number of biological responses as schematically indicated:

- a) the increase of intraerythrocytic 2,3-DPG level improves blood circulation and oxygen delivery to ischemic tissues,
- b) improve the general metabolism owing to an improved oxygen delivery,
- c) correct a chronic oxidative stress by upregulating the antioxidant system and inducing HO-1,
- d) induce a mild activation of the immune system,
- e) procure a state of well-being in the majority of patients by activating the neuro-endocrine system (Bocci, 2002; 2005) and
- f) do not cause acute or late noxious effects.

Clinical results so far available have shown that properly performed ozonotherapy appears useful in the following diseases:

- 1) Chronic osteomyelitis, pleural empyema, peritonitis, abscesses with intractable fistulae, infected wounds, bed sores, chronic ulcers and initial gangrena, diabetic foot, skin, mouth, vaginal and rectal bacterial, viral, fungine infections and burns (Matsumoto et al., 2001; Bocci, 2002; Menendez et al., 2002; Di Paolo et al., 2002; Bette et al., 2006). In these pathologies, simultaneous parenteral and topical therapy with ozonated oil (triolein triozone) solves the problem in spite of antibiotic resistance because of ozone’s potent disinfectant activity and capacity for accelerating the healing process. Valacchi et al. (2005) have discussed both the damaging effect of the skin exposed to ozone and the incredible effects of ozonated oil applied on skin lesions.
- 2) Advanced ischemic diseases (peripheral obstructive arterial disease and heart ischemia). A brief infusion of ozonated blood is more effective and free of side effects than the conventional 6 h infusion of a prostanoid. Enhanced and sustained vasodilation with an improved oxygen delivery is the key of success (Clavo et al., 2003; De Monte et al., 2005; Di Paolo et al., 2005).
- 3) A randomized controlled clinical trial performed at the Institute of Angiology and Vascular Surgery at the University of Havana, Cuba, with 101 patients with diabetic foot showed an improved healing and fewer amputations in

the ozone-treated group in comparison with the control group treated with conventional topical and systemic antibiotics (Martinez-Sanchez et al., 2005).

- 4) Age-related macular degeneration (ARMD, atrophic form only). In not too advanced cases, even a small increase of oxygen delivery at the foveola level, by acting on the photoreceptors and retinal pigment epithelium, is able to improve the visual acuity and the quality of life of 66% of patients. Orthodox medicine does not offer any viable option except the administration of antioxidants. The interested reader can examine Section 3 of Chapter 9 in my book (Bocci, 2005a, pp. 132–144), where the methodology and clinical data in 77 patients, including controls, are reported in details.
- 5) Lumbar and cervical hernial discs as well as localized osteoarthritis can be treated with a direct or intramuscular small injection of gas. Ozonotherapy is becoming one of the most successful mini-invasive approaches for these affections (Andreula et al., 2003). Over 120,000 treatments have been performed worldwide with a successful outcome over 80% and a record of safety superior to other mini-invasive procedures.
- 6) Dentistry, regarding primary root carious lesions, particularly in children. Ozone sterilizes the dental lesion and enhances the remineralization (Johnson et al., 2004; Baysan and Lynch, 2004). Prof. E. Lynch has edited a book of 300 pages, entitled: *Ozone: the revolution in Dentistry*, Quintessence, Copenhagen, 2004.

This brief summary points out the versatility of ozone due to its disinfectant activity and the ability to stimulate with several messengers a variety of cells. Space limits do not allow discussing the advantages of ozonotherapy associated with orthodox treatments in other diseases. So far, the validity of ozonotherapy in clinical practice is correctly criticized because only a few studies are available in comparison to large orthodox studies performed by thousands of clinical scientists well supported by pharmaceutical industries. Clearly, the fact that ozonotherapy is mainly performed by private practitioners and is not sponsored by anyone is not a valid excuse and the only hope is that Health Authorities would promote clinical studies because ozonotherapy will reduce medical costs. This possibility would be important because only ample and multicenter studies will provide a valid proof but, unfortunately, prejudice and an incomprehensible antagonism against this therapy, associated with a lack of funds, delays a crucial progress. However, on the basis of my personal experience, I can answer to a referee's comment that, following the established therapeutic dosages, there is at most 0.8% hemolysis (actually useful for stimulating HO-1 induction). Neither acute nor chronic adverse effects have been observed after millions of treatments. As official medicine, although too late, has reported that Viiox has provoked the death of some 50,000 Americans (Beardsley, 2005), I would be the first to denounce toxic effects, whereas I am glad to say that most of patients, during treatment, report a feeling of wellness. The state of well-being and euphoria the day after the infusion of ozonated blood in the donor is probably due to a stimulatory effect of LOPs on the

neuro-endocrine system with a supposed release of ACTH, cortisol, DHEA and perhaps β -endorphin. Unfortunately, this idea has not yet been experimentally validated.

Discussion

There have been several arguments for prohibiting the use of ozone in medicine: the first is that ozone is a strong oxidant and a toxic gas that should never be breathed. The second is due to the fact that several diseases are perpetuated by a chronic oxidative stress, and therefore a gas generating free radicals should be proscribed. The third has been the fault of unscrupulous quacks who, without any medical qualification, have injected the gas mixture (O_2-O_3), intravenously causing lung embolism. Thus, it has been easy to label ozonotherapy as a dangerous quackery, but the previous sections should have clarified why throposheric ozone is toxic and why a single pulse of a precisely measured ozone dose in blood is not. The success of ozonotherapy depends in using small and safe ozone doses, just above the threshold level, able to stimulate a number of biochemical pathways, finally responsible for the activation of the natural healing capacity. This concept echoes an old intuition by Paracelsus (1493–1541), who wrote that: “the body possesses the high art of wrecking but also restoring health”. We must admire his perspicacity because he had neither scientific knowledge of the chronic oxidative stress plaguing chronic diseases and even less of the adaptive phenomenon so critical to restore an effective antioxidant and protective response against pathogens nor could he envisage the beneficial influence of the psico–neuro–endocrine–immunological system. After our studies (described in details in Bocci, 2005, 2006) on the biochemistry, physiology and pharmacology of the compounds generated in blood ex vivo during the brief ozone reaction, we have a comprehensive framework for understanding ozonotherapy which, among complementary therapies, has emerged as a scientific discipline.

Although the putative physiological vasodilator was discovered in 1980 (Furchgott and Zawadzki, 1980), until 1987, nobody believed that NO, a toxic radical gas could be produced by cells and perform a crucial function. Moreover, we have another two gases: the far less reactive but potentially toxic CO derived with bilirubin by the breakdown of heme (Pannen et al., 1998; Brouard et al., 2000; Minetti et al., 1998) and the equally potentially toxic H_2S , synthesized naturally in the body from L-cysteine (Wang, 2003), which can coadjuvate NO in maintaining a correct vascular tone. These three gases, produced in trace amounts, have become indispensable mediators, but their excessive and continuous production during chronic inflammation is very deleterious. Even if ozone is a strong oxidant, I do not consider absurd to assimilate its behavior to these gases because, when either inspired or if continuously produced in atherosclerotic plaques, it is noxious (Wentworth et al., 2003) whereas, when it is used as a single small dose, it appears useful as a real drug able to elicit biological functions and therapeutic results. It is implicit that the idea “the more is better” is not appropriate for ozone because its concentration must be calibrated in relation to the blood volume and its antioxidant

capacity as well as to the target cells. In my latest book (Bocci, 2005), I defined ozone as a new medical drug and we know that any drug, depending upon its concentration and cumulative dose, can be either therapeutic or toxic. I am grateful to one referee reminding me that Paracelsus (1493–1541) wrote: “Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy”. His intuition, made in the Renaissance, appears valid still today.

My own elementary, but compelling observation is that essential molecules like oxygen and glucose can be deadly if their concentrations become too low or too high. It is hoped that this article, purposefully written to highlight the controversy regarding ozone toxicity or its beneficial activity, will interest the scientific community.

Final remarks and perspectives

Needless to say the toxicological problem of the photochemical smog is far more important than the controversy regarding ozone toxicity among chemists and ozonetherapists. The problem will worsen unless politicians, economists and scientists will make a serious effort to reduce pollution as soon as possible: obviously all possible preventive measures for reducing high urban ozone pollution and photochemical smog should be adopted and even partial remedies will help. It is encouraging to remember that, as soon as the problem was understood (Molina and Rowland, 1974), a good cooperation among scientists and Health Authorities successfully prohibited the dispersion of chlorofluorocarbons in the environment, a good first step for slowly recreating a normal ozone layer in the stratosphere.

I would like to plead Health Authorities to organize an ozonotherapy service for the parenteral and topical treatment of chronic wounds, bed sores and chronic ulcers (in diabetics) because they never heal using the most expensive pharmaceutical creams and distress millions of patients. The same could be done for ARMD's patients and for vascular ischemic diseases in combination with the best conventional treatments for reducing pain and improve the quality of life. Of the population at risk of continuous ozone-contaminated air in vast areas of the world, the worst off are children (Mortimer et al., 2000; Bell et al., 2005), patients with chronic pulmonary diseases, smokers and elderly people. What can be done to reduce the increasing incidence of serious complications such as lung cancer, emphysema, bronchopulmonary chronic obstructive disease, pulmonary fibrosis and heart failure? Studies suggesting the potential usefulness of a daily oral supplementation of antioxidants, namely vitamin C, E, *N*-acetyl-cysteine (Mudway and Kelly, 1998; Samet et al., 2001; Antonicelli et al., 2002; Asplund, 2002; Cross, 2003; Sienna-Monge et al., 2004; Polidori et al., 2004) or of inhalation of ozone scavengers (Keinan et al., 2005), have been published but, while they are harmless, their real therapeutic efficacy remains uncertain. Theoretically, it would be better to achieve a fairly constant upregulation of intracellular antioxidant enzymes and HO-1 in lungs and other organs, but is it feasible? One possibility would be to induce ozone tolerance (Rahman et al., 1991; Takahashi et al., 1997; Vargas et al., 1998;

McKinney et al., 1998; Christian et al., 1998; Otterbein et al., 1999; Li et al., 2000; Yang et al., 2003) by organizing an appropriate medical service in advance: this is easy to say but practically difficult in a large number of people because it will be necessary to program and evaluate an effective scheme consisting in a controlled, minimal inhalation of either ozone or ozone bound to unsaturated monoterpenes, before and at selected intervals during the period of high air pollution.

An interesting and practical approach is to induce anti-oxidant enzymes in subjects at high risk by administering a daily oral dose of a dietary supplement (675 mg) of a composition consisting of extracts of five medicinal plants including curcumin (Nelson et al., 2006). Although it may seem paradoxical, another simple way may be to perform a so-called “minor autohemotherapy” every 2 weeks. This is easily and quickly done by collecting in a 10 ml syringe 5 ml of the subject's blood, adding an ozone dose of 0.4 mg in 4 ml of gas, mixing it quickly and injecting it intramuscularly. This practice is as old as I because I learnt it when I was a medical student in 1953, and it positively works in improving the body's resistance to different stresses. It is used even without ozone addition as a sort of autovaccine in herpetic infections (Olwin et al., 1997). Moreover, it is currently used for treating patients with heart failure progression with the aim of reducing immune activation and inflammation, which contribute to the progression of chronic heart failure. Torre-Amione et al. (2005) treat blood at 42.5 °C with ozone and UV rays and have injected it intramuscularly in many patients. Personally, I do not think that it is necessary to use this harsh method and that the above indicated procedure is sufficient for upregulating the synthesis of antioxidant enzymes and HO-1.

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