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Editorial Review

Ozone therapy

Ozone in nature

Ozone, a gas discovered in the mid-nineteenth century, is a molecule consisting of three atoms of oxygen in a dynamically unstable structure due to the presence of mesomeric states (1). The gas is colourless, acrid in odour and explosive in liquid or solid form. It has a half-life of 40 min at 20°C and about 140 min at 0°C (2).

In nature it is abundant only in the stratosphere (20,000-30,000 m) where its concentrations reach 16-20 mg/m^3. In this layer, it is produced by the action of ultraviolet solar radiation and in turn, protects the earth from ultraviolet solar radiation. Ozone occurs at less than 20 µg/m^3 at the Earth's surface, concentrations that are perfectly compatible with life (2).

In recent decades, photochemical pollution of the lower atmosphere, caused by degradation of petroleum gas and volatile combustion products of oil, coal and a great variety of other compounds, ranging from gaseous mixtures prepared in chemical laboratories to forest fires, has led to much higher ozone levels, especially in cities. In the stratosphere, chlorofluorocarbons in liquid refrigerants and spray cans have destroyed part of the protective layer, causing a "hole" at the south pole. These events, widely reported in the mass media, have created considerable apprehension among the public and doctors, who see ozone as a dangerous toxic substance and have difficulty accepting evidence that it can have therapeutic effects (2).

Industrial production of ozone

The most widely used process is based on the reaction:

$$3O_2 + 68.4\ \text{Kcal} \rightarrow 2O_3$$

Ozone forms by this reaction when oxygen flows across an electric arc having a potential difference of about 10,000 Volt. This reaction is triggered by lightning and the ozone produced gives the air its typical smell after storms. Only 5% of pure oxygen is converted to ozone by the medical ozonator, producing a 95:5 mixture of oxygen and ozone. Ozone produced for medical use must be obtained from pure oxygen. If air (78% nitrogen) were used, the result would be a mixture of gases containing nitric oxide which is toxic (2).

Since a variety of ozone concentrations (0.5-80 µg/mL) are required for medical applications, it is necessary to be able to vary the potential of the arc by means of transformers and to modify oxygen flow across the arc. All components of ozone generators must resist oxidation, because ozone is one of the strongest oxidising agents known and attacks most plastics (except polyethylene, polypropylene, silicone and teflon) and most ferrous materials (except stainless steel 316 and titanium). Ozone generators require a photometer to monitor the ozone concentrations produced (1, 2). They must also have a system for destroying unused ozone, which cannot be released into the atmosphere. The most modern and efficient system is based on metal oxide catalysts (manganese, palladium and molybdenum) heated to about 80°C (1, 2).

Ozone toxicity

Ozone is toxic for animals and humans, affecting the lungs and eyes. It irritates the eyes, and its effects on the lungs depend on concentration, temperature, humidity and exposure time. Inhalation of low concentrations of ozone may cause coughing and irritation of the throat (3, 4).

Higher concentrations damage the bronchial mucosa and pneumocytes, and may lead to pulmonary edema (5). It has been calculated that breathing pure ozone at a concentration of 0.02 µg/mL leads to death in 4 h. No other toxic effects have been demonstrated. It should be recalled that oxygen, nitrogen and carbon dioxide, the main gases in the air we breathe, are also toxic and lethal if breathed in abnormal concentrations (2, 6).

Mechanisms of therapeutic action of ozone

In about 1940, Kleinmann (7) demonstrated bactericidal properties of ozone which is used today to sterilise water. Fish (8) observed that ozone has topical therapeutic activity in various skin diseases. In 1974, Wolff (9)

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described a method in which a certain quantity of blood was exposed to ozone in closed glass recipients and then reinfused into the patient, with interesting therapeutic responses. Since then, apart from sterilisation of water, ozone has been used in therapy in an empirical way, albeit with encouraging results (1, 10-13). Only recently has the medical literature begun to show serious interest in the topic, despite the fact that thousands of doctors throughout the world have been using ozone in various applications with positive and often surprising results. This use has occurred in the absence of codified procedures, specific rationale, scientific rigour or practical knowledge.

The main therapeutic use of ozone is that already recorded and described by Wolff, known today as ozone autohemotherapy (OAHT) (9). Recent studies to clarify the mechanism of action have shown that contact between ozone and blood gives rise to effects that can be exploited in medicine. Exposure of human blood to a mixture of oxygen and ozone is not toxic for blood, providing exposure times and concentrations are appropriate (14-17). Indeed, unlike the respiratory system, human blood, the components of which are in a highly dynamic state, is able to neutralise the oxidising power of ozone by a potent defence system. Like other gases (O_2, CO_2, ..), ozone must be dissolved in water in order to act at the biochemical level. On contact with blood, it dissolves in plasma and instantly decomposes in a cascade of reactive oxygen species (ROS), for example hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) (18). These compound are highly reactive and have a short half-life. Moreover, during peroxidation of plasma lipids, there occurs formation of late effectors denominated Lipid Oxidation Products (LOP_S). ROS are also produced by the body during cell respiration by mitochondria and during bacterial phagocytosis by leucocytes. Normally it is by virtue of production of hydrogen peroxide and hypochlorite that animals and humans defend themselves from continuous invasion by pathogenic agents (19, 20).

ROS have their own toxicity, however, and aerobic organisms have in turn developed an antioxidant system, consisting of substances in the plasma, such as uric acid, ascorbic acid, albumin, vitamin E and bilirubin, and of intracellular enzymes such as superoxide dismutase (SOD), catalase (T), glutathione peroxidase (GSH-Px), glutathione reductase (GSH R), glutathione transferase (GSH T) and the redox system of glutathione (GSH-GSSG), kept at optimal level by enzymes and the pentose cycle (via NADPH) (21, 22).

Most of the dose of ozone that comes into contact with blood is partly reduced by hydrosoluble antioxidants and partly transformed into ROS and LOPS, which are also checked by the antioxidant system of the body before they can damage blood cells. A first pharmacological effect of ozone is due to the slight excess of ROS acting as chemical messengers for membrane receptors and various biological functions (23, 24), while LOP_S act on practically all cells after blood reinfusion.

The oxidising action of ozone leads to the formation of hydrogen peroxide that enters cells with various effects: in red blood cells it shifts the hemoglobin dissociation curve to the right and facilitates release of oxygen (25, 26); in leucocytes and endothelial cells it induces production of interleukins, interferon, TGF, nitrogen oxide and antacoids (27, 28); in platelets it favours release of growth factors (29); in all cells (30, 31) it stimulates long term efficiency of antioxidant systems in adaptation to its oxidant action. Another likely effect, not yet demonstrated, is activation of endogenous stem cells.

On contact with blood, ozone therefore causes a very transitory imbalance between oxidants and antioxidants, as an acute, exogenous oxidative stress. With appropriate exposure time and ozone dose, the oxidative stress may be exactly calculated and transient with respect to endogenous toxicity of ROS produced over a lifetime. This calculated imbalance activates messengers that trigger biological effects, without exceeding the capacity of the antioxidant system (32). Ozone, therefore, acts like a drug with a precise therapeutic window: it is not toxic if administered within the therapeutic range, but it may be ineffective if the dose is too low (1) because totally quenched by antioxidants.

A further aspect of its action could be important and is currently being researched. It regards the capacity to positively regulate the antioxidant system (33). The body is besieged by continuous production of ROS. For example, production of ROS is high during respiration, in the metabolic cycle of fatty acids, in cytochrome P450 reactions to xenobiotics, in the presence of phagocytosis and in many pathological situations (34). There are situations in the course of a lifetime in which a vicious circle of imbalance between production and neutralisation of ROS develops: the former continue to increase while the antioxidant system becomes weaker. This happens during chronic viral infections, atherosclerosis, tumour growth, neurodegenerative diseases and aging (34). Excessive production of ROS and/or antioxidant deficit

may become chronic and irreversible at certain times, leading to death. Administration of exogenous antioxidants could, at best, slow down the process, but if the latter is not too advanced, prolonged ozone therapy with therapeutic and progressively increasing doses, may restore the balance between ROS produced and neutralised, inducing a potentiation of the intracellular antioxidant system, with adaptation to chronic oxidative stress (35). Indeed, we know that cells may react to oxidative stress in two ways: if the stress is excessive and continuous, the cell dies; if the stress is modest and transient, the cell has time to react and become resistant, activating expression of silent or rarely expressed genes and producing shock proteins, such as heat shock protein (HSP), glucose-regulated protein (GRP) and oxidative shock protein (OSP). Production of all these proteins is stimulated during ozone therapy (1, 36).

Monitoring of ozone therapy

It is technically impossible to measure ozone directly in the blood or assay ROS in ozonated plasma because of their very brief half-life (fractions of a second) (1). However, there are indirect methods of monitoring the oxidising action of ozone in the body through terminal products or biochemical modifications of the plasma antioxidant system. Indeed, it is possible to measure lipid peroxidation, antioxidant capacity, markers typical of oxidative status and enzyme activities in plasma. Many of these parameters are cumbersome to measure (for example, assay of isoprostanes and 8-hydroxyguanosine as markers of oxidative status) (37) or time-consuming (enzyme activities) or without commercially available kits (2-3 diphosphoglyceric acid) (38). Our group has been using two parameters of lipid peroxidation that are relatively easy and give reproducible results:

1) Assay of thiobarbituric acid reactive substances (TBARS) (1). Ozone in plasma reacts with unsaturated fatty acids to produce a vast range of aldehydes, including malonyldialdehyde (MDA). Determination of MDA gives an indication of the degree of peroxidation. The method, described by Buege & Aust (39), is a colorimetric determination based on reaction with thiobarbituric acid (TBA). This determination is useful in clinical practice, providing an indication of the degree of peroxidation of treated blood. The greater the peroxidation, the greater the concentration of TBARS.
2) Assay of protein thiol groups (PTG) (40). Plasma protein sulphydryl groups are the first line of defence against oxidants. PTG are released in the reaction and can be detected by the Ellman reagent which produces a coloured compound, measured by spectrophotometry. Ozone causes a decrease in PTG in plasma.

The patterns of TBARS and PTG provide sufficient indication of peroxidation status induced by ozone in clinical practice (1, 2).

Ozone autohemotherapy

OAHT is practised today in all countries of Europe, being first proposed, as we have seen, by Wolff in 1974 (9). *Minor O_3 autohemotherapy and major O_3 autohemotherapy* have been described; the former uses 5-10 mL and the latter 200-250 mL of blood. The technique is simple: blood is collected in a glass recipient containing either heparin or sodium citrate, placed in contact with an oxygen/ozone mixture at concentrations ranging between 15-80 µg/mL for 5-10 min and then reinfused into the patient. This is usually done twice a week for 7-8 weeks. Both methods are indicated for the following disorders:

- peripheral vasculopathy (11, 41, 42) Burger disease, atherosclerotic vasculopathy, diabetic vasculopathy)
- chronic ischemic cardiopathies (43, 44) not susceptible to surgical treatment, acute cerebral ischemia
- chronic virus infections (1, 45): hepatitis, herpes I and II, herpes Zoster
- chronic bacterial and fungal infections (46, 47), refractory to conventional therapy
- degenerative eye diseases such as retinal maculopathy of the elderly, diabetic ischemic retinitis, pigmented retinitis (with which Bocci et al have extensive experience: (1)
- orthopedic pathology (48)
- osteoarthritis (1, 2)
- various pain syndromes (1, 2).

To these major pathologies affecting a large number of patients we could also add the vast branch of aesthetic medicine. Here, however, we shall only consider clinical application for severe pathologies.

Although many papers have been published all over the world, there have been few studies with experimental animals confirming ozone efficacy. Controlled clinical studies have only just begun to appear in the literature (11, 41, 42, 49). OAHT is associated with induction of production

of interferon alpha, beta and gamma, TNF alpha, interleukin (1, 2, 50, 51) granulopoietin (GM-CSF) and transforming growth factor beta (TGF beta), and it seems likely that many other proteins are also stimulated (1). An increase in intraerythrocyte SOD activity has also been observed, suggesting an increase in antioxidant defences. These modifications can be observed for hours and days after OAHT, suggesting that once leucocytes are activated by ozone, they migrate into lymphoid environments where cytokine release triggers other immune cells (52, 53).

Extracorporeal blood oxygenation and ozonation (EBOO)

Although we consider the theory underlying OAHT to be valid, in our opinion the quantities of blood are small and more evident results can be obtained with larger quantities. To do this, we oriented towards a system of extracorporeal circulation. In the last 12 years, we have developed an O_2-O_3 exchanger and tested it *in vitro*, then with animals and finally humans. It took a long time to perfect a gas exchange device (GED) suitable for ozone, because it had to be impermeable to liquids, permeable to ozone, resistant to corrosion by ozone (membrane, housing and potting) and the surface in contact with blood had to prevent platelet adhesion (2, 54, 55). The solution turned out to be a biocompatible polypropylene membrane coated with either albumin or phosphorylcholine on the blood side. The housing and potting were built in materials inert to ozone. The GED was built by the company Dideco (Mirandola, Modena, Italy). It passed all *in vitro* tests, including those for release of plastic substances.

EBOO is based on contact of blood with ozone and is carried out by a method similar to that of hemodialysis, but with gas inside the hollow fibres and special filters or GED. The blood pumps, heparinisation, control systems and methods of connecting and disconnecting patients are identical to those used in hemodialysis.

In the blood circuit, the blood pump maintains a constant flow of 75-80 ml/min. Ozone is produced by an Ozonline International generator (Medica, Bologna, Italy) from oxygen obtained from the hospital distribution circuit. The generator can supply ozone at concentrations ranging from 1-20 µg/mL of oxygen, at a pressure of 0.2 bar. A specific photometer (Ozonosan 590, Iffezheim, Germany) controls the quantity of ozone supplied. The gas flows through the ozonator and thence to a system that destroys it with palladium salts heated electrically to about 80°C (Hansler Ozonosan, Iffezheim, Germany) so that no ozone escapes into the room (2, 56)

The return blood line is fitted with devices to remove bubbles. Clotting is inhibited by injecting 5000 IU (1 mL) heparin at the start of treatment. Once the extracorporeal circuit is stable, the ozone/oxygen mixture is allowed to flow into the ozone compartment and treatment begins. Since the method arose by chance in a nephrology department, it seems appropriate that it be provided by dialysis centres (2).

EBOO in animals

Once the GED had been perfected, we began experiments with sheep, which proved to be a good experimental animal for our purposes (54, 55). We first demonstrated that ozone is atoxic on contact with blood. Despite long attempts, we were unable to establish a DL50, or half lethal dose for sheep, when administered at high concentrations. Sheep whose blood was treated extracorporeally with ozone at a dose of 60 µg/mL oxygen did not show any changes in physiological parameters during treatment or in the following hours or days. The experiments were conducted in collaboration with expert veterinarians. The following conclusions emerged.

- EBOO is possible in sheep (2, 55).
- The treatment showed a complete absence of toxicity: besides unsuccessful attempts to establish a DL50, ozonation lasting more than 60 min with a blood flow of 100 ml/min exposed to oxygen/ozone mixtures containing 20-60 µg/mL oxygen of ozone (6 litres of blood treated per hour) did not cause clinical symptoms in sheep during treatment or afterwards.
- Both *in vivo* and *in vitro*, with ozone values greater than 20 µg/mL oxygen, blood at the GED outlet showed a slight increase in LDH which was no longer detectable in peripheral blood, and was not accompanied by significant changes in hematocrit or haptoglobin. No modifications were detected at ozone doses below 10 µg/mL oxygen.
- Both *in vivo* and *in vitro*, biological effects were observed at very low ozone doses (1 µg/mL oxygen).
- Testing by a specialist institute (19) showed that the equipment did not release any plastic substances into the blood, even at high ozone doses, suggesting that ozone did not attack the materials used to build the device.

EBOO and humans

The technique was the same as that tested in sheep. Treatment was carried out after fasting using the cubital vein of the two arms in about 90% of our series of patients. In 10% of patients (with unsuitable cubital veins) a jugular catheter was installed and left *in situ* until the end of the treatment cycle. Clotting was inhibited with an injection of 5000 IU (1 mL) heparin at the start of treatment. If the patient was taking anticlotting drugs, the dose of heparin was reduced after appropriate monitoring. Once the extracorporeal circuit was stable, the ozone/oxygen mixture was let into the gas compartment and treatment began. Treatment lasted an hour and was repeated twice a week to a total of 14 sessions. Ozonation was monitored by PTG and TBARS that decline and increase, respectively, with increasing ozone levels (2, 16).

Results of EBOO in humans

The single injection of heparin was appropriate for an hour of treatment. Extracorporeal circulation was successful with the cubital veins. Ozone doses greater than 4 µg/mL were never used (the current dose is 1 µg/mL) and no changes in LDH, hematocrit or haptoglobin were detected during or after treatment. Maximum oxygenation and maximum ozonation were obtained with blood flows of 75-85 ml/min in the first hour of treatment. Blood samples obtained at the GED outlet showed that pO_2 increased by a factor or 5 or 6, without significantly changing general arterial pO_2. TBARS and PTG, measured downstream of the GED, increased and decreased, respectively, by factors of 2-5, with respect to basal values, using ozone doses of only 1-2 µg/mL oxygen. Patients did not report any type of sensation during treatment. After several treatments, they reported a sensation of well-being and euphoria (2, 16, 57). No significant changes in the main blood chemistry or other parameters were observed after treatment or 1-2 months after the end of the cycle of 14 treatments. No side-effects of any type were experienced during or after treatment or in the course of the treatment cycle. In many cases, positive effects of EBOO manifested as much as 2-3 months after the end of the 14 sessions, a result we called “comet” effect, which is in line with the rationale of ozone therapy.

The first 1000 treatments carried out in 71 patients (2, 16, 56) showed benefits for severe peripheral arteriopathy, coronary disease, cholesterol embolism, severe dyslipidemia, Madelung disease, sudden deafness and osteoarthritis. So far there have been only two drop-outs, one due to infection of the permanent subclavian catheter and the other due to unavailability of the cubital veins.

We are currently conducting a controlled clinical study in patients with peripheral arteriopathy against patients treated with prostanoids.

Other medical uses of ozone

If venous access is lacking, ozonetherapy ca be performed using other administration routes that are not invasive such as rectal insufflation (2) or quasi-total body exposure (2).

Other medical uses include topical application of ozonated oil (58) and water (2) and intraarticular applications. The latter have proved especially effective for herniated vertebral discs (48). Ozone is now successfully used also in odontology for treating primary tooth caries (46).

CONCLUSIONS

Considered an alternative therapy, OAHT has been increasingly used in recent decades and has been found useful in various diseases:

1) It activates the immune system in infectious diseases (10, 15, 22, 28, 30, 36, 45-47, 50);
2) It improves utilization of oxygen and stimulates release of growth factors that reduce ischemia in vascular disease (4, 11, 25, 41, 44, 51, 52);
3) It activates the immune system and may kill cancer cells (2, 15, 35, 52).

EBOO is an extension of OAHT, and like the latter is atoxic when performed for an hour with a blood flow of 100 ml/min and a maximum ozone dose of 1-2 µg/mL ozone (6 L of blood per hour). Both treatments induce trace production of interferon alpha, beta and gamma, as well as TNF alpha, interleukins 1, 2, 4, 6, 8, 10, granulopoietin (GM-CSF), transforming growth factor beta (TGF beta) and probably many other proteins. An increase in intraerythrocyte SOD activity has also been observed, suggesting increased antioxidant defences (10, 20, 21, 56, 59). These changes continue for hours and days after OAHT, indicating that once activated by ozone, leucocytes migrate into lymphoid environments where cytokine release triggers immune cells. Controlled studies are needed to verify the good clinical results reported in various disorders.

N. DI PAOLO [1], V. BOCCI [2], E. GAGGIOTTI [1]

[1] Nephrology, Dialysis and Transplantation Department, University Hospital of Siena, Siena - Italy
[2] Institute of General Physiology of the University of Siena, Siena - Italy

Address for correspondence:
Nicola Di Paolo, MD
Department of Nephrology
University Hospital
Viale Bracci
53100 Siena, Itay
e-mail: N.DiPaolo@siena.toscana.it

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