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OZONE: A MULTIFACETED MOLECULE WITH UNEXPECTED THERAPEUTIC ACTIVITY.

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ABSTRACT

A comprehensive outline for understanding and recommending the therapeutic use of ozone in combination with established therapy in diseases characterized by a chronic oxidative stress is currently available. The view of the absolute ozone toxicity is wrong, because it has been based either on lung or on studies performed in artificial environments that do not correspond to the real antioxidant capacity of body compartments. In fact, ozone exerts either a potent toxic activity or it can stimulate biological responses of vital importance, analogously to gases with prospective therapeutic value such as NO, CO, H_2S, H_2, as well as O_2 itself. Such a crucial difference has increasingly become evident during the last decade. The purpose of this review is to explain the aspects still poorly understood, highlighting the divergent activity of ozone on the various biological districts. It will be clarified that such a dual effect does not depend only upon the final gas concentration, but also on the particular biological system where ozone acts. The real significance of ozone as adjuvant therapeutic treatment concerns severe chronic pathologies among which cardiovascular diseases, chronic obstructive pulmonary diseases, multiple sclerosis, and the dry form of age-related macular degeneration. It is time for a full insertion of ozone therapy within pharmaceutical sciences, responding to all the requirements of Quality, efficacy and safety, rather than as either an alternative or an esoteric approach.

INTRODUCTION

During the last decades, gases such as NO, H_2S, CO, H_2 and O_3 have shown to have different activities depending upon their concentrations and the biological substrates where they can solubilise and act. Ozone is even more typical because in the stratosphere there is an ozone layer reaching a maximal concentration of 10 ppmv, equivalent to 10 µg/ml. The production of ozone (Chapman mechanism) can be schematized as follows:

$$O_2 + \text{UV light} \rightarrow O + O \qquad \lambda < 242\text{nm}$$
$$O + O_2 + M \rightarrow O_3 + M$$

where any other molecule, M, present in the atmosphere performs the function of removing the excess energy. The lifespan of the ozone molecule is temperature dependent and at -50 °C it is halved only after about three months [1]. However, during the last hundred years the equilibrium between the synthesis and breakdown has been accelerated by progressively increasing pollutants such as chlorine derivates from chlorofluorocarbons (CFC), prevalently used as refrigerant fluids [2], and nitrogen oxide [3]. The excessive ozone destruction causes the thinning of the protective ozone layer. On the other hand, near ground level in the Earth's atmosphere, ozone is a harmful air pollutant joining the photochemical smog and being noxious for the lungs, eyes, nose and, to a lesser extent, skin [4]. There is concerns about this problem which has led to the wrong conclusion about the invariable ozone toxicity. It will be shown that the valid concept for at least some gaseous molecules is not only the right amount and the corresponding concentration that differentiates a toxic from a therapeutic agent, but also its concentration and the antioxidant activity as well as the hormetic-type response of the interacting biological system.

The toxicity of ozone

1) Human's odour perception threshold for ozone is about 0.01 ppmv (0.01 µg/ml), while the maximum work site concentration (WSC) over a breathing period of one hour is about ten times

higher. The World Health Organization (WHO) permits to work for 8 hours at 0.06 ppmv, when the ozone concentration causes a typical pungent smell. After breathing air contaminated by ozone, the gravity of symptoms and pathological changes are in relation to both ozone concentration and exposure time, which may vary from minutes to several months in heavily polluted areas [5,6]. As expected, there is an acute and a chronic toxicity. The effects of acute toxicity are reported in Table 1 and they are worse if the subject has breathed ozonated air contaminated with particulate matters, NO_2, acidic compounds and CO [5].

(insert Table 1)

On the other hand, the chronic toxicity due to ozone interaction with other compounds is a very serious problem because it involves the well-being of millions of humans either living in big metropolis or in industrial contaminated area. Ozone and other pollutants damage lung function, leading to severe ailments. There are many authoritative studies showing that chronic inhalation of ozone damages the respiratory system and extrapulmonary organs [7-12]. It is known that the release of noxious compounds such as substance P, NO [13], IL-1β, IL-8 and TNFα has been reported. Both Gohil et al. [14] and Last et al. [15] showed that mice, exposed to 1.00 ppmv ozone breathing for 8 h for three consecutive nights, gave rise to a systemic cachexic response. Such a result can be concurrently related to an up-regulation of the synthesis of specific pulmonary proteins – among which some pro-inflammatory cytokines - and to a down-regulation of a number of hepatic enzymes [16]. At the air-epithelium interface, the alveolar cells are constantly overlaid by a very thin (about 0.1 μm) film composed of water, salts and many biomolecules such as surfactant phospholipids together with small amounts of proteins, lipophilic and hydrophilic antioxidants. Generally, any inspired gas, firstly dissolves into the aqueous layer, then reaches the alveolar microcirculation and the erythrocytes, according to its relative concentration and pressure. In the course of evolution, aerobic organisms have developed a sophisticated antioxidant system against oxygen at the air–tissue barrier. Although

about 2% of the inhaled oxygen generates $^{-}O_2\cdot$, this is normally neutralized at an alveolar pO_2 pressure of 100 mmHg. In the case of ozone in contact with biological fluids although its solubility is higher than that of oxygen, it does not follow Henry's law. In fact, it immediately reacts with the biomolecules present in the epithelial lining fluid (ELF) and it is not transferred into the alveolar capillaries. As a result, ozone does not penetrate the cells but it oxidizes available substances [17,18]. On the other hand, its instantaneous reactions with polyunsaturated fatty acids (PUFA) present at the air–ELF interface form reactive oxygen species (ROS), such as hydrogen peroxide, ozonide radical ion ($O_3^{\cdot -}$), singlet oxygen ($^1O_2$) as well as a mixture of heterogenous lipid oxidative products (LOPs) including lipoperoxyl radicals, hydroperoxides, malonyldialdehyde, isoprostanes, and alkenals, mainly 4-hydroxy-2,3-trans-nonenal (HNE) [19-23]. Moreover, also cholesterol as a component of the ELF is readily attacked by ozone at level of double bond, generating biologically active oxysterols [24] among which 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (atheronal A) has been implicated in pulmonary toxicity, other than Alzheimer's disease and atherosclerosis [25]. So, ozone is far more reactive than oxygen, and breathing air containing 10.0 ppm ozone causes death within 4 h in rats [26]. On the other hand, the average environmental ozone levels vary considerably during the day for many reasons. The European air quality standards allows establishing an average environmental ozone concentration of 120 ppbv [27]. At peak time, the ozone levels can easily augment to 200-300 ppbv, reducing pulmonary functions and enhancing the risk of cardiovascular death [9]. The toxicity is augmented by the simultaneous presence of NO_2, CO, SO_2 and particles (PM10). It appears clear how the ozone-generated ROS and LOPs will be only partly quenched by the minimal antioxidants at the ELF level and they will act as cell signals able to activate nuclear factor-kappa B (NF-κB), NO synthase and some protein kinases. In this manner the synthesis and release of TNFα, IL-1, IL-8, IFNγ and TGFβ1 and the possible formation of nitrating species will be enhanced. The increasing inflow into the alveolar space of neutrophils and activated

macrophages gives rise to a vicious circle, achieving the production of an excess, among others, of hypochlorous acid, isoprostanes, tachykinins, cytokines and proteases all of which will self-maintain the inflammation after ozone exposure [28]. However, there is now ample consensus on the value of HNE as a cell messenger relevant to the induction of antioxidant enzymes in health and disease [29-30]. Specifically, mammals have developed a powerful enzymatic detoxification system (glutathione S-transferases, aldoketoreductases, aldose reductase, aldehyde dehydrogenases Cyp450 4A and β-oxidation enzymes) able to metabolize HNE and to minimize its toxicity. Moreover, HNE stress-preconditioned cells develop an efficient adaptive response by upregulating the synthesis of a variety of antioxidant enzymes together with γ-glutamate cysteine ligase, γ-glutamyltransferase, γ-glutamyltranspeptidase, HSP-70, and heme oxygenase-1 [22,31,32]. Summarily, for both controlling HNE toxicity due to oxidative stress and maintaining it at physiological plasma level of 0.1–0.7 μM mammalian organisms enact the processes of [33-37]: i) dilution; ii) detoxification; iii) excretion; iv) cell internalization. The latter is a crucial point because the resulting biological effects can be either negative or positive. In fact, chronic inflammation of lungs will maintain in the circulation a steady and high level of LOPs and pro-inflammatory cytokines for hours or days, leading to both cell degeneration and cachectic state [15]. Moreover, in spite of continuous detoxification, ozone can exert pathological effects as those observed in vitro via ozone in endothelial cells [38], Jurkat T cells [39] and cardiomyoblasts [40]. Interestingly, tolerance to ozone or HNE is far more easily achieved by small and repeated oxidative stresses than after a continuous and heavy oxidation. Under conditions of several months exposure to ozone, HNE increases its plasma levels up to 5–20 μM, analogously to a prolonged oxidative stress due to a chronic disease like atherosclerosis, diabetes, inflammation [41].

The behavior of HNE is an instructive example of how physiologic plasma levels of a toxic aldehyde produced by the usual peroxidation processes activate a number of efficient signaling

pathways [42,43]. In the second part of this paper it will be emphasized the role of HNE as a cell messenger relevant to the induction of antioxidant enzymes.

Finally, it must be mentioned that ozone skin exposition for hours can contribute to the overall toxicity of ozone. Both depletion of the skin antioxidants and induction of a remarkable oxidative stress have been shown in hairless mice after ozone exposure [44-49]. This is the reason why humans living in hot countries and during the summer season frequently become susceptible to ozone. In conclusion, ozone is not the only cause for adverse health effects, but it widely aggravates respiratory illnesses and enhances mortality in about 40% of the US inhabitants [9,10,50]. The overall ozone toxicity due to its direct continuous irritation effects on respiratory tract and skin, associated with a minimal efficiency of their detoxifying system gradually overwhelmed by the recurrent stress, sustain pathological effects such as inflammation and cell degeneration [51-54].

For Review Only

The therapeutic activity stimulated by ozone

An eminent American chemist has anticipated the view that “ozone is toxic any way you deal with it", supporting the thought that ozone should never be used in medicine [55]. Moreover, in the recent past, a number of additional negative factors such as the empiric use of ozone by quacks, lack of procedure standardization, ozone generators without adequate control instruments, paucity of scientific data have created good reasons for refusing ozone as therapeutic agent and these circumstances have generated an obstacle against ozone therapy in the US. Similarly, scepticism and antagonism exist also in the European medical establishment, mainly United Kingdom and France, so that highly qualified journals assign a low priority to papers dealing with this topic, perpetuating the trend to isolate it *in limbo*. This happens in contrast with other fields of ozone application like, for example, in water purification where ozone is considered one of the best drinking water agents capable of preventing the occurrence of infections [56]. However, only during the last years, a great effort to scientifically study the effect of low level of oxidative stress both at basic and clinical levels has been made [57-59]. Specifically for ozone, a groundwork description is available in books [60,61] and reviews [62-65]. Thus, the absolute ozone toxicity has been now shown as wrong because it does not take into account the potent antioxidant capacity of blood and interstitial fluids against gaseous ozone and the fact that the calculated ozone dose must be perfectly calibrated in such a way to only slightly and transitorily reduce the plasma antioxidant status [66,67]. In fact, when the antioxidant capacity present in the human ELF or in the blood is compared, it becomes evident how blood easily can buffer the ozone reactivity. By considering the spread of the alveolar surface (1 m^2/kg body weight) in a 70 kg human, it can be calculated that the normal volume of ELF ranges between 17 and 30 ml [68], whereas about 2.7 L of plasmatic environment are present in 5 L of blood. Moreover, in blood an enormous antioxidant capacity due to hydro-lipophilic antioxidants and enzymes able to neutralize any oxidant species is present. In detail,

erythrocytes, amounting to about 2.3 kg, can continuously supply NADPH-reducing equivalents via glucose-6-phosphate dehydrogenase activity in the pentose cycle. Moreover, they contain about 2.2 mM of GSH, representing therefore a huge reserve for this antioxidant [69,70]. As for plasmatic albumin, whose content is 99.9% higher than in ELF, it acts as a "sacrificial protein" against oxidants. To summarize, during the last four decades, ozone therapy has been used by practitioners in Europe in an empirical fashion, on the basis of pioneering Wolff's suggestions [71]. Figure 1 shows the updated mechanism of action of ozone therapy.

(insert Figure 1)

Unfortunately, even today, some ozone therapists have either a misconception or know only some technical tips for performing ozone therapy. Such a problem, associated with the paucity of controlled clinical studies, has delayed a real progress of ozone therapy. On the other hand, today the aspects of: i) how ozone is produced and used for medical purpose ii) how ozone acts; iii) how its toxicity can be controlled; and iv) how therapeutic effects can be exerted, have been fully clarified [72-76]. Overall, in Table 2 are summarized the main chronic diseases related to oxidative stress over which ozone therapy could be beneficial [77-80].

(insert Table 2)

Technical aspects for ozone generation

Generally, ozone cannot be stored and transported like other industrial gases, unless in the pioneering form of a clathrate hydrate [81]. In fact, ozone in the gaseous state reacts with itself and rapidly decomposes to oxygen [82]. The instantaneous ozone formation can be achieved by different methods, represented by corona discharge, cold plasma and UV-irradiation [83,84]. The technical aspects involved in design work and the overall quality requirements lead to the generation of ozone for therapeutic purpose starting from pure medical oxygen passing through a high voltage gradient (5-13 Megavolts) according to the reaction:

$$\tfrac{3}{2}O_2 + 163kJ \rightleftarrows O_3$$

Consequently, a gas mixture comprising no less than 95% oxygen and no more than 5% ozone is collected. Air must be excluded because of the formation of nitrous oxide and dinitrogen pentoxide from air-fed ozone generators [85]. Moreover, because medical ozone should never be inhaled, the clinics must be equipped with appropriate detectors, ozone destructors and emergency air depurators. Obviously, to be effective, ozone must be prescribed in sufficient concentration, for an adequate time and must be adequately delivered. Such a Quality framework will provide input in the development of the correct integration among technological aspects and clinical uses for optimizing patient's safety pertaining to the pharmacy and medical background, respectively.

Routes of ozone administration

Ozone can be administered with great flexibility by different routes of administration, as summarized in Table 3.

(insert Table 3)

However, the procedure that fully meets the requirements of security, standardization, reproducibility and completeness therapeutic is the so-called major autohemotherapy (M-AHT). In detail, a predetermined volume of blood (from 100mL up to 225mL) in an ozone-resistant container in the presence of either sodium citrate 3.8% (1 + 9mL blood) or heparin (20 IU/mL of blood) is exposed to an equal volume of oxygen/ozone gaseous mixture with the ozone concentration precisely determined and it is gently shaken. While sodium citrate solution (3.8%), in the right proportion (blood:citrate volume 9:1 ratio) is very safe, heparin may be used only after checking the patient's coagulation parameters, the type of disease and the ozone concentration. Thus, when heparin is used, the ozone concentration must be below 40 µg/mL per mL of blood.

Moreover, although ozone is one of the most potent disinfectants, a general misconception is that

ozone will effectively destroy pathogens in blood as it does in water [86]. In reality, ozone acts as a pro-drug and disappears within a few minutes and it is no longer present in the infused ozonated blood to inactivate bacteria, viruses and fungi *in vivo* because the pathogens are paradoxically well protected, particularly inside the cells, by the powerful antioxidant system.

The following sections aim to give the reader the critical information to operate as major methodological quality evaluation in the administration modality of therapeutic ozone.

ROS and LOPs as mediators of O_3 biological effects in blood

The water of the plasma is the critical phase where ozone promptly dissolves and reacts: hydrophilic and lipophilic antioxidants quench a small amount of the ozone dose but, if its concentration is correct, they allow formation of suitable amounts of ROS and LOPs while ozone disappears. The ozonation process is therefore characterized by these two classes of well-known chemical compounds acting in two phases. Such a process occurs mainly *ex vivo* (typically in the autologous patient's blood collected in a sterile glass bottle). In such a way, ROS immediately operate on blood cell as early and short-acting messengers, while LOPs, via the circulation, distribute all over the tissues and enter into the cells becoming late and long-lasting messengers. The transitory and small ozone-dose dependent decrease of the antioxidant capacity, ranging from 5 to 25%, return to normal values within 15-20 min, owing to the efficient recycling of oxidized compounds such as dehydroascorbate to ascorbic acid [69,70]. Among ROS, H_2O_2 rapidly diffuses into the cells and its appearance in the cytoplasm represents the activation process: different biochemical pathways can be activated in erythrocytes, leukocytes and platelets, resulting in several biological effects, among which the localized release of NO, CO and growth factors represents a contribution that must be kept in mind [64]. Moreover, between the plasmatic and the cytoplasm compartments there is a gradient and the intracellular H_2O_2 concentration is only about 1/10 of the plasmatic one [87,88] and its rapid reduction to water is

operated by the high concentration of reduced glutathione (GSH), catalase, peroxiredoxin and GSH-Px.

On the other hand, during the reinfusion of the ozonated blood into the donor, the vast expanse of the endothelial cells will be activated by LOPs – mainly HNE - resulting in an increased production of NO and S-nitrosothiols [89]. While NO has a half-life of less than one second, S-NO thiols can exert vasodilation also at distant ischemic vascular sites with relevant therapeutic effect. Therefore LOPs act as long-distance messengers transmitting to all organs the information of an acute, mild, well-balanced and well-tolerated oxidative stress. The improved hormonal or neurotransmitters activity [90] may stimulates both the endocrine and central nervous systems, partly justifying the feeling of euphoria and wellness reported by most of the patients during prolonged ozone therapy. The concept that ozone, acting as a mild stressor, can induce an antioxidant response capable of reversing a chronic oxidative stress is now well-established and there is consensus that this phenomenon is common also in the vegetal kingdom [91-93].

The behaviour and fate of the ozone messengers (after coming in contact with body fluids).

As previously shown, the lipid peroxidation by ozone leads to the concurrent formation of ROS and of LOPs. Radicals and aldehydes are intrinsically toxic and must be generated in very low concentrations. It is to be emphasized that, after an instantaneous reaction, the minimal amounts of ozone in the gaseous state do not exist any longer because it is fully exhausted by reacting with polyunsaturated fatty acids (PUFA), antioxidants such as ascorbic and uric acids, thiol compounds with -SH groups such as cysteine, reduced glutathione (GSH) and albumin (specifically at Cys34). Other proteins such as transferrin, ferritin and ceruloplasmin are important chelators of transitions metals and prevent formation of hydroxyl radicals, via either the Fenton or Haber-Weiss reactions. Therefore, the idea that ozone penetrates through both the skin and mucosae or that it enters as is into the cells is wrong. On the other hand, if the ozone

dose is wrong and too high, carbohydrates, enzymes, DNA and RNA can also be oxidized and broken down [66,94]. An excess of ROS can lead to the formation of other toxic compounds such as peroxynitrite (O=NOO), $^{\cdot}$OH (hydroxyl radical), hypochlorite anion (ClO^{-}) [95].

LOPs production follows peroxidation of plasmatic PUFA: they are heterogenous and can be classified as lipoperoxides ($LOO^{\cdot}$), alkoxyl radicals ($LO^{\cdot}$), lipohydroperoxide (LOOH), isoprostanes and alkenals, among which 4-hydroxy-2,3-hexenal (HHE) and malonyldialdehyde (MDA), in addition to HNE [96]. They are *in vitro* slightly more stable than ROS but, upon blood reinfusion, they undergo a marked dilution in body fluids, excretion (via urine and bile), and metabolism by GSH-transferase and aldehyde dehydrogenases [41] except than submicromolar concentrations as adducts bound to –SH groups that can reach all organs, particularly bone marrow, liver, lungs, kidneys, CNS, endocrine glands, where they act as signalling molecules of an ongoing acute oxidative stress. They are the most important messengers because, by entering a multitude of body cells, they bind to two critical cysteine (Cys272 and Cys288) of Kelch-like ECH-associated protein1 (Keap1), which is normally bound to nuclear factor E2-related factor2 (Nrf2), a transcription factor present in the cytoplasm with a normal half-life of 20 min [97,98]. At rest, this complex is continuously broken down in the proteasome but when alkenals, ROS and other agents bind to Keap1, they allow the release of Nrf2, which becomes free to translocate into the nucleus. Here, it heterodimerizes with small Maf protein and binds to the antioxidant response element (ARE) on DNA, thus allowing the transcription of about 230 genes [94,95]. If the stage of the disease is not too far advanced, a great number of cells will upregulate the synthesis of antioxidant proteins such as SOD, catalase (CAT), GSH reductase (GSH-R), GSH peroxidase (GSH-Px). GSH-Tr, UDP-glucuronosyl-transferase (UGT), NADPH quinone-oxidoreductase 1 (Nqol), heme oxygenase-I, heat shock protein 70 (HSP70) and phase II enzymes [99,100]. Moreover, it stimulates GSH regeneration via gamma-glutamylcysteine-synthetase, glutathione- and thioredoxin-reductase thus enhancing

the GSH levels. Indeed an increase of GSH and of antioxidant proteins has been determined in both experimental and human clinical studies, but until now the molecular mechanism of induction remained unknown [101,102]. Moreover, another highly protective enzyme as HO-1 is also up-regulated with increase of CO and bilirubin [103]. This compound is a significant lipophilic antioxidant and traces of CO cooperate with NO in enhancing vasodilation. By upregulating ferritin synthesis, the released Fe^{3+} is chelated and it is not noxious.

Modern medicine has elaborated a number of strategies for reducing chronic oxidative stress in inflammatory diseases [104,105]. However, on their own, they are scarcely effective in comparison with the activation of Nrf2 [106-108], and the consequent regulation of ROS production by mitochondria and NADPH oxidase [109]. Some mimetic drugs, such as oltipraz and bardoxolone methyl, have been already evaluated in clinical trials but they have worrying side-effects. Sulphoraphane, taken per os every day is useful but if it can display a real therapeutic activity in patients remains to be ascertained. Besides the dosage to be determined, it remains unknown if sulphoraphane is able to reverse a chronic inflammation. Consequently, the use of the best medical drug (statin, hyperglycaemia and hypertension inhibitors) integrated by ozone therapy has the capacity to defeat the chronic oxidative stress of different chronic pathologies. An antioxidant supplementation on itself is unable to increase the intracellular antioxidant capacity because only the cell has all the means to activate the adaptive response. It is emphasised that submicromolar LOPs levels can be stimulatory, while high levels can be cytotoxic. This conclusion reinforces the concept that optimal ozone concentrations are critical for achieving a therapeutic result: too low concentrations (below 10 µg/mL of gaseous ozone per ml of blood) are practically useless (at best elicit a placebo effect), while too high ones (above 50 µg/mL of gaseous ozone per ml of blood) 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 [72]. Thus, the ozone

concentration range between 10 and 40 µg/mL of blood well represents the concept of hormesis in agreement with Calabrese's hormetic mechanisms [72,110].

On the other hand, it must be clear that a correct ozonation process ranging between 10 and 40 µg/mL either carried out in blood, or at intramuscular, intraperitoneal, intradiscal, dental or even cutaneous level represents an acute but tolerable oxidative stress [111-115]. Provided that the ozone dosage is correct, not-inhaled gaseous ozone is not deleterious. In fact, acting as an acute oxidant stimulator, it is actually capable to elicit a multitude of useful biological responses and, possibly, reverse a chronic oxidative stress due to ageing, chronic infections, type-2 diabetes, atherosclerosis and degenerative processes.

Conclusions and perspectives

In spite of an obstinate obstruction of official medicine towards ozone therapy, both research and clinical studies must continue provided that using judicious ozone concentrations, there is neither acute nor chronic toxicity. The dogma of the absolute ozone toxicity is wrong, because it has been based either on pulmonary district or on studies performed in artificial environments unprotected by the physiologic plasma antioxidants. Ozone therapy is now intensively being used also in Asia and South America since studies, extensively reported in papers and books have clarified the main biochemical mechanisms of action and the real possibility of taming ozone toxicity. Obviously, a single ozone therapy treatment has a minimal effect and the therapeutic efficacy becomes subjectively and objectively evident after 6 to 10 treatments, with a frequency of 2-3 treatments per week. In other words, at least 1.5 L of blood within a period of 30 days must be ozonated. During this period, LOPs act as repeated stressors in all organs. Thus, the first comprehensive framework for understanding and recommending ozone therapy in combination with standard therapy in diseases characterized by a chronic oxidative stress is now available. The use of ozone in orthopedics has witnessed 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. In addition, topical therapy of chronic ulcers and infectious wounds with ozonated oil has been used in the last few years and has shown a clear positive effect, supported also by recent *in vivo* animal models [116]. Therefore, ozone treatment can be performed, based on the target, also at the topical levels confirming the wide range of potentiality that this molecule has in healing. Finally, the performance of controlled and randomized clinical trials in specific cardiovascular and/or metabolic pathologies will be critical in convincing the biased opponents of ozone therapy. It is the belief of the authors that it is time for a full insertion of the therapeutic use of ozone within pharmaceutical sciences with all the requirements of Quality, efficacy and safety, rather than as either an alternative or an esoteric approach.

CONFLICT of INTERESTS

The authors declare that there are no conflict of interests.

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FIGURE LEGEND

Figure 1. Mechanism of action of ozone therapy. *Top, right part.* Several pathologies have been linked to the presence of chronic oxidative stress (high levels of ROS and RNS) that leads to the formation of several bioactive molecules (aldehydes, isoprostanes, peroxidation products in general) that act as second messengers and might contribute to damage the cells at different levels (DNA, proteins and lipids). *Top, left part*. Treatment with O3/O2 in biological fluids that are characterized by an efficient non-enzymatic system (hydrosoluble molecules: uric acid, ascorbic acid, glucose, cysteine, cysteamine, taurine, tryptophan, hystidine, methionine, glutathione, plasma proteins) is able to induce a transient and mild oxidative stress that can activate the defensive enzymatic cellular system (mainly catalase, metal-dependent superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase, haem-oxygenase-1) and therefore decreasing the chronic oxidative stress related to the same pathologies. *Bottom.* The induction of lipid peroxidation with the formation of several intermediates as a result of oxidative stress.

Only

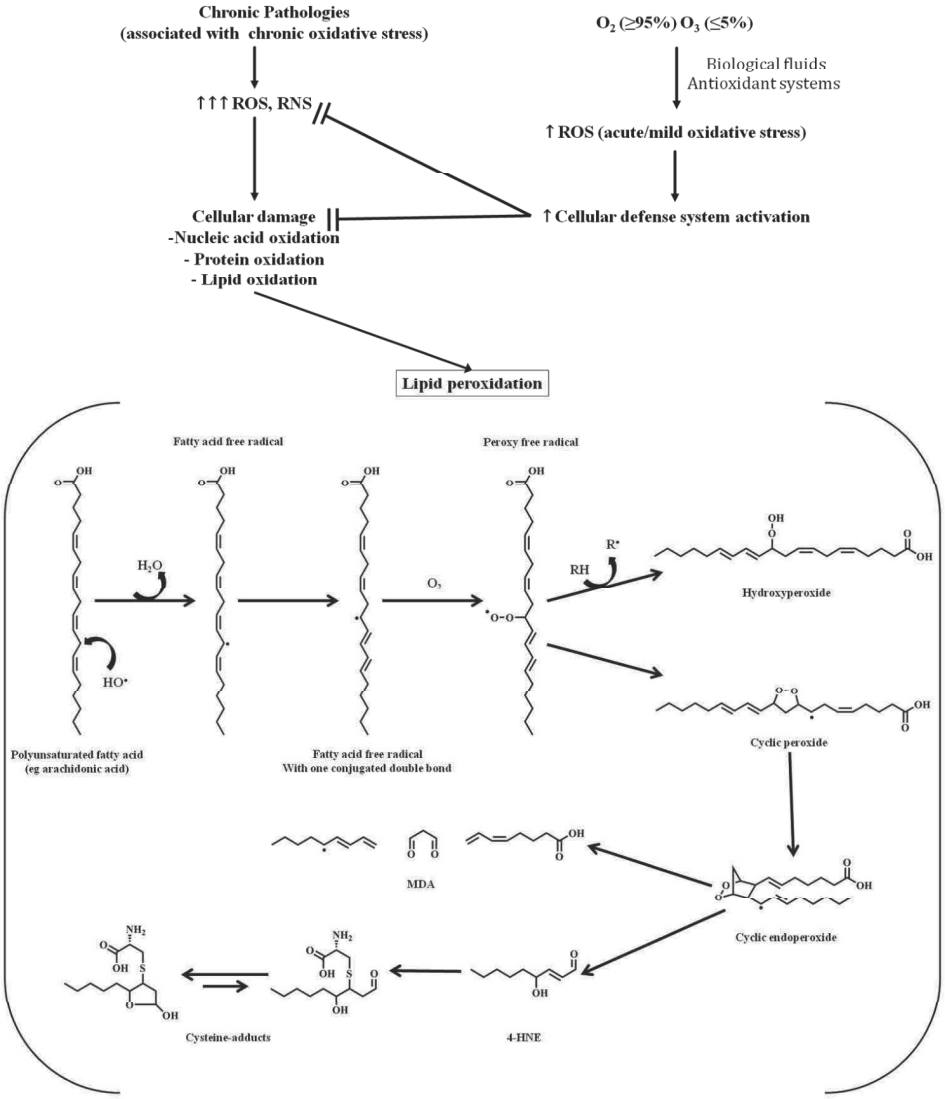

Figure 1. Mechanism of action of ozone therapy. Top, right part. Several pathologies have been linked to the presence of chronic oxidative stress (high levels of ROS and RNS) that leads to the formation of several bioactive molecules (aldehydes, isoprostanes, peroxidation products in general) that act as second messengers and might contribute to damage the cells at different levels (DNA, proteins and lipids). Top, left part. Treatment with O3/O2 in biological fluids that are characterized by an efficient non-enzymatic system (hydrosoluble molecules: uric acid, ascorbic acid, glucose, cysteine, cysteamine, taurine, tryptophan, hystidine, methionine, glutathione, plasma proteins) is able to induce a transient and mild oxidative stress that can activate the defensive enzymatic cellular system (mainly catalase, metal-dependent superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase, haem-oxygenase-1) and therefore decreasing the chronic oxidative stress related to the same pathologies. Bottom. The induction of lipid peroxidation with the formation of several intermediates as a result of oxidative stress.
469x550mm (96 x 96 DPI)

Table 1. Toxic effects of gaseous ozone in humans

O_3 concentrations in air (ppmv)	Toxic effects
0.1	Lachrymation and irritation of upper respiratory airways
1.0-2.0	Rhinitis, cough, headache, occasionally nausea and retching
	Predisposed subjects may develop asthma
2.0-5.0 (10-20 min)	Progressively increasing dyspnoea, bronchial spasm, retrosternal pain
5.0 (60 min)	Acute pulmonary oedema and occasionally respiratory paralysis
10.0	Death within 4 hours
50.0	Death within minutes

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Table 2 – Main pathologies related to oxidative stress over which ozone therapy could be beneficial

Diseases	References
Peripheral Arterial Occlusive Disease (PAOD)	[77] and references therein
Chronic heart failure (CHF)	
Stroke	
Age-related macular degeneration (AMD, Dry form)	
Chronic Obstructive Pulmonary Disease (COPD)	
Diabetes and its complications	[78-80]

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Table 3 – Routes for administering ozone as therapeutic agent

Parenteral routes	Topical or Locoregional routes
Intravenous (M-AHT)*	Nasal†
Intramuscular (m-AHT)*	Auricolar†
Rectal*	Buccal†,#
Subcutaneous*	Vaginal†
Transdermal*	Uretral and intravesical†
Intraperitoneal*	Cutaneous*,†,#
Intrapleural*	Dental†
Intraarticular*	
Intradiscal*	
Intralesional*	

Legenda: * gaseous mixture oxygen/ozone (≥95/≤5%); † gaseous ozone dissolved in aqueous solutions; # ozone derivatives of specific vegetable matrices

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