BASIC BIOLOGICAL AND THERAPEUTIC EFFECTS OF OZONE THERAPY IN HUMAN MEDICINE

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Contents

Summary

In this chapter we will expose the biochemical and pharmacological mechanism of action of ozone when dissolved in biological fluids. Although ozone is a strong oxidant, under controlled conditions, it can be therapeutically useful, in several human diseases. In fact ozone, once dissolved in the water or the blood, triggers a cascade of well-defined chemical compounds acting on multiple cellular targets. We will demonstrate that ozone is an extremely versatile drug and the therapeutic range has been defined precisely to avoid any acute and chronic toxicity. An interesting aspect is that prolonged ozone therapy allows an upregulation of the antioxidant enzymes and therefore ozone therapy represents a system for correcting the chronic oxidative stress present in many diseases.

1. Introduction

The authors have worked on this topic since the early 1990s and they believe that they can provide the reader all the information regarding the basic biology and explain the reasons why ozone can be a useful drug in human and veterinary Medicine.

Unfortunately ozone has a bad name because it is an important pollutant of the

tropospheric air and is also a strong oxidant and therefore potentially cytotoxic. Thus, most laypeople as well as clinical scientist and chemists have not yet either understood or learnt that the ozone reactivity can be perfectly tamed by the potent antioxidant system of blood and cells. However, it is absolutely necessary that any physician, before entertaining the use of ozonetherapy in patients, must know and fully understood how ozone acts on blood and other biological fluids and why it induces relevant biological effects leading to therapeutic results. Like other medical drugs, it is very much a question of dose and now we know exactly the therapeutic window within which ozone is useful and totally atoxic.

A full account of the ozone story will be given in this chapter but the methodology of production and measurements of ozone will not be discussed because this will be presented in another chapter.

Table 1 summarizes several reasons for refusing ozone therapy by orthodox medicine. However, problems 1-5 have been practically overcome, whereas the remaining 6 -9 are stumbling blocks hindering progress. During the last 14 years, we have made a great effort to examine ozone therapy in a scientific fashion both at a basic and clinical level, and we now have some ideas how ozone acts, how and why its toxicity can be controlled and how therapeutic effects can be exerted. There is no need to invoke philosophical speculations because the mechanisms of action are in the realm of classical biochemistry, physiology and pharmacology.

1	No precise ozone generator
2	Lack of standardization
3	Ozone toxicity
4	Lack of solid scientific biological and clinical data
5	The problem of charlatans
6	Lack of regulation and disinterest of health authorities
7	Lack of financial support
8	Skeptical and uninformed scientists

Table 1. The reasons why oxygen ozone therapy has not been accepted by orthodox medicine

2. Reactive oxygen species (ROS) are produced continuously during physiological conditions and are critical for cell survival.

During the last 2.5 billions year, oxygen (O_2) has become essential for the aerobic life. It is an unusual free radical because, in spite of having two unpaired electrons in the outer orbital, is unusually stable. However about 2-3% of oxygen used by mitochondria, via the complex I and III, during the process of oxidative phosphorylation will leak from the respiratory chain to form anion superoxide, $O^{\bullet-}_2$. NAD(P)H oxidases, present in cell membranes of fibroblasts, endothelial and vascular smooth muscle cells and particularly phagocytes, produce superoxide as a basic defensive process. Other enzymes such as nitric oxide synthase (NOS), xanthine oxidase, cytochrome P450, lipoxygenases and even heme oxygenases (HOs), during abnormal situation, as in ischemia–reperfusion or initial inflammation, may be implicated in superoxide

production. The reduction of superoxide, discovered by McCord and Fridovich in 1968, is performed by mitonchondrial (Mn), cytosolic (Cu/Zn) and extracellular(ec) superoxide dismutases(SODs), that catalyze the dismutation to hydrogen peroxide as follows:

$$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is not a radical molecule because it has paired electrons but it has been included among the reactive oxygen species (ROS) because it is an oxidant on its own right. As it is a unionized molecule, in the presence of an extracellular-cytosolic gradient, it passes through the cell membrane but the intracellular concentration is only about 1/10 of the extracellular one. Remarkably, it has a half-life of about 1-2" in plasma but less than 1" when generated in blood. Its relative stability allows measuring it in plasma: in normotensive subjects at a concentration of about 2.5 μM . In this case the intracellular concentration of hydrogen peroxide will be at the most of 0.25μM while the maximal intracellular concentration that can be generated for signaling purposes may reach 0.5-0.7 μM. It appears ubiquitous as it has been detected in urine and in exhaled air. When ozone induces a sudden production of hydrogen peroxide in plasma, its intracellular presence is always transitory because, as we shall describe, reductants and enzymes promptly reduce it to water. Depending upon its local concentration and cell-type, hydrogen peroxide can either induce proliferation or cell death. It can regulate vascular tone by causing constrictions of vascular beds or vasodilation although it remains uncertain if it acts as an endothelium-derived hyperpolarizing factor.

During blood ozonation, hydrogen peroxide, suddenly generated in plasma, permeates lymphocytes and, when it reaches the cytosol, by activating a tyrosine-kinase, it causes the phosphorylation of the NF-kB and the release and translocation into the nucleus of the heterodimer p50-p65, able to regulate the expression of over 100 genes. We need to emphasize that this process, checked by either a phosphatase or inhibited by intracytoplasmic antioxidants, is very transitory.

Anion superoxide can free and reduce Fe^{3+} from ferritin:

$$O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{++} + O_2$$

Obviously an excess of hydrogen peroxide in the presence of Fe^{++}, can give rise to the very reactive hydroxyl radical by way of the Fenton-Jackson reaction:

$$H_2O_2 + Fe^{++} + \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$$

Moreover hydrogen peroxide in the presence of anion superoxide can generate another hydroxyl radical via the iron catalyzed Haber and Weiss's reaction. Hydroxyl radicals, in spite of having one nanosecond half-life, can cause covalent cross-linking of enzymes or propagate deleterious free radical reactions in a variety of molecules such as DNA, proteins and lipids. It is almost needless to say that these types of dangerous reactions can be avoided by precisely calibrating the ozone dose against the antioxidant capacity

of blood. Similarly, in the presence of hydrogen peroxide, we should avoid the activation of the enzyme myeloperoxidase, which, by catalyzing the oxidation of halide ions, can form hypochloric acid (OCl^-). On the same vein, ozonation of physiological saline, not only generates H_2O_2 but also NaOCl as it has been shown by Ueno et al. (1998).

Nitric oxide ($NO^\bullet$) is a relatively unreactive free radical with a half life of 1-2" formed by NO synthase. We have shown that, during blood ozonation depending upon the ozone concentration, from pico to nanomolar concentrations of nitric oxide are generated. This physiological compound mediates relevant processes as vasodilation, platelet stability and host-defense. NO binds partly to cystein 67 in hemoglobin and to GSH with the formation of more stable nitrosothiols able to display useful pharmacological actions far distant from the synthesis site. During pathological situations, or using an excessive ozone dosage, micromolar concentrations of nitric oxide can be generated and can either aggravate an inflammatory state or, by reacting with anion superoxide, peroxynitrite ($ONOO^-$) and other reactive nitrogen species (RNS) are formed. They react with an array of biomolecules inducing lipid peroxidation, cross-linking and carbonyls. Furthermore either protonation or oxidation of peroxynitrite generate an oxydryl molecule and nitrogen dioxide (NO_2). These molecules are able to form nitro-adducts and carcinogenic nitrosamines.

Another series of compounds formed in different amounts in both physiological or pathological situations are the lipid oxidation products (LOPs). As an example, a hydroxyl radical, reacting with an unsaturated fatty acid (PUFA) as arachidonic acid (LH), bound to albumin or present in membrane phospholipids, produces a lipid molecule radical ($L^\bullet$):

$$OH^\bullet + LH \rightarrow L^\bullet + H_2O$$

The lipid molecule radical, by reacting with oxygen, forms a peroxyl radical, LOO•, which can be either reduced to a hydroperoxide, LOOH or to a final aldehyde such as malonyldialdehyde (MDA) or the typical 4-hydroxy-2,3-trans-nonenal (4-HNE). Needless to say that among plasma lipids, there is a heterogenous abundance of polyunsaturated fatty acids (PUFA) which, during ozonation, may in part be transformed into a bewildering mixture of aldehydes. These compounds are intrinsically toxic because they can inactivate enzymes, other lipids and nucleic acids. Unlike ROS, they are fairly stable in vitro as we observed their constant concentrations after incubating at 37°C several samples of ozonated blood. Once again, their toxicity depends upon their final concentration and location because in vivo, after the slow reinfusion of carefully ozonated blood, they undergo a marked dilution in the blood and extravascular fluids, detoxification via aldehyde-dehydrogenases and GSH-transferases, and excretion via the bile and urine. Thus, after diffusing all over the organism, the remaining molecules that eventually enter into the cells are very few, most likely at submicromolar levels. Interestingly, in line with the concept of a dynamic balance, the physiological plasma level of 4-HNE ranges between 0.3 and 0.7μM. At these concentrations, 4-HNE displays useful functions and stimulates the synthesis of GSH-transferases and aldehyde dehydrogenases. The problem of detoxification of aldehydes has been extensively discussed.

Owing to the presence of oxygen, evolution has allowed the formation of interacting mechanisms for protecting living beings against the threat of ROS. Thus we cannot omit mentioning the critical role of hydrophilic (~50 μM ascorbic acid, ~300 μM uric acid, GSH, thioredoxin and other electron donors) lipophilic (vitamin E, bilirubin) compounds, proteins like albumin acting either as oxidant scavenger and/or Fe^{++}, Cu^{+} chelator (transferrin, ferritin, ceruloplasmin) and a large series of antioxidant enzymes like SOD, catalase, GSH-peroxidases, GSH-reductases, peroxiredoxins, not to forget glucose-6-phosphate dehydrogenase as one of the key enzyme of the pentose phosphate pathway supplying the constantly required NADPH as a reductant. The maintenance of an optimal balance of GSH/GSSG, NAD+/NADH and NADP+/NADPH is critical for the cell.

The constant collaboration of the various components of the antioxidant system, made quite effective by the recycling of its components, is sufficient to keep at bay the offence due to ROS, LOPs and RNS for long periods of the life of any organism. However aging and particularly chronic inflammatory diseases cause an often irreversible disruption of the control of the redox state that progressively aggravates the pathology. On the other hand, a judicious ozonation of blood implies a precisely measurable and small perturbation of the oxidant-antioxidant balance that, within a few minute is re-equilibrated, within a few minute. Moreover the pharmacologically induced acute oxidative stress activates a number of biochemical pathways on different cells able to explain biological and therapeutic effects.

2.1. When and why we begun to study the Biological Effects of Ozone in Human Blood?

About eighteen years ago we were studying the induction of interferon-gamma by oxidizing agent when, by a mere coincidence, a hematologist asked to one of us an explanation of the apparently beneficial effect of ozonated blood re-transfused in donor patients affected by chronic hepatitis C. We only knew that ozone was a potent oxidant but we remembered that periodate and galactose-oxidase could induce in blood mononuclear cells (BMC) the synthesis of IFN: thus, we felt compelled to evaluate whether human BMC, briefly exposed to small ozone doses, could produce this cytokine. It took some time to learn how to precisely handle ozone because this labile gas must be produced extempore and represents about 2% of the gas mixture made up with medical oxygen. Indeed we demonstrated the ozone dose-dependent production of IFN-gamma. Our observation, extended to other cytokines, was confirmed by other Authors, evaluating the ozone as an inducer of proinflammatory cytokines in the lung. However we learnt that ozone therapy was a poorly known and empirical complementary approach and that orthodox medicine was skeptical about it. Actually a distinguished ozone chemist has declared that “ozone is toxic, no matter how you deal with it and it should not be used in medicine”.

We soon realized that ozone was an excellent generator of free radicals: in the 1990s, there was a general consensus that ROS and LOPs were involved in many human pathological conditions and, at the very least, they could perpetuate a chronic oxidative stress. Thus the idea of using ozone in medicine appeared wrong but this did not deter us in starting a scientific program for objectively clarifying if ozone can really be

always toxic. During the Renaissance, Paracelsus (1493-1541) wrote that "poison is in everything and nothing is without poison: the dosage makes it either "a poison or a remedy". In 2005, John Timbrell entitled his book "The poison paradox; chemicals as friends and foes" reminding us two essential facts: firstly, it is the dose that makes a chemical toxic and secondly and more important, toxicity results from the interaction between chemicals and biological defenses. Thus, throughout the last 16 years, we have noticed that prejudice weighs more than knowledge and we start to wonder how the attempt to introduce ozone therapy within orthodox medicine will end. Encouragingly, we have noticed that recently a more objective view has been taken by considering that hydrogen peroxide and two gases such as NO and CO, produced in normal conditions, have an essential role in physiology and they can become toxic when produced in excessive amounts overwhelming the antioxidant defenses. The experience gained in these years taught us that ROS and LOPs are produced continuously and participate in a variety of crucial physiological functions although they can also display negative effects when critical determinants such as location, time of exposure and concentration are responsible for pathologic effects (Bocci,1999). We will then briefly describe our results that show how judicious ozone doses trigger a number of biological activities without any adverse effects.

2.2. A Detailed Description of the Action of Ozone on Whole Human Blood

Today there is no doubt that, under appropriate conditions, the blood's antioxidant system can neutralize ozone within the therapeutic dosages ranging from 0.21 µmol/mL(10µg/ml of gas per ml of blood) up to 1.68 µmol/mL (80µg/ml of gas), without preventing the fulfillment of biologic activities and no toxicity. What is the behavior and fate of ozone after coming in contact with body fluids?
The essential concepts to bear in mind are the following:

(a) As any other gas, ozone dissolves physically in pure water according Henry's law in relation to the temperature, pressure and ozone concentration. Only in this situation ozone does not react and, in a tightly closed glass bottle, the ozonated water (useful as a disinfectant) remains active for a couple of days.

(b) On the other hand, at variance with oxygen, ozone reacts immediately as soon as it is dissolved in biological water (physiological saline, plasma, lymph, urine). Contrary to the incorrect belief that ozone penetrates through the skin and mucosae or enters into the cells, it is emphasized that, after the mentioned reaction, ozone does not exist any longer.
In order of preference, ozone reacts with abundant PUFA, bound to albumin, antioxidants such as ascorbic and uric acids, thiol compounds with –SH groups such as cysteine, reduced glutathione (GSH) and albumin, particularly rich in –SH groups. If the ozone is overdosed, carbohydrates, enzymes, DNA and RNA can also be affected and because all of these compounds act as electron donor, they would undergo oxidation and serious damage.

(c) The main reaction:
$$R\text{-}CH{=}CH\text{-}R' + O_3 + H_2O \rightarrow R\text{-}CH{=}O + R'\text{-}CH{=}O + H_2O_2$$
shows the simultaneous formation of one mole of hydrogen peroxide (included

among reactive oxygen species, ROS) and of two moles of LOPs.

The fundamental ROS molecule is hydrogen peroxide, which is a non radical oxidant able to act as an ozone messenger responsible for eliciting several biological and therapeutic effects. As we have mentioned, the concept that ROS are always harmful has been widely revised because, in physiological amounts, they act as regulators of signal transduction and represent important mediators of host defense and immune responses. In normal conditions, the formation of hydroxyl radicals is practically impossible because all the iron is chelated and none is released free. While exposure to oxygen is ineffective, ozone causes the generation of hydrogen peroxide and of the chemiluminescent reaction in both physiological saline and plasma. However, while in saline there is a consistent and prolonged increase, in the ozonated plasma both chemiluminescence and hydrogen peroxide increase immediately but decay very rapidly with a half-life of less than 2 min. suggesting that both antioxidants and traces of enzymes rapidly quench hydrogen peroxide. Its reduction is so fast in ozonated blood that it has been experimentally impossible to measure it. Consequently we feel confident that the extremely transitory gradient of hydrogen peroxide in plasma may generate only a submicromolar gradient in the cytosol, which nonetheless is indispensable for the activation of biochemical pathways in blood cells.

Interestingly, we have also determined the formation of nitrogen monoxide ($NO^{\bullet}$) in human endothelial cells exposed to ozonated serum. We feel confident that using ozone within the therapeutic range neither peroxynitrite, nor other RNS, nor hypochlorite anion is formed.

Although ROS have a lifetime of less than a second, they can damage crucial cell components and therefore their generation must be precisely calibrated to achieve a biological effect without any damage. This can be achieved by regulating the ozone dose (ozone concentration as μg/ml of gas per ml of blood in 1:1 ratio) against the antioxidant capacity of blood that can be measured and, if necessary, strengthened by oral administration of antioxidants before and throughout ozone therapy. A very enlightening finding (Bocci and Aldinucci, 2006), was achieved by evaluating the variation of the total antioxidant status (TAS) in plasma after ozonation and 1 min mixing of the liquid-gas-phases of either fresh blood or the respective plasma withdrawn from the same five donors: We have shown that, after ozonation of plasma with either a medium, or a high (40μg/ml or 80μg/ml of gas per ml of plasma, respectively) ozone concentration, TAS levels progressively decrease at first and then remain stable after 20 min: The decrease was ozone dose-dependent and varied between 46 and 63%, respectively. Interestingly, TAS levels in blood treated with the same ozone concentrations decreased from 11 to 33 %, respectively, also in the first minute after ozonation but then 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. Mendiratta et al. (1998) have found that dehydroascorbate can be recycled back to ascorbic acid within three 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. All of

these data clearly show that the therapeutic ozonation modifies only temporarily and reversibly the cellular redox homeostasis. There is now a general consensus that ascorbic acid, GSH, α-tocopherol and lipoic acid, after oxidation, undergo a continuous reduction by a well coordinated sequence of electron donations.
d) LOPs production follows peroxidation of PUFA present in the plasma: they are heterogenous and can be classified as lipoperoxides (LOO•), alkoxyl radicals (LO•), lipohydroperoxides (LOOH), isoprostanes and alkenals, among which, 4-HNE and MDA. Radicals and aldehydes are intrinsically toxic and must be generated in very low concentrations. They are in vitro far more stable than ROS but fortunately, upon blood reinfusion, they have a brief half-life owing to a marked dilution in body fluids, excretion (via urine and bile), and metabolism by GSH-transferase) and aldehyde dehydrogenases. Thus only submicromolar concentrations can reach all organs, particularly bone marrow, liver, Central Nervous System (CNS), endocrine glands, etc., where they act as signaling molecules of an ongoing acute oxidative stress.

If the stage of the disease is not too far advanced, small amounts of ROS and LOPs can elicit the upregulation of antioxidant enzymes on the basis of the phenomenon described under the term of "hormesis". The oxidative preconditioning or the adaptation to the chronic oxidative stress has been now demonstrated experimentally. The increased synthesis of enzymes such as superoxide-dismutase (SOD), GSH-peroxidases (GSH-Px), GSH-reductase (GSH-Rd) and catalase (CAT) has been repeatedly determined in experimental animals and in patients. Interestingly, it was recently demonstrated that HNE, by inducing the expression of glutamate cysteine ligase, causes an intracellular increase of GSH, which plays a key role in antioxidant defense. Furthermore LOPs induce oxidative stress proteins, one of which is heme-oxygenase I (HO-1 or HSP-32) which, after breaking down the heme molecule, delivers very useful compounds such as CO and bilirubin. 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 upregulated ferritin. The induction of HO-1 after an oxidative stress has been described in thousands of papers as one of the most important antioxidant defense and protective enzyme.

Although it remains hypothetical, it is possible that LOPs, throughout the treatments, acting as acute oxidative stressors in the bone marrow microenvironments activate the release of metalloproteinases, of which, particularly MP-9 may favor the detachment of staminal cells. These cells, once in the blood circulation, may be attracted and home at sites where a previous injury (a trauma or an ischemic-degenerative event) has taken place. The potential relevance of such an event would have a huge practical importance and it will avoid the unnatural, costly and scarcely effective practice of the bone marrow collection with the need of the successive and uncertain reinfusion.

It is emphasized that submicromolar LOPs levels can be stimulatory and beneficial, while high levels can be toxic. This conclusion, based on many experimental data, 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.

In conclusion, it must be clear to the reader that the ozonation process either happening in blood, or intradiscal or in an intramuscular site represents an acute oxidative stress. However, provided that it is precisely calculated according to a judicious ozone dosage, it is not deleterious but it is actually capable of eliciting a multitude of useful biological responses and, possibly, reversing a chronic oxidative stress due to ageing, chronic infections, diabetes, atherosclerosis, degenerative processes and cancer. Indeed the ozonotherapeutic act is interpreted as an atoxic but real "therapeutic shock" able to restore homeostasis (Bocci, 2002: 2005).

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Biographical Sketches

Emma Borrelli, physician, is actually referred physician for the Postgraduate Course on Oxygen Ozonetherapy and Director of the Laboratory of Cardiopulmonary Pathophysiology, Department of Surgery and Bioengineering at the University Hospital in Siena, Italy. She was awarded the Medical Doctor Degree from the Faculty of Medicine at University of Siena in 1986, and she obtained her Ph.D in Cardiopulmonary Pathophysiology in 1992. In 1996 she became specialist in Pulmonary Disease. After a fellowship in Switzerland and England, she served as Resident and Consultant in the Department of Surgery and Bioengineering at Siena University. In 1997 she began her collaboration with Prof. V. Bocci in the field of ozonetherapy. She is member of FIO (Italian Federation of Ozonetherapy) and author and co-author of chapters and articles on ozonetherapy in national and international books and journals. Her research is focoused in the clinical application of ozone therapy.

Velio Bocci, physician, was born in Siena in 1928. He was awarded the Medical Doctor Degree at the University of Siena in 1954 an the Specialty in Respiratory Disease and Clinical Haematology.After a brief training in Surgery, he went back in 1956 to the Institute of General Physiology at the University of Siena where, with some intermissions, he has worked since then. He got further training in Biochemistry in London (UK) and at the State University of Buffalo (USA). Since 1971 he was Professor of General Physiology and since 1978 Director of the Institute at the Faculty of Pharmacy at the University of Siena, Italy. His fields of research include plasma protein separation and the pharmacology of interferons. Since 1991 he has contributed crucial research papers regarding the biological effects of ozone. He is author of about 60 publications on ozone therapy, mostly published in international journals, and three books. From 2003 he is Emeritus Professor at the Department of Physiology, University of Siena.