BASIC BIOLOGICAL AND THERAPEUTIC EFFECTS OF OZONE THERAPY IN HUMAN MEDICINE

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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.

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, $O_2^{\bullet -}$. 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:

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

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

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:

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

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

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:

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

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

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

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):

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

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

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:

$$\text{R-CH=CH-R'} + O_3 + H_2O \rightarrow \text{R-CH=O} + \text{R'-CH=O} + H_2O_2$$

R-CH=CH-R' + O3 + H2O → R-CH=O + R'-CH=O + H2O2
R-CH=CH-R' + O3 + H2O → R-CH=O + R'-CH=O + H2O2

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 ($NO^{\cdot}$) 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).

2.3. An Evaluation of the Biological Effects Elicited by ROS and LOPs

The ozonation process is therefore characterized by the formation of ROS and LOPs acting in two phases. This process happens either ex vivo (as a typical example in the blood collected in a glass bottle) or in vivo (after an intramuscular injection of ozone) but, while ROS are acting immediately and disappear (early and short-acting messengers), LOPs, via the circulation, distribute throughout the tissues and eventually only a few molecules either bind to cell receptors, or enter into the cell. Their complex pharmacodynamcs allows minimizing their potential toxicity and allows them to become late and long-lasting messengers.

During the first phase, hydrogen peroxide diffuses from the plasma into the cell cytoplasm and represents the triggering stimulus: depending upon the cell type, different biochemical pathways can be concurrently activated in erythrocytes, leukocytes and platelets resulting in numerous biological effects. The rapid reduction to water is operated by the high concentration of GSH, CAT and GSH-Px but, nonetheless, H_2O_2 must be above the threshold concentration for activating several biochemical pathways and acts on the different blood cells as follows: the mass of erythrocytes mops up the bulk of hydrogen peroxide: GSH is promptly oxidized to GSSG and the cell, extremely sensitive to the reduction of the GSH/GSSG ratio, immediately corrects the unbalance by either extruding GSSG, or reducing it with GSH-Rd at the expenses of ascorbate or of the reduced nicotinamide adenine dinucleotide phosphate (NADPH), which serves as a crucial electron donor. Next, the oxidized NADP is reduced after the activation of the pentose phosphate pathway, of which glucose-6-phosphate dehydrogenase (G-6PD) is the key enzyme. We have determined a

small but significant increase of ATP formation but, whether this is due to the activation of the pentose cycle or to phosphofructokinase or to both remains to be clarified. Moreover the reinfused erythrocytes, for a brief period, enhance the delivery of oxygen into ischemic tissues because of a shift to the right of the oxygen-hemoglobin dissociation curve, due either to a slight decrease of intracellular pH (Bohr effect) or/and an increase of 2,3-diphosphoglycerate (2,3-DPG) levels.

Contrary to what has been observed by performing unphysiological experiments using saline-washed erythrocytes resuspended in saline, or, even worse, using distilled water-lysed erytrocytes, we and Shinriki et al. (1998), have neither observed a loss of K+ or an abnormal increase of methemoglobin. It is very unfortunate that these types of unphysiological studies have created an unjustified concern that ozone damages blood cells. In agreement with Shinriki et al., data (1998) we are sure that ozone does not act on the phospholipids or cholesterol of the erythrocytic membrane. Further, more recent studies completely exclude any action of ozone on erythrocytic membranes by using ozone concentrations within the prescribed therapeutic window (Travagli et al. 2007). Thus it also remains unlikely that ozone is ever able to activate sphingomyelinases and release ceramide and its derivatives.

Obviously one autohemotherapeutic treatment has a minimal effect and we need to ozonate at least 2.5-4 Litres of blood within a period of about 60 days. During this period, LOPs act as repeated stressors on the bone marrow and these frequent stimuli cause the adaptation to the ozone stress during erythrogenesis with upregulation of antioxidant enzymes. As a consequence, a patient with chronic limb ischemia undergoing ozone therapy can have a clinical improvement due to the formation of successive cohorts of erythrocytes progressively more capable of delivering oxygen to his ischemic tissues. However the final improvement is also due to the localized release of NO, CO and growth factors released from platelets.

Although ozone is one of the most potent disinfectants, it cannot inactivate bacteria, viruses and fungi in vivo because, paradoxically, the pathogens are well protected, particularly inside the cells, by the powerful antioxidant system of blood. The fact that ozone can destroy pathogens dispersed in water has created this diffused misconception and the illusory idea that ozone therapy can cure HIV infection and AIDS. Thus, as we proposed a long time ago, ozone acts as a mild enhancer of the immune system, by activating neutrophils and stimulating the synthesis of some cytokines. Once again the crucial messenger is hydrogen peroxide, which, after entering into the cytoplasm of blood mononuclear cells (BMC), by oxidizing selected cysteines, activates a tyrosine kinase, which then phosphorylates the transcription factor Nuclear Factor kB allowing the release of an heterodimer (p50+p65) eventually responsible for causing the synthesis of several proteins, among which, the acute-phase reactants and numerous interleukins. In the past, we have measured the release of several cytokines from ozonated blood upon in vitro incubation. Once the ozonated leukocytes return into the circulation, they home in lymphoid microenvironments and successively release cytokines acting in a paracrine fashion on neighboring cells with a possible reactivation of a depressed immune system. This process, described as the physiological cytokine response, is a part of the innate immune system and helps us to survive in a hostile environment.

During ozonation of blood, particularly if it is anticoagulated with heparin, we have noted an ozone-dose dependent increase of activation of platelets with a consequent release of typical growth factors, which will enhance the healing of chronic ulcers in ischemic patients. Whenever possible, the use of heparin as an anticoagulant is preferable to sodium citrate because, by not chelating plasmatic Ca++, reinforces biochemical and electric events.

During the reinfusion of the ozonated blood into the donor, the vast expanse of the endothelial cells will be activated by LOPs resulting in an increased production of NO, plasma S-nitrosothiols and S-nitrosohemoglobin. While NO has a half-life of less than one second, protein-bound-NO can exert vasodilation also at distant ischemic vascular sites with relevant therapeutic effect.

Moreover, on the basis of the phenomenon of ozone tolerance, that says the exposure of an organism to a low level of an agent, harmful at high levels, induces an adaptive and beneficial response, we have postulated that LOPs, by acting as long-distance messengers, can transmit to all organs the information of an acute oxidative stress. The bone marrow is particularly relevant because it can upregulate antioxidant enzymes during erythrogenesis and allows the release of staminal cells for possibly regenerating infarcted organs. Moreover the stimulation of the endocrine and central nervous systems may help to understand why most of the patients during prolonged ozone therapy report a feeling of euphoria and wellness probably due to an improved metabolism as well as to an enhanced hormonal or neurotransmitters release.

The paradoxical concept that ozone eventually induces an antioxidant response capable of reversing a chronic oxidative stress is common in the animal and vegetal kingdom and there is good experimental evidence that this phenomenon is a characteristic of all living beings. Moreover it is already supported by our findings of an increased level of antioxidant enzymes and HO-1 during ozone therapy. It also suggests that a judicious use of ozone, in spite of acting as an oxidant, enhances the antioxidant capacity, which represents the critical factor for overcoming chronic viral infections, ischemia and cell degeneration. On the other hand, it is acknowledged that a continuous inhalation of tropospheric ozone is deleterious for the unprotected pulmonary system and increase the death rate in exposed populations. The apparent discrepancy between the ozone toxicity for the respiratory system, but not the blood, has been clarified on the basis of the cumulative ozone dose in the lungs in comparison to a minimal, very brief and calculated exposure for blood.

3. Which are the Routes of Ozone Administration?

Table 2 shows that ozone can be administered with great flexibility but it should not be injected intravenously as a gas because of the risk of provoking oxygen embolism, given the fact that the gas mixture contains always no less than 96 % oxygen.

Table 2. Routes of ozone administration

So far the most advanced and reliable approach has been the major ozonated AHT because, on the basis of the patient's body weight, a predetermined volume of blood (200 -270 mL) can be exposed to an equal volume of gas (O2- O3) in a stoichiometric fashion, with the ozone concentration precisely determined. Figur1 shows a schematic drawing of the components necessary to perform AHT with an ozone resistant glass bottle (plastic bag must be avoided because they are not ozone resistant and contaminate blood with phtalates and plastic microparticles). Blood, drawn from a cubital vein via a G19 Butterfly needle, is rapidly sucked inside the bottle under vacuum via Segment A. Then a precise volume of gas is delivered via segment B. With gentle mixing to avoid foaming, ozonation of blood is completed in 5-10 min and the ozonated blood is reinfused, via suitable tubing with blood filter, into the donor in about 15 minutes. This simple procedure has already yielded therapeutic results in vascular diseases superior to those achieved by conventional medicine.

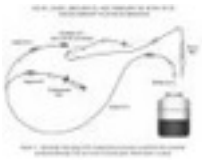

Figure 1. Schematic drawing of the components necessary to perform the ozonated autohemotherapy with an ozone-resistant glass bottle under vacuum.

Moreover, the therapeutic modalities, until now restricted to major AHT and to the empirical and imprecise rectal insufflation of gas, have been extended: they include the quasi total body exposure to O_2-O_3 (Bocci et al 1999) and the extracorporeal blood circulation against a similar gas mixture. The latter procedure is rather invasive because blood collected from a vein circulates through an ozone-resistant gas exchanger device and, with the help of a peristaltic pump, returns into the circulation via a controlateral vein. On the other hand, the partial cutaneous exposure to oxygen-ozone (only the neck and the head are excluded to avoid ozone inhalation) does not need any venous puncture and owing to the vast expanse of the skin, allows a generalized and beneficial effect. Clearly, today we can select the most suitable method for different pathologies, their stage and the patient's condition. A discussion on its own is needed for the minor AHT, which basically consists of withdrawing 5 mL of blood to be immediately and vigorously mixed for 1 min with an equal volume of O_2-O_3 at an extraordinarily high ozone concentration ranging between 200 and 400 µg/ml of gas per mL of blood. The strongly oxidized blood, including the foam and some free hemoglobin, is promptly injected into the gluteus muscle without the need of any anesthetic. As an unspecific immunomodulatory approach, physicians have used this treatment since 1953 and, during the last two decades, several ozone therapists have successfully treated herpetic infections. We have speculated that the partly hemolysed blood, infiltrated into the muscular tissue, will undergo coagulation due to platelet and protrombin activation. Although patients rarely report a slight swelling and pain at the injection site, a mild sterile inflammatory reaction may take place with infiltration of monocytes and neutrophils scavenging denatured proteins, lysed erythrocytes and apoptotic cells. If plasma contains some free virions (HCV, HBV, HHV, HIV and so on), these may undergo inactivation by the high ozone concentration and may act as an autovaccine. At the same time a moderate release of cytokines will modulate the physiological response, and the abundance of heme will upregulate the synthesis of both antioxidant enzymes and oxidative stress proteins, particularly of heme oxygenase I. It is wonderful that such a simple and autologous treatment can act as a powerful enhancer of several biological responses. A variant and unnecessarily complicated procedure proposed in the 1990s consists of treating a similarly small volume of citrated blood with ozone, ultraviolet light (obviously generating more ozone and ROS) and heat (42.5 °C) for 3 min. To my knowledge, without clarifying the rationale of using three psychochemical stresses, this method appears superfluous because ozone, as an oxidizer, is more that enough and the addition of other stresses makes the interpretation of the response very difficult. A first pilot study by Garber testing this technique in HIV patients was badly conceived and showed neither toxicity nor efficacy, but it has amply discredited the use of ozone. This approach has been subsequently used in patients with either vasculitis or advanced chronic heart failure. As might have been expected, two biological studies have shown the possibility of controlling a chronic oxidative stress and of activating regulatory T cells for downregulating a chronic inflammation. In conclusion we suggest coupling the major and minor AHT as above described in all patients to potentiate the biological and therapeutic effects.

On the basis of experimental data obtained during the last decade and on the average antioxidant capacity of human blood, we have determined the so-called therapeutic window, which is the range of ozone concentrations (expressed as µg/mL of gas per mL of blood) within which ozone can exert therapeutic effects without toxicity with regard to major AHT. The range is surprisingly wide: 10-15 µg/mL as a minimum and 80 µg/mL as a maximum. Above 90 µg/mL, an incipient hemolysis (4-5%) warns about toxicity. The threshold level varies between 15 and 20 µg/mL, depending upon the individual antioxidant capacity. The scheme presented in Figure 2 is meant to illustrate the breadth of action expressed by the ozonated blood throughout the whole organism. It is clear that the ozone oxidative activity is efficiently counteracted by the wealth of plasmatic and intracellular antioxidants so that an ozone concentration of 5 -10 µg/mL per mL of blood is practically neutralized: only a trace of ROS and LOPs become detectable and therefore, at this very low level of ozonation, AHT may only have a placebo effect. As we are particularly conscious of ozone toxicity, we always apply the strategy "start low, go slow" and, depending on the stage of the disease and the patient's condition, we usually scale up the concentration from 15, then 20, 30 and 40 µg/mL, and more when necessary, during the 1st,2nd , 3rd and 4th weeks, respectively. By using this strategy, after many thousands of autotransfusions, we have never recorded any acute or chronic toxicity. The venous puncture is usually well tolerate because it is performed with a G19 butterfly needle (quite suitable for withdrawing blood into the glass bottle under vacuum) that remains inserted throughout the 35-40 min treatment. However, a small percentage of women have a very poor venous access: in this case we can select one of the following three options: rectal insufflation of gas, body exposure to gas, or slow infusion into a visible vein on the hand dorsum, via G25-27 needle, of an isotonic glucose solution containing a final concentration of 0.03%-0.06% (8.8-17.6 mM) hydrogen peroxide. This last approach cannot be effective as the classical ozonated AHT but is useful. We absolutely discourage the use of ozonated saline because it contains some sodium hypochlorite and can cause phlebitis. Normally we perform two treatments weekly but if necessary, we can do it every day or even three times daily.

Figure 2. Ozonated blood, after reinfusion into the donor patient, is distributed throughout the whole organisms

4. The Problem of Ozone Toxicity. How we have explained the Ozone Toxicity for the Pulmonary System and its Atoxicity for the Blood

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 showing that prolonged inhalation of ozone damages the respiratory system and extrapulmonary organs. "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. It is not surprising that the release of noxious compounds such as substance P, NO, IL-1beta, IL-8 and TNF alpha has been demonstrated. Recent reports are particularly instructive because they have further shown that mice, exposed to 1.00 ppm ozone breathing for 8 hours for three consecutive nights, upregulate the synthesis of a new 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 lungs and, thirdly, their distribution and fate in body fluid and organs.

4.1. 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 and small amounts of proteins, lipophilic and hydrophilic antioxidant. 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 tenfold 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, 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 heterogeneous LOPs including lipoperoxyl radicals, hydroperoxides, malonyldialdeyde, isoprostanes, the ozonide radical, and alkenals, particularly 4-HNE.

As cholesterol is a component of ELF and because its double bond is readily attacked by ozone, it can give rise to biologically active oxysterols of which 3-beta-hydroxy-5-oxo-5,6- secocholestan-6-al (CSeco) has been implicated in pulmonary toxicity, Alzheimer disease and atherosclerosis.

In Table 3, 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 meter/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 of 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. 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 compounds" against oxidants is impressive (99.9.% higher than ELF),and only free GSH appears higher in ELF than in plasma. However erythocytes have a GSH content of about 2.2 mM (almost 700 fold higher than plasma) and therefore they contain a huge reserve. 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 superoxide, this is normally neutralized at an alveolar pO_2 pressure of 100 mmHg. It is useful, however, to bear in mind that rats inhaling pure oxygen (alveolar pressure at about 700 mmHg) die within 60-66 hours. Ozone is far more reactive than oxygen, and breathing air containing 10.0 ppm ozone causes death within 4 hours in rats. In order to understand the effects of a daily 8-hours 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 μg/m^3) as an 8-hours mean concentration to protect the health of workers. The evaluation of recent studies allows establishing an average environmental ozone concentration of 90±10 ppb. However, ozone concentrations in urban air can exceed 800 ppb in high pollution conditions. 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 of 21.0 -23.1 mg monthly. This is likely the minimal ozone intake because physical activity increases the volume of inhaled air and at the peak time, the ozone levels can easily augment to 200-300 ppb, reducing pulmonary functions and enhancing the risk of cardiovascular death. Moreover, the toxicity is certainly augmented by the presence of NO_2, CO, SO_2 and particles (PM10). On this basis, it appears clear how the ozone generates ROS and LOPs at the ELF level, after being only partly quenched by the scarce antioxidants, will act as cells signals able to activate nuclear factor-kappa B, NO synthase and some protein kinases, thus enhancing the synthesis and the release of TNF alpha, IL-1,IL-8, IFNgamma and TGFbeta1 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 hypoclorous acid, LOPs, isoprostanses, 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 no 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: HNE-adducts have been detected in the bronchoalveolar lavage fluid (BAF) of human subjects exposed to 0.4 ppm ozone for 1 h after exercise and the presence of F2-isoprostane has been demonstrated in the bronchoalveolar lavage fluid of hamsters exposed to 3.0 ppm (but not to 0.12 ppm) ozone for 6 h. Moreover 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. and another group measured H_2O_2, 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 ozone for only 2 h and yet a subgroup of “susceptible” subjects showed a significant increase of H_2O_2 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. These data reviewed by Bocci (2006) were 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 because when mice were exposed to air containing an ozone concentration of 1 ppm for 8 h during three consecutive nights, 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 g/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, where the medium contains a lower level of antioxidants than plasma, 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. TNFa, IL-1, IL-8, IFN and TGFß1 can easily reach their respective receptors in any organ and, in spite of a half-life of a few hours, 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: it had been reported that about 70% of [3H]HNE was excreted in urine within 2 days after its intravenous (IV) administration in rats. Another investigation, regarding the metabolism of HNE in several mammalian cells and organs, has demonstrated that HNE, at a concentration of 100 M, 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 M. 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. 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. As far as the cholesteryl ester hydroperoxide is concerned, it has been 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, aldoketoreductases, aldose reductase, aldehyde dehydrogenases, Cyp450 4A and ß-oxidation enzymes, important in the metabolism of HNE, but they and other Authors 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.

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 M, enact the following processes:

(a) *Dilution*, a simple calculation indicates that a bolus injection of a dose of 500 MHNE in 1 ml plasma once diluted in a plasma-extracellular fluid volume of 12 l of a normal human,irrespectiveof any other process, yields a concentration of as low as 0.04 M.

(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.

(c) *Excretion*, into bile and urine after hepatic detoxification and renal excretion 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. 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 M and, in spite of continuous detoxification, they can exert pathological effects as those observed in vitro studies performed with endothelial cells, leukemic cells, lens epithelial cells, Jurkat T cells and when testing CSeco in cardiomyoblasts. Interestingly, tolerance to ozone or HNE is far more easily achieved by small and repeated oxidative stresses than after a continuous and heavy oxidation.

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.

On the other hand, a normal endogenous HNE level (0.1 — 0.7 M) appears to act as a defensive agent against itself and other toxic compounds. 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 can activate a number of useful signaling pathways.

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. 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 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. The problem is linked to the abnormal ozone concentration of trophospheric 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 in 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 lung, liver (fibrosis), heart, kidneys and brain. Obviously, the knowledge of these phenomena has popularized the idea of ozone toxicity but, in the next section, it will be clarified that the generalization of this concept is incorrect.

5. Ozone can be used as a Real Drug in Medicine

When human blood is exposed to a gas mixture composed of medical oxygen and ozone (about 96 and 4 % respectively), both gas present in the phase overlying a superficial layer of about 10µ 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 the intraerythrocytic water before becoming bound to hemoglobin until it is fully oxygenated, as shown by the rapid increase of the pO_2 from about 40 up to 400 mmHg. 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 molecules and albumin thiol groups. Ozone doses, within the therapeutical range (10-80 µg/ml of gas per ml of blood), are partly neutralized by well — known sacrificial reactions: However it must be mentioned that when the oxidative action

of ozone on plasma proteins was investigated, no electrophoretic modification of lipoproteins was detected. Albumin-SH groups undergo oxidation and in fact albumin is considered the main sacrificial molecule and surely prevents lipoprotein damage. As the small amount of oxidized albumin cannot be reduced, it is rapidly removed from the circulation and does not affect the plasma level. Evidence has been provided that oxygen-ozone behaves similarly when this gas mixture comes in contact with a moist human skin and the rabbit colon-rectal mucosa: ozone dissolves immediately in the water overlaying in 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 LOPs. These are absorbed via lymphatics and venous capillaries and reach first the liver and then enter into general circulation where these have been measured so that the concept that ozone is absorbed into the circulation is absolutely wrong. 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 (antioxidant and PUFA) the newly formed hydrogen peroxide and a heterogeneous number of LOPs represent the chemical mediators of the totally extinct ozone. 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. The behavior and of hydrogen peroxide 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 water by free GSH, catalase and GSH-Px. Its half-life is less than one second 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 ug/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 can 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 that has favored the concept of ozone toxicity. Provided that the ozone dose is within a well defined, experimentally determined range (10-80 μg/ml or 0.21-1.68 microM per ml of blood), there is only a transitory decrease (no more than 25 %) of the potent antioxidant capacity of plasma, fully reconstituted within 20 min owing to the efficiency of the redox system. There is neither damage to erythrocytes: hemolysis is negligible (from 0.4 up to 1.2 %) and methemoglobin remains normal, 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 oxyhemoglobin to the right, confirming the observation of an improved delivery of oxygen in peripheral obstructive arterial disease. Extensive data have been reported in reviews and two books. It is now clear that a “physiological” ozone dose (most frequently ranges between 10 and 40 ug/ml or 0.21 and 0.84 microM per ml of blood) triggers an acute and precisely calculated oxidative stress able to activate several biological processes summarized in Figure 2.

What happens during the rapid reinfusion of the hyperoxygenated-ozonated blood into the donor? The hyperoxygenation of blood (pO_2 about 400 mmHg) is irrelevant because, during the 15 min infusion period, it mixes with about 75 L venous blood so that the final venous pO_2 relative pressure

is hardly modified. LOPs (mainly 4-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 a variance with the high and fairly constant LOPs levels generated by lungs chronically exposed to ozone, HNE can act as useful and not injurious signals 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 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. Another important biological effect is the amply demonstrated induction of adaptation to oxidative stress, a phenomenon decribed also as "ozone tolerance" or "oxidative preconditioning". This interesting process is universally present from bacteria to fungi to plants and mammals and the term "hormesis" was designed to indicate "the beneficial effect of a low level exposure to an agent that is harmful at high levels". The repetition of a small ozonated autohemotherapies in patients upregulates the synthesis of several antioxidant enzymes (SOD, GSH-Px, GSH-Rd, GSH-Tr and G6pd) and HO-1 which is one of the most protective enzymes catalyzing the release of useful compounds such as bilirubin and CO from heme. The 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, degeneration typical of the chronic oxidative stress. The so-called "major ozonated autohemotherapy" was invented in Germany and until now millions of treatments have been performed in patients all over the world without any acute or chronic toxicity. However, a few deaths have been caused by malpractice performed by quacks, who, at the height of HIV infections, either injected the gas, intravenously provoking pulmonary oxygen embolism, or injecting excessive volumes of gas in women with cellulite. These unfortunate episodes caused a justifiable outcry and greatly helped to condemn ozonetherapy. 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 it follows: a) the increase of intraerythrocytic 2,3-DPG and of NO levels increases the blood flow 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 and do not cause acute or late noxious effects.

5.1. An Updated Account of Clinical Results

The therapeutic potential of ozone scientifically using precise ozone generators, which allows continual checking of the ozone concentration in real time by a photometer calibrated using the classical iodometric method have been performed during the last decade. Some reviews and two critical books have reported the first comprehensive framework for understanding and recommending ozone therapy in some diseases. Today, ozone is considered to be a real drug and thus it is used with caution after having carefully defined its therapeutic window Thus, it is important to calibrate precisely the ozone dose used against the antioxidant capacity of the patient's blood, thereby limiting potential ozone toxicity. Clinical applications demonstrate that the classical treatment, denominated major ozonated autohemotherapy (O_3-AHT), stimulates several biochemical pathways without producing acute or chronic toxicity. The potential antioxidant capacity of blood tames the reactivity of a calculated ozone dose and readily reconstitutes the antioxidant titre. In addition, the concept that ozone is always toxic is inconsistent with the knowledge that another two potentially toxic gaseous molecules (nitrogen monoxide, NO and carbon monoxide, CO) can co-operate as crucial cell activators after short exposure to low concentrations of ROS and LOPs in particular cells and tissues. On the other hand, during chronic inflammation typical of viral and autoimmune disease, diabetes, atherosclerosis and cancer, excessive and constant release of ROS, NO and peroxynitrite are detrimental and perpetuate pathological state. Thus, it is reasonable that precise and brief (2-3 min) oxidative stress induced by "physiological" ozone concentration cannot be equated to the pathological chronic oxidative stress caused by excessive and constant release of ROS unchecked by antioxidants. Contrary to expectations, the judicious application of ozone in infectious disease, the atrophic form of age-related macular degeneration (ARMD), vasculopathies, diabetes, wound healing disorders, orthopaedics and dentistry has yielded striking results. Therefore, it would seem appropriate to consider the therapeutic potential of ozone in some diseases. The versatility of ozone is due to the generation of a number of chemical compounds, some of which have oxidant activity, while others, acting on cells with different functions, exert a number of biological responses. This explains why ozonetherapy, in combination with conventional medicine, can be applied only in specific diseases and should not be seen as a panacea for all ills. In reality, it may be specifically useful in only a few pathologies where orthodox medicine has proved inadequate. The following examples aim to clarify this concept.

5.2. Age-related Macular Degeneration

Owing to a continuous increase of the life-time, only in Western Europe, there are more than a million patients affected by the dry form of ARMD suitable for treatment with the major ozonated-HAT. These patients, unless properly treated, although they are reasonably well, are condemned to blindness within 5-10 years with a shocking social cost. Nonetheless, ophthalmologists can only prescribe antioxidants and zinc, which are only minimally effective. Since 1995, almost 750 patients with the dry form of ARMD have been treated with ozonated-AHT and three quarters have shown an improvement of one to two lines on the visual acuity chart. Usually 16-20 treatments, at an initial ozone concentration of 20 mcg/ml of gas per ml/blood, slowly upgraded to 50 mcg/ml (twice weekly), followed by two monthly session as a manteinance therapy, permit continued visual acuity. Although only partially controlled, this study emphasises that ozone therapy can improve the patient's quality of life dramatically (Bocci, 2005). In this disease there is progressive degeneration and death of the fovea centralis photoreceptors and of the pigmented retinal epithelium (PRE) as a consequence of several factors, one of which is a chronic hypoxia. Although ozonetherapy induces a pleiotropic response, the main advantage is an increased delivery of oxygen to the retina. It must be said that, contrary to other reports, performed by practitioners who exploit desperate patients, ozone therapy mixed with surgery and acupuncture is useless, even harmful, in the exudative form

of ARMD and in multigenic and progressive disorders (e.g. retinitis pigmantosa and recessive Stargardt's disease). The exudative form, characterized by an aberrant choroidal vascular growth and a vascular hyperpermeability beneath the retina and the PRE, is treated with several experimental therapies, such as photodynamic therapy with verteporfin or with periocular or intravitreal administration of angiostatic inhibitors. It is also emphasized that orthodox therapies (in the exudative form) and ozonetherapy (in the dry form) not only improve visual acuity but also the quality of life.

5.3. Vascular Disease

In comparison to pentoxyfylline and prostanoids (the gold standard of orthodox treatments), ozone therapy has proved more effective and less toxic in ischemic vascular disease. In one of our small trials, 28 patients were randomized to either receive their own ozonated blood with the method of extravascular circulation of blood against ozone, or to undergo to thirty IV infusion of prostacyclin. All patients continued conventional treatment with statins, antihypertensive and antiplatelet aggregation drugs. Ozonetherapy proved more effective than a prostacyclin analogue in terms of pain reduction and improvement in the quality of life, but no significant difference was seen in vascularisation of the lower limbs in either group, possibly due to the short duration of treatment (14 treatments in seven weeks) and to the late stage (IV) of the disease. Since 1982, several studies have confirmed the validity of ozonetherapy in this complex pathology, but it is a mistake to stop therapy too early in these patients because ozonetherapy, as with other conventional drugs, must be continued for life. An improved schedule, as yet to be fully evaluated, consists of two ozonated-AHT (225 ml blood plus 25 ml 3.8% sodium citrate solution), given weekly for at least six months, with topical therapy with ozonated olive oil, may be useful when initial dry gangrene or ulcers are present. Millions of people suffer from chronic limb, brain and heart ischemia, which represent the first cause of death worldwide. This represents an enormous socioeconomic burden, particularly in the developing world. Despite the present lack of a proof of concept study in this patient group, it is possible that ozonetherapy as an adjunct to conventional treatment may prove very useful.

5.4. Metastatic Cancer

Cancer cells are notoriously up-regulating glycolysis, even in aerobic conditions, where they thrive in hypoxia. The greater the hypoxia in the neoplastic environment, the more clinically aggressive is the cancer. It is now well known that hypoxia favors metastasis, and thus administration of anti-angiogenic proteins or anti-vascular endothelial growth factor (VEGF) antibodies should halt tumor growth. However, after massive investments in time and of money and energy, this approach has been rather disappointing. For example, survival of colon cancer patients treated with chemiotherapy and bevacizumab was prolonged for just five months. From a physiological perspective, it would seem logical to try restoring normoxia in the neoplastic environment. Preliminary study on a small number of preterminal patients has been performed, consisting of two ozonated-AHTs and two minor AHTs (via intramuscular administration) weekly for at least six months. At the very least, improvement in oxygen transport and delivery should enhance the effect of radiotherapy and chemotherapy. Furthermore, ozone therapy exerts an anti-immunosuppressive effect and reduces the symptoms of fatigue, which plague almost 90% of patients. As soon as chemoresistance becomes evident, chemotherapy should be stopped and replaced by ozonetherapy, which, in our experience, improves the quality of life due to a feeling of wellness and euphoria. If chemotherapy is continued, the patient becomes totally disabled, with a Karnofky status below 40%. At this point even ozonetherapy becomes useless.

5.5. Diabetes Mellitus

A controlled and randomised clinical trial was performed recently at the Institute of Angiology and Vascular Surgery, University of Havana, Cuba, in which 101 patients with diabetic foot were recruited. Fifty-two patients were treated 15 times in 20 days with ozone (local and rectal insufflation of the gas mixture, including about 96% oxygen and about 4% ozone, with a fixed ozone dose of 10 mg). Forty-nine patients were treated with systemic antibiotics and conventional topical treatment. The efficacy of the treatments was evaluated in both groups after 20 days of treatment but, regrettably, not later on and this is a serious drawback. Ozonetherapy improved glycemic control, prevented oxidative stress, normalized levels of organic peroxides, increased intraerytrocytic SOD, enhanced ulcer healing and significantly reduced amputation rate. The authors concluded that medical ozone treatment could be an alternative therapy in the treatment of diabetes and its complications. The Cuban study reports too good data that should be replicated in a much large controlled study in other clinics as soon as possible. If rectal administration of ozone, which is an imprecise and biochemically less-effective procedure than ozonated-AHT, produces such incredible improvements in advanced diabetes, then health authorities worldwide should evaluate the enormous potential of this therapy.

5.6. Lung disease

Lung diseases, such as chronic obstructive pulmonary disease (COPD), will soon become the fourth most common cause of death, which, with emphysema and asthma, cause significant disability. Using corticosteroids, long-acting beta2 agonists and antibiotics, orthodox medicine has certainly proved helpful, but it cannot change the course of COPD. However, in a series of elderly patients simultaneously affected by macular degeneration and either emphysema or COPD, a remarkable improvement has been observed by us combining ozone therapy (using the schedule adopted for vasculopathies) with the best conventional treatments. Ozonetherapy also appears to be effective in asthma. A trial performed in Cuba recruited as many as 113 patients underwent three cycles' treatment during one year of either 15 ozonated AHT (applied at doses of 4 mg and 8 mg) or rectal insufflation of gas. In Cuba ozonetherapy is used in all hospitals and rectal administration has proved to be both practical and quick, although some patients have refused rectal administration of gas. Used a fixed ozone concentration of 40 mcg/ml per ml of blood (8 mg dose) and after completion of the last cycle of 15 treatments, a significant reduction in IgE and HLA-DR levels was observed, together with increased blood antioxidant capacity, as determined by increased GSH and GSH peroxidase levels. They also noted a significant improvement of lung function and symptoms. On the other hand, rectal insufflation of gas (10 mg for each treatment per 20 sessions) in one group of patients was found less effective indicating that ozonated AHT was the most effective treatment. This result is somewhat in contrast with the diabetes's trial previously discussed. The comparison of ozone therapy with conventional therapies with respect to improvements in lung function will be very important but the lack of sponsors represents a major impediment.

5.7. Chronic Infectious Disease

Ozone is universally regarded as the best topical disinfectant because bacteria, viruses, fungi, and protozoa, when free in water, are more or less oxidized and inactivated. However, destruction of free pathogens in plasma by ozone, ex vivo, is hampered by soluble antioxidants such as albumin, ascorbic acid and uric acid and they are virtually unassailable when intracellular. This is a critical distinction and, hopefully, should eliminate the diffused misconception that ozone therapy can easily cure viral diseases and particularly HIV infection and AIDS. However, ozone therapy still

deserves attention because, by improving metabolism and operating as a mild cytokine inducer, it can have a beneficial influence on infectious diseases. Thus, there remains a place for the application of ozone therapy as an adjuvant in chronic viral infections (e.g. HIV herpes and hepatitis), in combination with highly active anti retroviral therapy (HAART), pegylated interferon-alpha plus either lamivudine or ribavirin and the acyclovirs. Bacterial septicaemia must be treated with the most suitable antibiotics to prevent toxaemia and multisystem organ dysfunction. However, it should be kept in mind that ozone generates in blood the same ROS produced by granulocytes and macrophages during infection, and this is one of the reasons for the efficacy of ozone therapy. Particularly important is the topical application of ozone as a mixture (about 4% ozone and 96% oxygen) as ozonated water or ozonated olive oil (where ozone is stabilized as a triozonide) for the treatment of bacterial, viral and fungal infections, burns, abscesses and chronic osteomyelitis. Topical therapy is most effective when combined with major ozonated-AHT owing to oxygenation of hypoxic tissues. Radiodermitis and wound healing have been enhanced because ozonated solutions display a cleansing effect, act as disinfectant and stimulate tissue reconstruction. In 1996, 6.5 million people in the USA suffered from diabetic ulcers, at an annual cost of about $ 21 billion. As previously discussed, it seems now possible to improve the prognosis of diabetes by combining ozonated topical therapy with the simple, inexpensive and risk free rectal insufflation of oxygen-ozone that, ideally, could be carried out by the patient at home under the supervision of a physician. Chronic ulcers and/or putrid wounds are one of the most distressing and difficult medical problems with which to deal, and are caused by ischemia, diabetes, immunosuppression and malnutrition. During the past decade the use of ozone in such cases has proved very beneficial. With the current increase in medical costs, ozone therapy deserves attention because it reduces hospital assistance and is cheap but, unfortunately and incredibly, Health Authorities of advanced countries are not interested or neglect this therapy. Another exciting finding is that ozone, when properly used with ozonated-AHT, can upregulate the intracellular synthesis of antioxidant enzymes and the most protective stress protein, haeme oxygenase-1. Thus, ozone can induce an adaptive response and is the only drug able to correct the chronic oxidative stress observed in several diseases. In comparison with the inconclusive usefulness of oral antioxidants, experimental and clinical data show that the cautious and prolonged use of ozonetherapy can arrest or delay the progression of these diseases and improve the quality of life. However, some patients respond less well to repeated and minimal oxidative stress, which may be due to an advanced stage of disease or to genetic polymorphism, which is an essential component of the NADPH oxidase complex.

5.8. Dentistry

Ozone gas has been used in dentistry for the sterilizing of cavities, root canals, periodontal pockets, herpetic lesions. Ozonated water has been shown to be a powerful antimicrobial agent against bacteria, fungi, protozoa and viruses and its use was useful in reducing the number of infections caused by oral microrganisms.Ozone seems to stop the action of the acidogenic and aciduric microorganisms responsible for the tooth decay. It is consequently alleged to be able to reverse, arrest or slow down the progression of dental caries.

5.9. Orthopaedics

The application of ozone in low back pain has proved very effective. It can be administered directly (intradiscal) or indirectly via intramuscular administration into the paravertebral muscles. Ozone exerts a multiplicity of effects, such as the activation of the anti nociceptive system, and it has anti inflammatory action due to lipid peroxidation products, with the consequent inhibition of

cyclooxygenase-2. Because ozone in orthopaedics is so important and effective, we consider important to expand this concept.

Borrelli and Bocci (2002) have evaluated the usefulness of ozonated HAT in patients with chronic fatigue syndrome and fibromyalgia obtaining marked improvement in about 60% of the patients without any adverse effect.

5.9.1 Organization of Pain Pathways and Speculative Ideas about the Ozone Ability to Quench Pain.

Sensory stimuli able to activate free nerve endings in various tissues and viscera can be elicited by:

(a) Mechanical pressure corresponding to the compression of the spinal ganglions in the case of intraforaminal and extraforaminal herniation and deformation of nerve fibres disrupting the myelin nerve sheat.

(b) Vasculomediated factors due to either ischemia with either possible trophic nerve impairment or venous stasis with oedema caused by blockage of venous reflux, particularly occurring in intraforaminal herniations.

(c) Infections or rather sterile chronic inflammation affecting neural and perineural structures.

The pathogenesis is complex and frequently linked to immune-mediated reactions with inflammatory cells infiltration and release of a number of toxic compounds such as ROS, LOPs, excessive NO and peroxynitrite formation, release of bradychinin after kallikrein activation, prostaglandins, especially PGE_2 (after phospholipase A_2 and cyclooxygenase, COX 2 activation), proinflammatory cytokines such as Tumor Necrosis α, Interleukins 1, 6, 8, 15, interferon gamma and matrix metalloproteinases (MMP) able to hydrolyse proteins of the intercellular matrix.

Nociceptive signals are conveyed to the spinal cord by unmyelinated and small myelinated sensory axons. Moreover chronic mechanical and inflammatory stimulation of the nerve root may stimulate the ganglionic and periganglionic nociceptors (mainly polymodal type C) responsible for hyperalgesia, a condition presenting allodynia (perception of a non-nociceptive stimulus as painful), characterized by a lowering of the pain threshold and an increase in the intensity of pain even following subliminal stimuli. Damaged tissues as well as local nerve endings can release a variety of noxious agents such as histamine, prostaglandins, potassium, bradykinin and substance P.

The axons of nociceptive dorsal horn neurons form the ascending spinothalamic tract of which the direct system carries sensory discriminative information about pain to thalamic level within the nucleus ventralis posterolateralis (VLP), while the phylogenetically older spinoreticulothalamic system terminates more diffusively in the brainstem reticular nuclei or, more precisely, in the nucleus centralis lateralis and nucleus parafascicularis.

Besides the major ascending pain pathways, the brain contains the potent descending circuits able to suppress nociceptive inputs. The midbrain periaqueductal gray neurons project to nucleus raphe magnus and can produce generalized analgesia without other sensory or motor responses. A descending projection from raphe magnus inhibits the nociceptive responses of dorsal horn neurons. A separate inhibitory pathway from the nucleus locus coeruleus also directly ends in the dorsal horn. Interestingly endogenous opiate peptides are intensely synthesized by neurons present in the periaqueductal gray region, the medullary raphe and the dorsal horn. Moreover a high density of

opiate receptors are found in those areas as well as in the medial thalamus and limbic forebrain and this fact explains their important role in the analgesic response.

Besides the release of endorphins, neurons present in the descending pathways modulate, or/and reduce nociception by releasing neurotransmitters such as a) serotonin, typically present in many raphe neurons that end in dorsal horn and b) norepinephrine produced by neurons present in the nucleus coeruleus of the pons.

5.9.2. Discussion on the Mechanisms Underlying the Effects of Ozone in Orthopedic Diseases.

Ozone acts in different ways depending upon the route of administration. In the case of a gas injection directly into the nucleus pulposus, we have some evidence that ozone dissolves in the intradiscal water and reacts with the complex macromolecular components such as proteoglycans and glicosoaminoglycans. The reaction entails an oxidation of these substrates (galactose, glycuronic acid, glycine, 4-hydroxyprolin) and the breakdown of intra- and intermolecular chains leading to a disintegration of the three- dimensional structure. Its collapse frees the entrapped water that, after reabsorption, allows a decrease of intradiscal pressure and possibly a disappearance of pain due to the reduced pressure on the nervous root. However, because ozone is very often released also along the injection path (i.e., intraforaminal), the final therapeutic effect is due to the combination of a vasculomediated and biochemical effects (improved oxygenation, correction of local acidosis, disappearance of venous and lymphatic stasis). It seems important to postulate that in the intraforaminal space, the presence of lipids, an excellent substrate for ozone, may favor the release of oxidized phospholipids to be included among LOPs: surprisingly, during inflammation, these compounds can inhibit inflammation as it has been shown in mice undergoing a lethal endotoxic shock. Thus, ozone appears to display paradoxical and unexpected useful effects such as inhibition of the release or inactivation of proteinases and likely enhancement of the release of immunosuppressive cytokines such as TGFß or/and IL-10. Even more surprising is the recent evidence suggesting that ozone, by inhibiting COX-2, blocks the synthesis of proinflammatory prostaglandin E_2. Another important analgesic effect may be derived by the induction of antioxidant enzymes resulting in the adaptation to chronic oxidative stress. These novel and surprising aspects have allowed formulating the concept of the ozone-paradox.

The indirect approach consisting in the injection of 10-20 ml of gas in 1-4 sites of the paravertebral muscles in patients with back-ache, has become very popular in Italy, China and Spain. We defined this procedure as a “chemical acupuncture” because both the needle and oxygen-ozone must have a role in eliciting a complex series of chemical and neurological reactions leading to the disappearance of pain in the majority (70-80% of good response) of patients with low back-ache. It has been clearly established that the ozone concentration must be neither below 18-20 mcg/ml, nor higher than 25-28 mcg/ml. If it is too low, it is hardly effective but higher than 20 mcg/ml can be, especially during the initial treatments, too painful and may even cause lipothymia and a risky vasovagal reflex. On the other hand, it has been often observed that, after 5-7 treatments, the pain threshold raises and therefore we can carefully increase the ozone concentration.

What happens to ozone injected into the paravertebral muscle? Ozone dissolves mostly into the interstitial water and reacts immediately with antioxidants and PUFA generating hydrogen peroxide and LOPs, as it has been amply described. These compounds stimulate local C-nociceptors and cause a transitory but usually tolerable pain that is an essential requirement for achieving the final therapeutic effect. It is always useful to warn patients by saying: “no pain, no gain”! Moreover another overlooked effect is caused by the mechanical factor induced by injecting oxygen that is

about 98% of the gas volume. This certainly is another stimulus causing a feeling of tension and pressure on the muscle and it is, at least in part, responsible of the final therapeutic effect. Unfortunately, in spite of several formal requests, only once the effect of oxygen alone had been systematically ascertained when, unintentionally, it was injected into the lumbar muscles of 30 patients against 66 experimental (ozone: 20 mcg/ml) Interestingly, albeit slightly less, even control patients improved. All others reported studies have NO CONTROL and this, from a scientific point of view, is a pitfall. This serious drawback has been recently emphasized by an American scientist, who has specified that the FDA will not approve the use of ozone unless a randomized and controlled study is performed. A sham injection with normal saline has been proposed but this is not correct and should be substituted by the injection of oxygen that represents the bulk of the gas mixture.

The stimulation of nociceptors is able to elicit the elevation of pain threshold and an antalgic response via the well-known descending antinociceptive system. As it occurs during a cutaneous stimulation, or acupuncture, albeit at a far lower level, the introduction of the needle, reinforced by the pressure of the gas plus the generated chemicals, induces a sort of prolonged stunning of nociceptors. It is known that an algic stimulation of the skin and muscles can reduce pain through the mechanism of diffuse noxious inhibitory control (DNIC). Recently it has been shown that even minimal acupuncture that is the superficial needling of NON-acupuncture points, has a similar benefit than real acupuncture on patients with tension-type headache. Thus, although we know that the placebo effect is important, we wish to ascertain its relevance.

In conclusion, the probable mechanisms playing a role follow:

(a) Activation of the descending antinociceptive system.

(b) Release of endorphins blocks transmission of the noxious signal to the thalamus and cortex.

(c) Hypostimulation (elevation of the activation threshold) linked to the oxidative degeneration of C-nociceptors.

(d) Simultaneous psychogenic stimulation of the central analgesic system induced by the gas injection, somehow due to a placebo effect.

(e) The localized oxygenation and analgesia are most important because they permit muscle relaxation and vasodilation, thus a reactivation of the muscle metabolism, by favoring oxidation of lactate, neutralization of acidosis, enhanced synthesis of ATP, Ca^{2+} reuptake and edema reabsorption.

Some of the ideas exposed above could be experimentally verified as the injection of oxygen alone or of solution of hydrogen peroxide (60-80 mM) in a 5% glucose solution. It would be nice to help anyone interested in this sort of investigation.

6. Conclusions and Perspectives

In spite of an obstinate obstruction of official medicine and disinterest by Health Authorities towards ozone therapy, owing to clear beneficial results, research must be continued. The compliance is excellent and the patients, as soon as the therapeutic effect declines, ask for a new cycle. This is an excellent proof that, provided we are using judicious ozone concentrations, there is

neither acute nor chronic toxicity. The dogma of ozone toxicity is wrong and has been also based firstly on unphysiological studies performed in washed erythrocytes or diluted blood, hence unprotected by the plasma antioxidants and secondly, in unrecognizing the profound difference between the endogenous chronic oxidative stress, occurring every day during a lifetime or during a chronic disease and the calculated, extremely brief and exogenous oxidative stress that we induce on blood by using a precise ozone dose.

We know that any drug, depending upon its dosage, can be either therapeutic or toxic and these properties often overlap in the case of chemotherapeutics. The following elementary observation is even more compelling: the normal glucose concentration in the plasma ranges between 0.7 and 1 mg/ml and it is essential for survival. However, when this concentration falls below 0.4 mg/ml, the consequent hypoglycaemic coma can be deadly; on the other hand, if the glucose concentration remains constantly above 1.3 mg/ml induces the metabolic syndrome, as it is well exemplified by the current diabetic epidemic. Thus the dogma about ozone toxicity is absolutely futile because, after millions of treatments, we have never observed any acute or chronic toxicity. Moreover most of the patients report a feeling of wellness. It cannot be omitted to mention that as many as 55,000 Americans (how many Europeans?) may have died as a result of taking the now infamous VIOXX, a member of the COX-2 inhibitors! In contrast, the number of reported deaths in Italy caused by ozone are 6 in two decades (still too many for treating cellulite!) and have been caused not by ozone, but they were due to oxygen embolism owing to quacks malpractice. It is therefore deplorable that official Medicine and Health Authorities are so heavily biased against ozone therapy. Ozonetherapy is slowly capturing increasing attention in Europe and Asia, since our studies have clarified the main biochemical mechanisms of action and the real possibility of taming ozone toxicity. We really have now the first comprehensive framework for understanding and recommending ozone therapy in a few diseases as a first choice and in combination with orthodox therapy in many others. Indeed, one important characteristic of ozonetherapy is that, in comparison to other complementary approaches, it can be experimentally verified both at the biochemical and clinical levels.

The most exciting aspect of ozonetherapy relies on inducing an adaptation and possibly a re-equilibration of a chronic oxidative stress; moreover, different mechanisms of action elicited by different cell types are obviously important in different settings but it is also quite possible that they act concurrently. This line of thought can explain why a simple gaseous molecule like ozone (that probably it is even be produced naturally in vivo!) can have superior therapeutic effects than ordinary drugs.

Regarding the orthopedic problem, we have seen that pain has a multifactorial origin and that ozone can surprisingly displays a number of beneficial effects ranging from the inhibition of inflammation, correction of ischemia and venous stasis and finally inducing a reflex therapy effect by stimulating anti-nociceptor analgesic mechanisms. The intradiscal and intramuscular injection of oxygen-ozone is a successful approach comparable to other mini-invasive procedures but the elucidation of the mechanisms of action remains to be completed. Is really ozone the crucial factor or, like other procedures involving thermocoagulation, is the external stimulus, which, by acting as a shocking event, is able to stimulate the natural healing capabilities? It seems probable that, unless we perform appropriate control studies, we will not solve the problem. However it must be said that the use of ozone in orthopedics has witness a far swifter success than the practice of ozonated autohemotherapy: this discrepancy can be explained by the rapid disappearance of pain achievable

in most cases after a single intradiscal injection of ozone! The patient's satisfaction generates a lot of publicity.

Moreover the potential and real drawbacks when many autohemotherapy treatments are needed, it must be mentioned. Although the cost of ozone is very low, it represents an unpractical drug because it is unstable and cannot be stored in any form. It must be prepared from medical oxygen and used at once only by a knowledgeable physician. Domiciliary treatment, useful for old and chronic disease' patients, can only be done if the patient, under the ozonetherapist supervision, performs the rectal insufflation of gas, but this happens rarely. This administration route is the least precise but it is still useful.

In contrast, topical therapy of chronic ulcers and infectious wounds with ozonated oil is very practical and easy because we have standard and stable preparations. The last, but certainly not the least, problem is the lack of financial support for performing controlled and randomized clinical trials, whose results are critical and urgently needed to prove the validity and atoxicity of ozone therapy in various diseases. Objective results from clinical studies represent the unique possibility of convincing the biased opponents of this approach.

Although our actual understanding of ozonetherapy is still incomplete, if we can continue our research, we may find interesting and useful surprises.

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Glossary

Antioxidant	**:** Is a molecule capable of slowing or preventing the oxidation of other molecules.
Blood	**:** This is a specialized biological fluid consisting of red blood cells (also called RBCs or erythrocytes), white blood cells(also called leukocytes) and platelets (also called thrombocytes) suspended in a complex fluid medium known as blood plasma.
Earth's atmosphere	**:** This is a layer of gases surrounding the planet Earth and retained by the Earth's gravity. It contains roughly (by molar content/volume) 78% nitrogen, 20.95% oxygen, 0.93% argon, 0.038% carbon dioxide, trace amounts of other gases, and a variable amount (average around 1%) of water vapor. This mixture of gases is commonly known as air.

Oxidation	**:** In chemistry, oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent.
Oxidative stress	**:** This is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. In chemical terms, oxidative stress is a large increase (becoming less negative) in the cellular reduction potential, or a large decrease in the reducing capacity of the cellular redox couples, such as glutathione.
Oxygen (O	**:** This is a chemical element with the chemical symbol O. It is a gas at standard conditions, consisting of 2-atom molecules. Elemental oxygen is most commonly encountered in this form, as 21% of Earth's atmosphere.
Ozone	**:** This is a triatomic molecule, consisting of three oxygen atoms.
Lipid peroxidation	**:** This refers to the oxidative degradation of lipids. It is the process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage.
Radicals (often referred to as free radicals)	**:** These are atomic or molecular species with unpaired electrons on an otherwise open shell configuration. These unpaired electrons are usually highly reactive, so radicals are likely to take part in chemical reactions.

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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.

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