Mechanisms of Action and Chemical-Biological Interactions Between Ozone and Body Compartments: A Critical Appraisal of the Different Administration Routes

MECHANISMS OF ACTION AND CHEMICAL-BIOLOGICAL INTERACTIONS BETWEEN OZONE AND BODY COMPARTMENTS: A CRITICAL APPRAISAL OF THE DIFFERENT ADMINISTRATION ROUTES

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ABSTRACT

After a long initial stage obscured by empirism and misconceptions, oxygen-ozonetherapy has now become a scientific discipline where the reactions between ozone and human blood are within the realm of orthodox biochemistry, physiology and pharmacology. Most of the basic mechanisms of action have been clarified and ozone can be considered a pro-drug, which almost instantaneously reacts with antioxidants and unsaturated fatty acids. These reactions generate the actual ozone messengers represented by either hydrogen peroxide as a fast acting compound or a variety of lipid oxidation products as late effectors. While ozone is totally consumed, micromolar amounts of these messengers are able to enhance the delivery of oxygen via erythrocyte activation, the immune system by a bland leukocyte stimulation and most of the remaining body cells by up-regulating the antioxidant system. The hazard of ozone toxicity has been dispelled by using the gas only within a dose range perfectly calibrated against the potent blood antioxidant capability. Ozonetherapy can be very useful in patients with chronic vascular disorders and ischemic problems and should became extensively used by official medicine. An extraordinary facet of ozone is its medical application versatility, as represented by several administration routes, and the minimal cost of this drug.

KEYWORDS: Ozonetherapy, Oxidative stress, Antioxidants, Ozone tolerance, Hormesis, Vascular diseases

INTRODUCTION

Ozonetherapy is almost a century old as it was firstly used as a potent disinfectant for treating gaseous gangrene in German soldiers during Wold War I. At about the same time, Stoker [1] reported the ozone treatment of several medical cases. It was unfortunate that Christian Friedrich Schönbein, who discovered ozone in 1840, could not take advantage of ozone when he contracted and died for a *Bacillus anthracis* infection in 1868. A leap forward was made by the physicist Joachim Hänsler when, in the 70s, invented the first medical ozone generator thus allowing the possibility of using ozone in medicine. Hans Wolff [2] deserves the credit for having developed the methodology of the ozonated autohemotherapy by exposing human blood in a disposable, ozone-resistant glass bottle to a volume of gas composed by a mixture of medical oxygen (about 95%) and the extemporaneously generated ozone (about 5%). However, only at the end of the century, modern ozone generators, including a UV photometer (252.6 nm) measuring in real-time the ozone concentration, became available and permitted a real progress. In 1995 the National Institutes of Health (Bethesda MD, USA) included ozone and hydrogen peroxide therapy among the pharmacological and biological treatments as alternative and complementary therapies [3]. In spite of this, only about fourteen States of the USA permit to practice ozonetherapy while the Food and Drug Administration continues to deny permission to use ozone in medicine. The FDA is slow in adjourning its decision because ozone has been badly used in the 90s by directly injecting the gas mixture intravenously into HIV patients, thus procuring deadly oxygen embolism. However, in the patients' interest, it is time that the FDA revises its negative position.

During the last decade a number of scientific studies [4-6], reviews [7-9] and books [10,11] have allowed to clarify that ozone dissolves in the water of plasma very rapidly and switches on a number of chemical reactions, which, on one hand, lead to its exhaustion, while, on the other hand, generate new chemical compounds able to trigger a number of biochemical reactions. Thus now ozone is considered a prodrug implying that pharmacological or eventual side effects are due to its generated messengers. At variance with other complementary approaches, where the mechanisms of

action remain hypothetical or scientifically not verifiable, oxygen-ozonetherapy, after some initial difficulties due to misconceptions and empirism, has reached a stage where the reactions elicited by ozone in human blood and other biological fluids are within the realm of orthodox biochemistry, physiology and pharmacology. This is so true that ozone, in itself a strong and dangerous oxidant, can be used as a real medical drug provided that both its precise concentrations (hence the dose equal to the product of the precise ozone concentration with the gas volume) and the antioxidant capacity of the biological substrates are known. The actual understanding of the various biochemical reactions, the chemical characteristics of the generated messengers, their interaction with physiological components and their pharmacodynamic allow to recommend oxygen-ozonetherapy in vascular disorders characterized by ischemia and chronic oxidative stress where orthodox medication, although useful, still present limitations [8,11].

The main aim of this review is to critically analyze all the involved compounds, their biological activities and potential toxicity for justifying the use of ozone by different administration routes in some pathologies. It has become obvious that ozonetherapy cannot be explained by a straightforward interaction between a molecule and its receptor because of the number of messengers and of their different fate and life-time in the organism. This complexity is not a disadvantage and it seems to result in the synergism of different biological activities. Therefore ozonetherapy is now considered a modifier of the biological response, that is needed in chronic pathologies usually complicated by a chronic oxidative stress. Moreover it must be emphasized that ozonetherapy represents another example of stimulatory responses following a precise stimulus below the toxicological threshold. The concept of “hormesis” has been masterly exemplified by Calabrese [12] and, interestingly, the ability of some medical gases (NO, CO, H_2, H_2S. Xe and O_3) used at very low concentrations to ameliorate oxidative stress has been further stressed by Nakao et al., [13].

THE DOGMA OF OZONE TOXICITY IS WELL DISPROVED IN THE CASE OF OZONETHERAPY

Ozone is a very reactive gas and it has an oxidation potential of 2.07 Volts. In the stratosphere the ozone layer with an average concentration of 10 ppm (0.01 μg/ml) is very useful for avoiding an excessive UV solar radiation on living beings. Chemists, lacking an ample knowledge of the biological system, are concerned about its medical use and tend to create a diffused scepticism. Moreover, ozone toxicity for the pulmonary system during prolonged inhalation of photochemical smog containing 0.2 ppm (0.0002 μg/ml) ozone is well known. Any possible leakage of ozone must be avoided and not only children, asthmatics, smokers and elderly people but also normal adults can be adversely affected [14,15]. Chronic oxidative stress induced by ozone in the lungs causes a steady release of a huge amount of peroxidative products and proinflammatory cytokines which, by easily overwhelming the antioxidant defences present in the very thin film of surfactant (about 0.1 μm), layered over as many as 70 m^2 of alveoli, not only damage the respiratory surface but, after entering the circulation, cause chronic inflammations in several organs [16].

How then ozone can be medically useful? Luckily, after about 2.5 billion years of terrestrial life in oxygenated air, a very potent and multiform antioxidant system (Table 1) has developed in biological fluids and cells and it has become almost completely protective.

Table 1. The antioxidant nonenzymatic and enzymatic systems in biological fluids.

NONENZYMATIC
Hydrosoluble: uric acid, ascorbic acid, glucose, cysteine, cysteamine, taurine, tryptophan, hystidine, methionine, glutathione, plasma proteins
Liposoluble: vitamin E, vitamin A, carotenoids, coenzyme Q, α-lipoic acid, bilirubin, thioredoxin, bioflavonoids, melatonin, lycopene
Chelating proteins: transferrin, ferritin, caeruloplasmin, lactoferrin, haemopessin, albumin
ENZYMATIC
Superoxide Dismutases (SOD): Cu/ZnSOD, MnSOD, CuSOD $2O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + O_2$
Catalase (Cat) $2H_2O_2 \longrightarrow 2H_2O + O_2$
Glutathione Peroxidases (GSH-Px) $2GSH + H_2O_2 \longrightarrow GSSG + H_2O$ $2GSH + ROOH \longrightarrow GSSG + H_2O + ROH$
Glutathione Reductases (GSH-Rd) $GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$ $NADP^+ + \text{Glucose 6-Phosphate} \longrightarrow NADPH + \text{6-Phosphate Gluconic Acid}$
Glutathione Transferases (GSH-Tr) It detoxifies electrophilic xenobiotics, unsaturated aldehydes (e.g. 4-hydroxynonenal, HNE) and hydroperoxides formed as secondary metabolites during oxidative stress
Heme-oxygenase-I (HO-I) $\text{Heme} \longrightarrow \text{Bilirubin} + CO + Fe^{2+}$

A comparative evaluation of the antioxidant system in the human lungs and blood has been very instructive [17]: the enormous respiratory surface is covered with only about 20-40 ml of endothelial lining fluid (ELF) containing a modest amount of hydrophilic antioxidants, and less than 100 mg of albumin to neutralize the ozone insult. On the other hand, about 2.8L of plasma and a further 10-12 L of interstitial fluids contain as many as about 470 g of albumin, which is one of the most protective protein. Similar huge differences have been calculated [17] for several other

antioxidants. On this basis and using only the minimal, yet sufficient ozone dosage, it has become clear why we can safely use ozone as a medical drug and it will be further demonstrated how a precisely calculated ozone dose can be promptly tamed by the natural antioxidant system.

WAYS AND ROUTES OF OZONE ADMINISTRATION

After the preliminary clarification of the previous critical issue, the aim of this paper is to make a critical revision of the several ways currently used for ozone administration in patients, to evaluate the pros and cons of:

1) The major and minor ozonated autohemotherapy
2) The extracorporeal circulation of blood against oxygen-ozone
3) The intravenous infusion of ozonated physiological saline
4) The intravenous infusion of ozonated water
5) The quasi-total body exposure to oxygen-ozone
6) The administration of the gaseous oxygen-ozone mixture via several routes: subcutaneous, intramuscular and intradiscal.
7) The intracavitary and intralesional administration of ozone.
8) The administration of oxygen-ozone via the colon-rectal route.

1) THE MAJOR AND MINOR AUTOHEMOTHERAPY (AHT)

These techniques represent the classical, old methods of blood ozonation. Major and minor AHT are referred only to a large (100-250 ml of blood) or to a small volume (5 ml) of blood, respectively. However, also their routes of administration are different: the former is intravenous and the latter is intramuscular. Moreover, there are fundamental differences regarding the preparation, the biochemistry, pharmacokinetic and the therapeutic aim. Both these procedures represent the paradigmatic example of ozone administration and they are also the best model for understanding the chemical reactions of ozone with blood components. The gas mixture is always

composed of medical oxygen (about 95%) and ozone (about 5% or less). Oxygen is of medical grade and the gas mixture must be always sterilized by passing through an ozone-resistant filter (0.2 μm).

Is oxygen therapeutically important? Not in this case, even though oxygen must be used as the generator of ozone. As the 250 ml of the gas mixture contain no less than 95% oxygen, the pO_2 in the glass bottle containing 250 ml of anticoagulated (either with 3.8 % Na citrate or with heparin 10-20 IU/ml) blood, at a pressure of 700 mmHg allows the solubility of about 1.5 ml oxygen in the plasma, that is almost 7-fold higher than the physiological one, in the pulmonary veins. All haemoglobin is fully saturated with oxygen and exists as Hb_4O_8 only. However the relevance of the high oxygen tension is irrelevant because the infusion of the ozonated blood into the donor takes about 20 min (about 12.5 ml/min) and within this period, it mixes with as many as 100 litres of venous blood with a pO_2 of about 40 mmHg. As a consequence the extra oxygen has a negligible value.

The real drug is ozone which appears to be 10-fold more soluble than oxygen in water, in relation to the experimental conditions [18]. The ozone dissolves very rapidly in the plasmatic water and immediately reacts with both hydrosoluble antioxidants (ascorbic acid: about 50 μM, uric acid: about 400 μM, reduced glutathione - GSH: about 6 μM) and polyunsaturated fatty acids (PUFA), mainly omega-6, bound to albumin. Normal human plasma contains about 5 mg/ml lipids but phospholipids and cholesterol in lipoproteins are not readily accessible to water-dissolved ozone [19]. Moreover, of the about 70 mg/ml proteins in plasma, about 40 mg are constituted by albumin (67 KDa) that contains one free cysteine (Cys34) and 17 pairs of disulfide bridges (S-S linkages) in its three homologous domains. During ozonation, the Cys-SH group of albumin could also allow the formation of Cys-SOH group, typical of sulfenic acid [20,21]. Thus, the potent antioxidant capacity of plasma is partly responsible for taming the strong oxidant properties of ozone. During its rapid decomposition, electrons donated to ozone by ascorbic acid transform it to dehydroascorbate, when the highest ozone concentration of the therapeutic range is used. Moreover,

uric acid cooperates with ascorbic acid, but it is irreversibly oxidized to allantoin. The reaction with PUFA [22] occurs at the same time as follows:

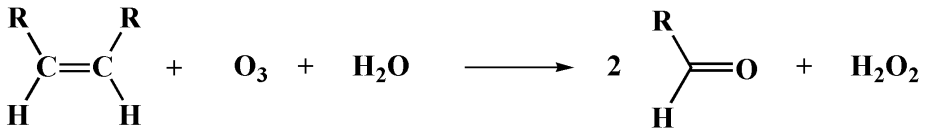

Figure 1. Simplification of the PUFA ozonation process in aqueous environment.

This reaction has a fundamental importance because it generates two types of compounds, the first of which is H_2O_2 (included among Reactive Oxygen Species, ROS) with a very brief half-life and the second represented by a variety of aldehydes, relatively more stable.

Upon the variable characteristics of PUFA, several types of aldehydes are formed although HNE represents the main species. The free Cys(34) of albumin can be either readily oxidized or it can bind HNE. Moreover eleven accessible nucleophilic residues, constituted by Lys(199) and His(146) can also readily bind up to eleven HNE molecules. The small amount of cysteine, but especially free GSH in plasma can also act as an electron donor either being oxidized to sulfonates [23], or they can form an adduct with HNE at a slower rate constant than the nucleophilic albumin residues.

It is now clear that the ozone dose, calibrated against the antioxidant capacity of blood, is partly consumed by the readily available antioxidants and partly is used for generating the ozone messengers, H_2O_2 and HNE, necessary to elicit the biochemical reactions leading to therapeutic effects. It becomes understandable that a too small ozone dose will be totally quenched by antioxidants, while an excessive dose may damage blood cells.

For some time it has been known that the reducing compounds act as “sacrificial” molecules and albumin, besides being one of the most potent antioxidant [24], acts also as endogenous detoxifying

agent of circulating reactive carbonyl species transporting them in the three hydrophobic pockets [25-27].

Physiological levels of transferrin, ceruloplasmin and metallothioneins prevent the formation of $^{\cdot}OH$. Both uric acid and ascorbic acid are valuable scavenger of $^{\cdot}OH$, $O_2^{\cdot -}$, O-NOO- and lipoperoxides [28-30]. H_2O_2 and peroxinitrite may also allow the formation of sulfenic acid in albumin [20,21] or induce its dimerization [31,32]. While allantoin is excreted, heavily oxidized albumin can be taken up by the reticulo-endothelial system without any negative effect owing to the large albumin pool and an intensive hepatic synthesis [33]. On the other hand, dehydroascorbate is reduced within a few minutes by recycling in the erythrocytes via the cooperation of thioredoxin, GSH-Rd and the continuous formation of reducing equivalents by glucose-6-phosphate dehydrogenase [9,10]. The scheme suggested by Parker (personal communication to V.B.) is very enlightening (Figure 2).

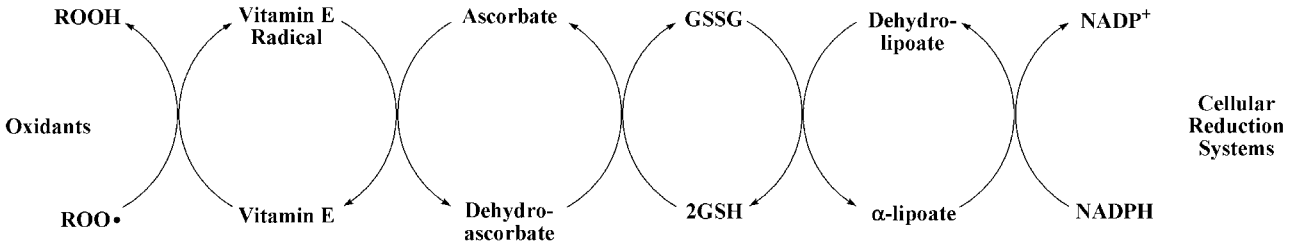

Figure 2. Pathways of the cellular reduction systems during oxidant insult.

During this rapid initial phase, ozone becomes extinct but it causes the formation of the messengers such as hydrogen peroxide and HNE as the most substantial aldehyde. The valid range of ozone dosages has been determined between 0.42 µM or 20 µg/ml and 1.68 µM and 80 µg/ml of gas per ml of blood) against the antioxidant capacity of blood (between 1.28 and 1.83 mmol/L plasma) [34]. At the usual therapeutical doses, ozone is unable to peroxidize phospholipids of erythrocytic membranes [5] and/or modify metahemoglobin levels, to increase hemolysis or cause a leakage of K^+ and lactic dehydrogenase from erythrocytes, simply because the ozone dose is totally consumed in the plasma [35,36]. The electrophoretic mobility of erythrocytes as well as their osmotic fragility

remain unmodified [36]. Moreover plasma levels of fibrinogen, cholesterol, triglycerides, HDL and LDL as well as enzymatic levels of superoxide dismutase, glucose-6-phosphate dehydrogenase, GSH-Px and GSH-Rd (U/g Hb) in erythrocytes do not vary after ozonation of blood within the therapeutic range [36]. Ozone dosages above 2.1 μM or 100 μg/ml gas per ml of blood can slowly start to affect all of these parameters. On the other hand, very extensive alterations have been observed during ozonation of saline-washed human erythrocytes suspended in either saline or distilled water and it has been unfortunate that these artifactual and unphysiological studies have been performed reaching the wrong conclusion that ozone irreversibly damages erythrocytes [37,38]. However, these studies have been useful in proving the great importance of plasma antioxidants in neutralizing the deleterious ozone effects.

The next problem was to evaluate the biochemical and toxicological relevance of the most important ozone messengers: the peroxidative decomposition of the PUFA [39], on the basis of their varied and complex composition, lead to the release of a non-radical, reactive oxygen species such as hydrogen peroxide and a variety of lipid oxidation products (LOPs) such as lipoperoxides ($LOO^{\cdot}$), alkoxyl radicals ($LO^{\cdot}$), lipohydroperoxides (LOOH), isoprostanes and a group of 4-hydroxylated-2,3-*trans*-alkenals of which the most quantitatively important is HNE. This aspect has been extensively reviewed by Barrera *et al.* [40] and Poli *et al.* [41].

As ozone dissolves into the water of plasma, hydrogen peroxide is generated within the first few minutes and, while it is partly quenched by antioxidants, it selects the PUFA as a preferred substrate. The establishment of a H_2O_2 gradient between the plasma and the cytoplasmatic water of blood cells makes this oxidant a very early effector. Its concentration depends upon the ozone concentration but at a concentration of 1.68 μmol/ml is no higher than 40 μmoles [42]. This concentration is very transitory because firstly, in the plasma, it is inactivated by antioxidants and secondly, by quickly passing through the cell membrane, undergoes dilution and further inactivation in the cytoplasmic water. Its intracellular concentration (at most 2-4 μmoles) has been assessed to be about 1/10 of the plasmatic one because it is quickly reduced to H_2O by free GSH, Cat and GSH-

Px [4,6,42-46]. Its half-life is of about 1 sec and yet its intracellular concentration has a critical importance because activates the pentose cycle in erythrocytes [9,11], a tyrosine kinase in lymphocytes [9,47], and induces the release of growth factors from platelets [48]. Although the threshold is only of a few micromoles, it is physiologically important and means that an ozone dose below 0.21 μmol/ml of gas per ml of blood, can be biologically ineffective because the ozone dose, hence the generated H_2O_2 is totally neutralized by the plasma antioxidants. In other words, the concept of a threshold helps to understand that a too low ozone dose may be ineffective (placebo effect), while a dose higher than the therapeutic one can be toxic. It is interesting that the ozonation process, characterized by the consumption of antioxidants, deeply differs whether it occurs in the plasma alone or in blood. Within the ozone therapeutic dose, when it occurs in the plasma, during the following 20 min the antioxidant capacity is reduced by 52±4 % and it does not recover, while in blood is reduced by 26±3% within one min, but it rapidly return to the initial value after 20 min. The rapid reconstitution of the antioxidant capacity was well shown to be due to the recycling process of dehydroascorbate to ascorbic acid, mainly operated by GSH and thioredoxin reductases [49,50]. Moreover the erythrocytes mass, via glucose-6-phosphate dehydrogenase activity, can continuously supply NADPH-reducing equivalents. Notably an increase of this enzyme has been determined in very young erythrocytes during ozonetherapy [9].

Given the toxicity of aldehydic LOPs, particularly regarding HNE [41], it is important to know their distribution, metabolism and fate.

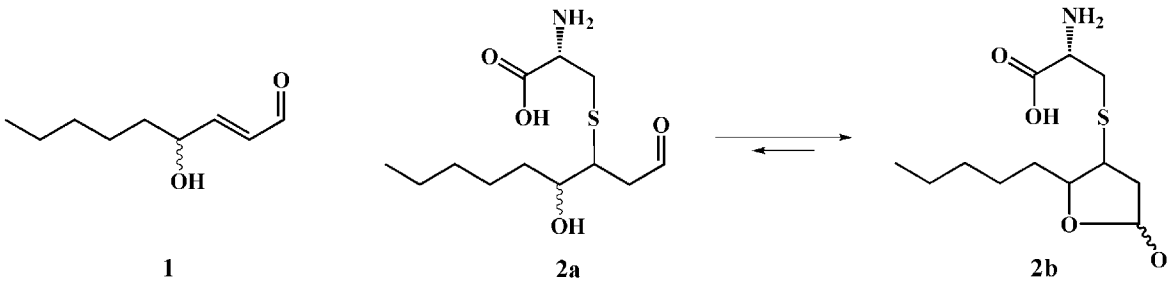

Figure 3. HNE (1) and HNE-Cys-adducts (2a,2b). The lipid aldehyde HNE is an electrophile highly reactive with nucleophils (e.g. Cys, His and Lys).

HNE is a normally detectable molecule (0,7-1,0 μM) and on its own, is very unstable and toxic. At first it was surprising to observe that LOPs' levels (generated by ozonation of human plasma samples and measured as thiobarbituric acid reactive substances, TBARS) incubated *in vitro* at +37°C and pH 7.3, hardly declined during the next 9 hours indicating their stability in a acellular medium. On the other hand, the same samples, infused intravenously in the respective macular degeneration's patients, disappeared rapidly from the circulation with a half-life of 4.2±1.7 min [51,52].

This result can be explained by the following five processes:

a) *formation of albumin-HNE adducts*

Assuming to ozonate 200 ml of blood with an ozone dose of 8 mg, the presence of about 5.4 g of albumin, particularly Cys(34) can form an adduct with HNE (see Figure 3). Moreover HNE binds easily to the albumin nucleophilic groups [53].

b) *dilution in the plasmatic and extravascular albumin pool*

During the infusion of the ozonated blood into the patient, the albumin-HNE adducts will firstly dilute within the intravascular albumin pool represented by about 130 g albumin and then with the extravascular pool containing about 340 g albumin. Thus, in a total body pool of about 470 g albumin, the ozonated aliquot is less than 1%

c) *detoxification*

This is operated very rapidly inside billions of cells by available GSH and at least three enzymes such as aldehyde dehydrogenase CYP450, aldose reductase and GSH-Tr [54,55].

d) *excretion*

HNE metabolized as mercapturic acid had been detected in urine and in bile after hepatic detoxification [56].

e) *HNE as a signaling molecule*

It is most interesting that traces of HNE or other alchenals-albumin adducts and sulfenic acid-albumin, in submicromolar concentrations, can act on a variety of organs as signaling molecules able to activate a number of biochemical pathways [53,57,58].

Processes a), b) and e) can explain that LOPs and particularly HNE, at submicromolar levels produced during calibrated blood ozonation will eventually inform cells from the bone marrow to the hypothalamus, of an acute oxidative stress. If this interpretation is correct, LOPs will have the role of late-acting messengers able to induce an upregulation of antioxidant enzymes and HO-I. Previous clinical data have shown that patients, if not already overwhelmed by the chronic oxidative stress, can express this positive response during prolonged ozonetherapy [9,59].

The major AHT has already proved to be very useful in vascular disorders complicated by ischemia because of an improved vasodilation in ischemic areas and an increased delivery of oxygenated blood. No evidence of side effects has ever been shown [7,9-11] but it remains impellent to perform a multicenter trial in thousands of patients for eliminating the persisting prejudice and convince sceptical angiologists that this approach is preferable to the intravenous infusion of prostanoids, which are expensive and often procure side effects.

By now, several million major AHT have been performed in many Countries, but one practical problem is the need to collect blood, to ozonize it *ex vivo* and to reinfuse it in the donor. Although the cost of the material is negligible and the whole procedure can be done in less than an hour, it needs to be performed at a hospital (or in a private clinic) and, when the patient is not auto-sufficient, a family's help is required. There is no legal problem for the physician to collect the patient's blood in a glass bottle provided that the blood is reinfused in the donor, who accept the procedure having signed an informed consent and with a compliance of almost 100%. The physician specialized in ozonetherapy cannot in any case infuse the blood in any other patient but this, to our knowledge, has never been done but it would infringe the law regulating blood transfusions. Collection of blood is easy and quick in most men with a G-21 or a G-19 angiocath for withdrawing either up to 100 or 250 ml blood while some women have a poor venous access and

therefore only minor AHT can be done in this case. Up to 50 autotransfusions have been performed in the same patient in six months with no problem. However the search for a valid and really satisfactory blood substitute has being going on in our and other laboratories but so far has been unsuccessful. It is not surprising that the search of a blood substitute for the safe administration of ozone has led to some problematic approaches such as the intravenous infusion of either ozonated physiological saline or ozonated water.

The minor AHT deeply differs from the major AHT because the small volume of blood, without anticoagulant, after receiving an equal volume of gas, with an ozone concentration of 80-100 μg/ml (dose 0.4-0.5 g) is rapidly mixed and injected intramuscularly at once. It is meant to cause a sterile inflammation able to elicit a mild stimulation of the immune system and to activate an upregulation of the HO-I after the local breakdown of erythrocytes with the release of heme. It is worth noting that about six decades ago, this procedure was used without ozone as a sort of aspecific immunotherapy and remains very useful for the treatment of herpes I, II and zoster infections as well as atopic dermatitis [60].

A similar example of minor AHT, where ozone had been improperly used was published in 2008 [61]. It clearly showed the failure of excessively oxidized blood with ozone (as many as 75 mg ozone per 10 mL of blood), UV radiation and heat stress for improving the clinical conditions of chronic heart failure in 1213 patients compared with controls treated only with orthodox medication. This ill-conceived procedure, denominated Celacade, was intended to procure a specific reduction of the vascular inflammation, hence of a chronic oxidative stress which progressively aggravates the heart function. Although this methodology for achieving immunosuppression has been criticized [62-64], the future of ozonetherapy has been jeopardized because incompetent scientists have conceived and used such an enormous ozone dose to completely denature blood, obviously neglecting the fundamental concept that any drug, in relation to its concentration, can be useful or very deleterious.

2) THE EXTRACORPOREAL CIRCULATION OF BLOOD AGAINST OXYGEN-OZONE (EBOO)

This approach was devised as a possibly more efficacious way to deliver ozone than major AHT. It is basically a dialysis-like system with the substantial difference that blood flows outside hollow fibres while oxygen-ozone flows inside the fibres in a counter-current direction. Dialysis filters have been firstly used [65], but, after a long search, polypropylene hollow fibres coated with phosphorylcholine have been selected because this material is ozone-resistant and biocompatible, while common dialysis filters under ozone may release toxic materials in the patient's blood [66]. The actual Gas-Exchange Device (GED) has been well characterised and is very effective [67,68]. At this stage:

a) the extracorporeal circulation of blood against oxygen-ozone has become a reality. The main characteristic is that ozonation levels must be kept at very low levels because one treatment corresponds to about twenty conventional AHT simultaneously performed.

b) technical and methodological aspects have been satisfactorily resolved but, obviously, they are more complex than the classical AHT.

c) owing to the improved oxygenator efficiency, up to 5 L of blood/hour can be exposed to very low ozone concentrations, just above the thresholds of the therapeutic window. To enhance ozone tolerance, the first and the second EBOO last only 30 and 45 min, respectively;

d) as it occurs in the pulmonary circulation, the hollow fibre efficiency allows a quantitative gas - exchange in one min. The usual blood flow running on the GED is of 80 ml/min, hence far less than in dialysis.

e) both oxygenation and ozonation remain effective without any increase of venous pressure because the phosphorylcholine coating prevents adhesion of platelets and leukocytes.

f) in arteriopathic patients (grade III and IV), both subjective and objective clinical improvements have often been noted after the first treatment [69]. Classical treatments usually do not provide such

a rapid improvement unless they are repeated during the day. In fact, this approach has been developed for the treatment of critical patients.

g) neither metabolic derangement, nor changes in blood chemistry, nor any toxic effect have been observed during the cycle or months later.

Some possible disadvantages must be taken into due considerations:

i) the cost of the device, including ancillary materials, is now near 700 euros, but it could decrease once the application will be used world-wide;

ii) the cost of a qualified technician expert in extracorporeal blood circulation;

iii) the potential deterioration of the necessary two venous accesses;

iv) the occasional need of inserting a catheter into a central vein to continue the cycle, with the related risk of infection. The last problem may be reduced by using improved catheters impregnated with antibacterial substances [70].

So, the therapeutic benefits should be ascertained in the following areas: a) critical, inoperable ischemic limbs (stage III and IV, Leriche-Fontaine) when amputation remains the only option. Medical treatments help but are rarely successful [71]. The surgical procedure of distal venous arterialization is also a complex and experimental procedure [72]; b) end-stage ischemic myocardiopathies, previously operated on with no success; c) acute cerebral ischemia, to be treated with both thrombolytic therapy and EBOO as soon as possible to re-oxygenate the ischemic (penumbra) and infarctuated areas, thus limiting neuronal death and favouring a more rapid recovery. Neurologists prefer thrombolytic approach and are afraid of testing ozonetherapy. It would be also of great interest to compare in at least a thousand patients the efficacy of the classical major AHT with EBOO in a selected pathology. Regretfully, improvement of our knowledge is slow because neither funds nor sponsors supporting this research have been so far available. Finally we have to denounce the use of common dialysis filters in the place of the appropriate GED in Asian countries. Although good clinical results have been claimed, dialysis filters are not ozone-resistant and can release toxic compounds in the patient's blood.

3) THE INTRAVENOUS INFUSION OF OZONATED PHYSIOLOGICAL SALINE

In 1994, it was demonstrated that ozonation of medical physiological saline (0.9% NaCl) with various ozone concentrations (50-70-100 µg/ml ozone) induced at the same time formation of hydrogen peroxide and chemiluminescent effects indicating the generation of free radicals [4]. The production of H_2O_2 is progressive and by using an ozone concentration of 100 µg/ml reached the value of 20 µM after 60 min ozone insufflation (Figure 4).

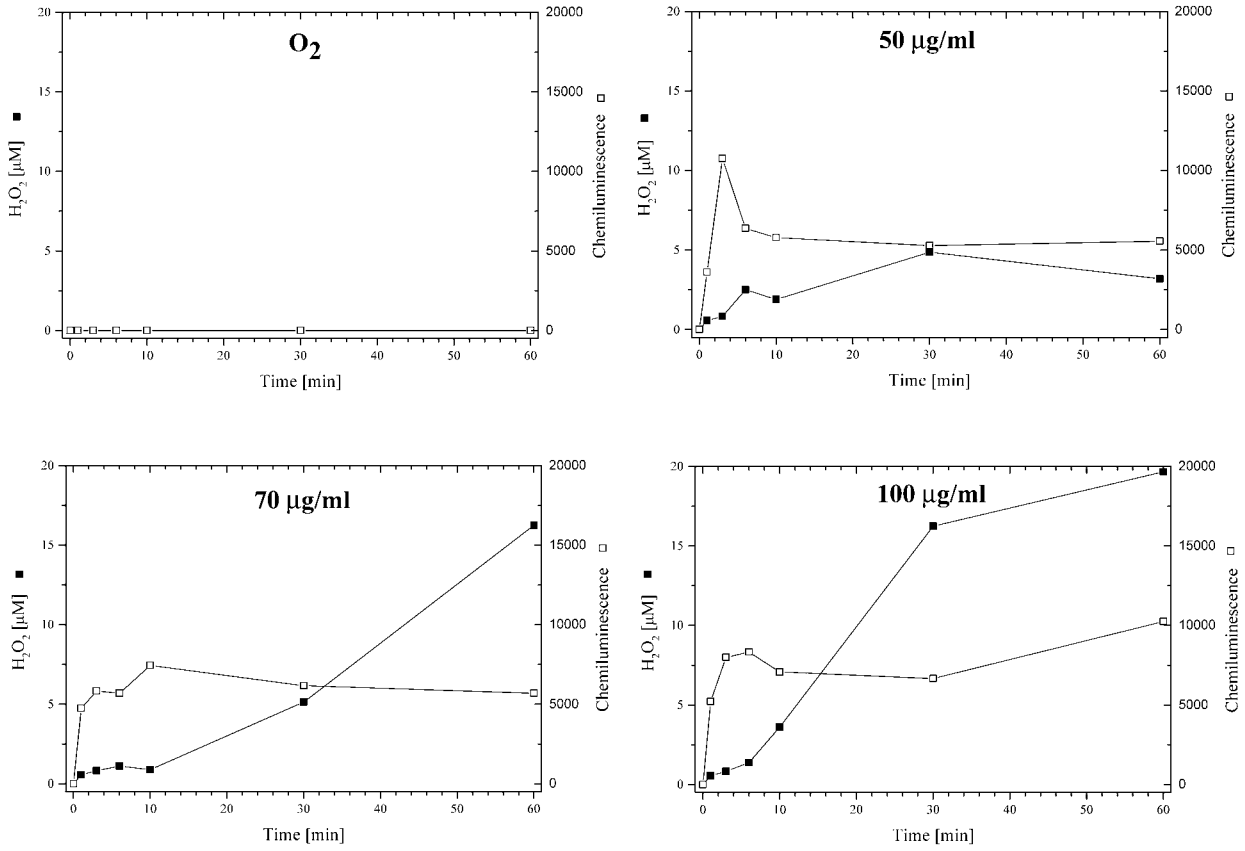

Figure 4. Kinetics of the chemiluminescent signals and of the H_2O_2 production after exposing saline to oxygen alone or to three concentration of ozone (50, 70, 100 µg/ml per ml of solvent).

Infusion of 250 ml of this solution in healthy volunteers caused considerable pain along the venous path of the infused arm after about 24 hours. This indicated that the solution has irritated the endothelium with the risk of a phlebitis and we were concerned that, besides H_2O_2, a transitory

formation of HOCl may be the noxious agent. Although chloride could be oxidized by ozone to perchlorate [73], the saline solution containing traces of Fe^{2+} may lead to the Fenton's reactions [74]:

$$H_2O_2 + Fe^{2+} \longrightarrow HO^{\bullet} + OH^- + Fe^{3+}$$

$$H_2O_2 + Cl^- + HO^{\bullet} \longrightarrow HClO + 2H_2O$$

Hypochlorous acid constitutes an inflammatory agent of the endothelium during an infusion, even at a concentration of 10 µM. Moreover, it may activate platelets and induce a microcoagulation. Although it is well known that ClO^- is physiologically produced by phagocytic cells and it is an efficacious bactericidal compound, it remains either confined in phagosomes or released in plasma near endothelial cells [75]. However, ClO^- is one of the most noxious reactive oxygen species (ROS) during a chronic inflammation.

It is unfortunate that the practice of using ozonated saline has become common in Russia and is widely used because it is inexpensive and less time-consuming than major AHT and simultaneously applicable to many patients. As it could be foreseen, physicians have started to use it also in Italy, Spain, Greece and Turkey. Ikonomidis *et al.* [76] have reported that they maintain the saline solution under a constant flow of ozone during transfusion but they warned that the maximum amount of ozone daily administered is usually 4-5 mg and should never exceed 8-10 mg. In their publication they also stated "if we exceed these rates, the over coagulation syndrome starts" and they strongly recommended to perform coagulation tests before starting therapy. These precautions reinforce our preliminary objection to this approach. Moreover, to the best of our knowledge, Russian physicians ozonize the saline with very low ozone concentrations (2-3 µg/ml) and this precaution may reduce toxicity. Clinical advantages have been claimed but results have never appeared in peer-reviewed journals; thus there is a serious concern that the advantage is due to a placebo effect not to be compared with the therapeutic advantage of properly ozonated blood. In comparison to the multiform effectors generated by ozone in blood, the ozonated saline appears as a palliative solution. Moreover, in contrast to ozonated saline, 0.03% (9 mM) H_2O_2 in isotonic

glucose solution (5%), which does not contain traces of ClO^- have been safely infused [77]. Obviously, this solution should not be used in diabetic patients.

4) THE INTRAVENOUS INFUSION OF OZONATED WATER

Recently, a new technique based on a central vein infusion of ozonated water has been proposed and tested in a few cancer patients [78]. The definition of either "liquid polyatomic oxygen" infusion or "ozone in liquid form" is wrong because liquid ozone at ordinary pressure conditions exists at below -111.9 °C, and it cannot be infused. Moreover, the term polyatomic oxygen is an euphemism because the corona-discharge method, one of the most effective procedures, practically generates only ozone.

Either ozone polymers (O_4) [79,80] or clusters (O_6 and superior) [81,82] can be generated. Thus, at this stage, it seems that the idea of superactive oxygen polymers/clusters must be further examined as well as if they have any therapeutic impact. Minimal details of the procedure have been reported [78] except the detail of ozonated water infusion via a Groshong type central venous catheter positioned in the patient's subclavian vein. It has been stated to perform a continuous administration for months of a mixture of "liquid polyatomic oxygen" useful for boosting the activity of some cytotoxic drugs. As we have already mentioned both ozone and oxygen are physically soluble in pure bidistilled water. When the gas mixture composed of oxygen (95%) and ozone (5%) is bubbled in pure water, ozone in relation to its relative pressure, temperature and solubility coefficient will dissolve as a gas in the water and will saturate it within 5-10 min up to 26% [83]. However ozone, even if kept in a tightly-closed ozone-resistant container spontaneously will decompose to oxygen during the following 11 hours at +20°C. If the container is worn by a patient at the body temperature, ozone decays rapidly and the half-life is about 2.5 hours; this disadvantage, however, has not been mentioned. Provided that the gas mixture is dissolved in pure water at ordinary pressure, it is theoretically possible to infuse water-dissolved oxygen and ozone **very slowly** into the blood circulation. To the best of our knowledge, this technique had been firstly used by Belianin

[84] in order to decrease the resistance of multiresistant mycobacteria in TBC patients. This is a technique for ozone administration probably less dangerous than the direct IV administration of gas correctly prohibited in 1984, owing to serious side effects and the risk of oxygen embolism. However, it appears obvious that the infusion of "liquid polyatomic oxygen" will catastrophically freeze the blood and this term should be proscribed.

The proposed technique still present several disadvantages: firstly, there is no documented proof that a continuous deliver of solubilized ozone in pure water into the central venous system is more effective than the classical, practically risk-free, infusion of blood ozonated *ex vivo* into a cubital vein. Even in skilled hands, complications such as pneumotorax and sepsis are low but do happen. Venous thrombosis is also a risk always well emphasized by expert anesthesiologists. Noticeably, the patient must be informed about the implantation procedure, accept the procedure and sign an informed consent. The system can run smoothly only if the ozonetherapist procures the daily infusions, checks the pump programming, trains the patient and assiduously checks the safety of the system. Another crucial objection is that so far the usefulness of the solubilized ozone infusion without the integration of temozolomide (or other antitumoral agent) has not been demonstrated.

Although the direct intravenous injection of water-soluble oxygen and ozone is a very unusual and peculiar method of ozone administration, it deserves to be theoretically evaluated [9]. In order to prevent a pathological hemolysis (at the tip of the catheter, local hypotonicity cannot be lower than 100 mM NaCl) and in consideration that cancer patients undergo a chronic oxidative stress, we cannot infuse more than 360 ml (depending upon the body weight) of pure water during 24 hours. In other words, water can be cautiously infused at a rate of 0.50 ml/min. By using the currently available ozone generators, 360 ml of water at 20 °C may dissolve no more than 9.1 mg ozone. However, as ozone decomposes rapidly at about 30°C. it is likely that, at best, we can deliver a negligible amount of oxygen and no more than about 6 μg/ml ozone per minute. Thus the daily dose of ozone is below the average dose of 8.0 mg administered by ozonating 200 ml of blood *ex vivo* with an equal volume of gas containing 40 μg /ml ozone. This reasoning questions the validity and

usefulness of the direct infusion of water-soluble ozone in blood because ozone will immediately react with plasma components and it will never reach and kill neoplastic cells *in vivo*.

On the other hand, ozonated water can be rationally used if applied intratumorally, i.e., by using the Radial Expansible Retractor (RER) invented by one of us (D.M.) [85]. After placing the RER in the area of a glioblastoma multiforme (GBM), if the RER has been equipped with a silicon chamber supplied with an inlet and outlet tubings, it is possible to establish a constant flow of oxygen/ozone directed against the neoplasm. The outlet tubing leads to an ozone destructor to prevent air pollution. A second possibility is that ozonated water can be delivered very slowly directly into the neoplastic tissue for several days. Ozonation of medically pure, injectable water can be achieved by using the mixture oxygen-ozone under 2 atm pressure, possibly obtaining a concentration of 30-50 μg/ml ozone physically dissolved in water. A third important possibility is the application of sterile ozonated olive or sesame oils of which we have good experience and have successfully used in deep necrotic ulcers [86]. The main characteristic of these preparations is that ozone, while on itself very unstable, remains fixed and stable as a trioxolane between two carbons along the aliphatic chain (Figure 5).

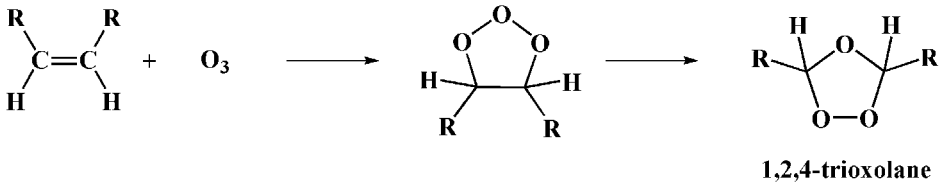

Figure 5. Trioxolane formation by chemical reaction of ozone with PUFA in absence of water.

When ozonated oil, placed in a neoplastic tissue, comes in contact with the exudate, it slowly release peroxidic compounds that will easily kill GBM cells uncapable to resist oxidation. As an example, application of ozonated oil could be performed during the night or when necessary.

GBMs are known to be the most aggressive and lethal tumors resistant to orthodox therapies. Whenever possible, after an initial surgery to remove the bulk of the tumor, the slowly but

continued administration of ozonated water or of ozonated oil will release of ozone in swollen cells and since 1980 [87] and our experience of cancer cells kept *in vitro* under ozone, leaves little doubt of a progressive killing of glioblastoma cells until we reach normal vascularized tissue where ozone activity is rapidly neutralized. The use of ozonated water and oil appears greatly advantageous over the use of ozone as a gas because not only the atonic water has a swelling effect but it allows the release of ozone inside the cells and prevents also the risk of polluting the surrounding air. Moreover the successive use of temozolomide and a vaccine against epidermal growth factor receptor variant III (EGFRvIII) [88,89] may lead to a prolonged remission, if not a cure.

The same procedure can be evaluated in patients with cerebral abscesses, notoriously difficult to be treated owing to antibiotic resistance and to the local difficulty of achieving an effective bactericidal activity.

A very much experimented and useful application is the topical use of pure ozonated water in chronic ulcers (diabetic foot), decubitus and many other cutaneous and mucosal infections. This is a problem of great socio-economical interest because millions of patients suffer of chronic cutaneous infections that never heal and cause pain and depression. An extensive review on the topical use of gaseous ozone and derivatives as anti-infective agents is being published [86]. Finally, a great progress has been achieved by the topical application of gaseous ozone, ozonated water and oil derivatives in dentistry [90,91].

5) THE QUASI-TOTAL BODY EXPOSURE TO OXYGEN-OZONE

An extensive experience in treating localized chronic infections or ulcers in the skin has been gained during the last 25 years by exposing the lesion to oxygen-ozone in a totally humidified area enclosed in a tightly-closed polyethylene bag. The so-called “bagging system” depending upon the availability of an ozone generator with an aspirating pump can be dynamic in the sense that there is a continuous input and a corresponding gas output where ozone is decomposed by a suitable destructor. The environmental air must never be contaminated with ozone. More frequently, the

system is static with the bag filled with gas for about 20 min, a period sufficient to disinfect the lesion and enhance healing. Also in this occurrence, ozone must be aspirated and destroyed. This procedure is valuable for a variety of infected and necrotic lesions caused by either ischemia, or venous stasis or diabetes, or trauma, burns and bed sores. In this cases, ozone concentration can be as high as 70 μg/ml because that is needed for highly infected lesions and because ozone at about 30 °C quickly decomposes. As soon as the lesions progressively improve lower ozone concentrations are used to enhance cell proliferation and healing. As no damage has been observed on the surrounding normal skin, it has been obvious to examine the value of ozone in other pathologies with generalized lesions such as atopic dermatitis, eczema, psoriasis and seriously advanced lipodystrophy.

As a large polyethylene bag is only a temporary solution, suitable ozone-resistant cabins with an ergonomic seat and back rest have been built where the body of the patient except than the head remains perfectly insulated inside, without risk of breathing ozone. Moreover, a thermostatic controlled system allows to regulate the internal temperature (from 36 to 42 °C) and saturated water vapor, essential for maximal ozone activity. The patient can sit inside the chamber for 20-30 min but before opening the cabin, the internal gas (maximal ozone concentration is no more than 1 mg/L) is again aspirated and destroyed. This is indeed an interesting system not only for treating ample cutaneous lesions, but also for treating systemic diseases. As usual ozone is not absorbed through the skin because it immediately reacts with the water film covering about 1.7 m^2 of the skin surface. Sweat and sebum secreted compounds enhance the ozone reactions and it was of great interest to examine the modification of several metabolic parameters [92,93]. After 20 min permanence in the cabin, body weight decreased of almost 1 kg and systolic pressure also decreased of about 10 mmHg. Pressure of either oxygen or $PvCO_2$ markedly increased or decreased, respectively, indicating oxygen absorption through the skin. While ozone was not absorbed, peroxidation compounds were and doubled in the venous blood. On the basis of evaluation of several other parameters, the conclusion was reached that the quasi-total body exposure to oxygen-

ozone mixture involves a reaction of the whole body and it represents a very promising procedure for modifying the biological response in different pathologies. We have experimented it ourselves and many physicians have volunteered to test the system and conclusions have been enthusiastic. The treatment is simple, inexpensive to perform and not-invasive (no venous punctures are required) and does not involve the handling of potentially infected blood, an aspect much appreciated by medical personnel. It requires only to be well-organized with a small room where to undress and a room for a comfortable one-hour rest for the patient with a final shower, if desired. No side effects have been noted and everyone who has tested the system reports a feeling of well-being next day. This approach for which an extensive report was published in 2005 [94], may therefore will compete with major AHT, EBOO and certainly other administration ways of gaseous ozone, like the rectal insufflation.

6) THE ADMINISTRATION OF THE GASEOUS OXYGEN-OZONE MIXTURE VIA SEVERAL ROUTES

a) The gaseous mixture oxygen-ozone collected with an ozone-resistant syringe can be injected into various body compartments. As an example, the subcutaneous (SC) injection of several small gas volume (1-2 ml) with an ozone concentration between 2-3 μg/ml of gas has been amply used for the treatment of lipodistrophia (cellulitis). On the whole, no more than 40 small injections (total volume ~ 80 ml) should be injected for each treatment. At the end of the session, a gentle massage for about 10 min should enhance the lipolytic activity and accelerate the gas reabsorption. The treatment is effective, atoxic and the risk of a pulmonary embolism due to oxygen is minimal provided to avoid injection of over 80 ml gas in about 40 SC areas [10,11]. Indeed larger volumes (up to 250 ml) are dangerous and have caused three deaths in Italy.

b) The intramuscular (IM) injection of ozone as a substitute of major AHT is not performed today because a minimal 4 mg ozone should be injected by using 200 ml gas with an ozone concentration of 20 μg/ml that would be dangerous and painful.

On the other hand, the IM injections in the paravertebral muscle of about 10-20 ml of the gas mixture (with an ozone concentration ranging from the initial 15 up to 25 μg/ml, total ozone dose of 300-500 μg) are in wide use. The route of infiltration of the gas is located in the paravertebral muscle, about two cm bilaterally to the spinous process, just above the transiliac line, points useful for identifying the L4 spinous process in case of a hernial disk between L4-L5. The procedure has been defined as a "chemical acupuncture" or the indirect approach [95]. This methodology is hardly fifteen years old but, by now, many patients have been treated in Italy, China, India, Spain and Germany during an episode of low back-ache.

It is an easy approach consisting in one or two injections of 5-10 ml of gas per site. The ozone concentration normally must not exceed 20 μg/ml because it is painful except when, after several injections, the pain threshold has markedly gone up. At first, it is wise to test the patient's reactivity with an injection of sterile saline and then start with 10 μg/ml of ozone. The injection must be done very slowly into the trigger points corresponding to the metamers of the herniated disk. The length of the needle varies from gauge 22 to 25, depending on the patient's obesity. Usually, two symmetrical injections (total dose 10-20 ml gas with at most 200-400 μg ozone) repeated twice per week for about 5-6 weeks (10-12 sessions) are sufficient. If not, the patient is unresponsive to this approach. This point remains controversial because some ozonetherapists continuous treatments up to 30 sessions. The pain at first elicited with an ozone concentration of 20 μg/ml tends to subside because of a progressive elevation of the pain threshold. In such a case, the ozone concentration can be increased up to 25 μg/ml. It appears that the stimulation of nociceptors, hence of a tolerable and transitory pain is an essential requirement for achieving the final therapeutic effect. Indeed, the patient must be reminded that "no pain, no gain".

Indeed the injection of oxyge/ozone elicits a fairly sharp pain lasting a few minutes and the injection must be done very slowly to avoid any risk of embolism. Serious adverse effects, such as sudden hypotension, bradycardia, mydriasis, intense perspiration and cardiac arrest (vasovagal reflex) can be also avoided by a very slow injection.

An important question is: how does intramuscularly injected ozone work? The gas infiltrates the muscle and after 24 hours some gas bubbles (residual oxygen) move towards the vertebral canal, as can be radiologically seen. It was postulated that ozone will reach the site of the herniated material and will lyse it. This is an untenable idea: ozone is water-soluble and it dissolves so quickly into the interstitial water of the muscle that within 20-40 sec will generate a gradient of ROS and LOPs able to inhibit amyelinic fibres (C-nociceptors), which are able to elicit the elevation of pain threshold and an antalgic response via the descending antinociceptive system. The introduction of the needle, reinforced by the pressure of the gas, induces a prolonged stunning of nociceptors due to ROS and LOPs. It is known that an algic skin and muscle stimulation can reduce pain through the mechanism of diffuse noxious inhibitory control (DNIC) [96]. That is why the needle, in combination with ROS, LOPs and oxygen pressure can be translated into a chemical acupuncture. This mechanism is likely correct because too low ozone concentration (3-10 μg/ml) or small gas volumes (1-2 ml) are ineffective, whereas too high concentrations or excessive gas volumes can cause lipothymia. It is unclear whether pre-infiltration with an anaesthetic reduces the effect of ozone but probably it is counterproductive.

In conclusion, the probable mechanisms [95] playing a role are the following:

i) release of endorphins and endocannabinoids blocks transmission of the noxious signal to the thalamus and cortex;

ii) hypostimulation (elevation of the activation threshold) linked to the oxidative degeneration of C-nociceptors. ROS and LOPs may act as capsaicin;

iii) activation of the descending antinociceptive system;

iv) simultaneous psychogenic stimulation of the central analgesic system induced by the gas injection (elicitation of a placebo effect);

v) the localized oxygenation and analgesia are most important because they permit muscle relaxation and vasodilation, thus a reactivation of muscle metabolism, by favoring

oxidation of lactate, neutralization of acidosis, increased synthesis of ATP, Ca^{2+} reuptake and absorption of edema.

The reader may be interested to know that after performing about a dozen IM injections, up to 75% of patients have a good and long-lasting response. It appears that this approach is far more valid than either the peridural injections of desamethasone or the paravertebral injection of 0.25% buvipicaine.

c) The other relevant breakthrough regarding the frequent problem of back-ache is the direct injection of the gas mixture (~ 2-6 ml of gas with an ozone concentration up to 25-30 μg/ml; ozone total dose 50-180 μg) into the nucleus pulposus related to the hernial disk site [11]. During life-time, low-back pain is a very disturbing symptom that can affect, at least for a while, up to about 80% of the world population [97,98]. Luckily, in most cases physical therapies (exercises, manipulation therapy) can solve the problem [99], but if a hernial disk is present it must be removed with the least invasive procedure. One of the most obvious mechanism of action is that ozone dissolves in the interstitial water of the nucleus and immediately reacts, generating a cascade of ROS among which H_2O_2 and $^{\cdot}OH$, that is a most reactive radical. Indeed, this radical is likely to react with carbohydrates and amino acids composing proteoglycan, a major component of the nucleus pulposus, leading to its breakdown [100-102]. Consequently, reabsorption of hydrolytic products and water appears to lead to progressive shrinkage and disappearance of the herniated material that is responsible for radicular pain. Detection of free radicals is difficult because they are very reactive and short lived. Indeed, the half-life at 37 °C of the $^{\cdot}OH$ radical is extremely short, equivalent to about 10^{-9} sec. Electron Spin Resonance (ESR) is the only analytical technique currently capable of directly measuring free radicals. The combined technique of ESR and spin trapping is highly selective and sensitive for the detection of free radicals. A specific study [103] has shown that during the exposure of a nucleus pulposus gel material to oxygen-ozone mixture, the hydroxyl radical is the unique radical species produced. This radical is so reactive that can start a

chain reaction leading to oxidation and degradation of proteoglycans able to explain the rapid disappearance of herniated material.

There is no doubt that hydroxyl radicals can cause the depolymerization of hyaluronic acid, synovial fluid as well as collagen and that the degradation could be inhibited by free radical scavengers. Moreover, this is likely to happen because the natural antioxidant system, composed of hydrophilic, lipophilic substance and enzymes [104], is hardly present in the discal material. However, also in this case there is evidence of the ozone paradox [11]: although hydroxyl radicals can degrade the degenerated material and reduce pressure, they surprisingly exert a rapid "anti-inflammatory action", particularly because only a few ml of gas can be introduced inside the nucleus pulposus and some of the gas invades the intraforaminal space. This may mean that ozone rapidly blocks inflammatory reactants and stimulates the "restitutio ad integrum". What is even more surprising is that this change remains stable (unlike corticosteroids) and it does not necessarily coincide with the disappearance of the herniated material. In fact, CAT or NMR controls 5 months after treatment in 612 patients showed that the hernia disappeared in 226 (37%), was reduced in 251 (41%) and was unmodified in 135 (22%). After another 5 months, CAT/NMR controls were again performed in 200 (of 251) patients in whom the hernia was reduced: a further reduction and improvement was noted in 44 patients (22%). In 120 patients (of 135) in whom the hernia was unmodified, there was an improvement in 11.6% [105] Thus, the ozone effect is deployed in successive phases: there is an initial rapid change, probably with disappearance of edema and improvement of circulatory and metabolic conditions, followed by a stasis and then a further improvement possibly due to release of TGFβ1 and bFGF [106] favoring the reorganization of the residual nucleus pulposus with incipient fibrosis. Moreover, ozone may inactivate proinflamatory cytokines, inhibit proteinases and cyclooxygenase-2 thus blocking the synthesis of prostaglandin E_2. All of these surprising aspects have contributed to formulate the concept of the ozone paradox [107].

A few problems have been reported. In young patients, it is often very difficult to introduce more than 1-2 ml of gas inside the nucleus pulposus, so that the gas is mostly released into the intraforaminal space. In these cases, a preliminary aspiration of the nucleus followed by the gas introduction might improve the result. Apparently, the intraforaminal administration of gas yields good results even in the case of sclerotic hernias [108] Side effects are very rare: one patient had a transient lipothymia and one reported by Alexander *et al.* [109] presented amaurosis fugax (bilateral blindness which reversed after about 24 h) after cervical discolysis in a young athlete.

Dr Kieran J. Murphy, an interventional neuroradiologist at the University of Toronto, at the annual meeting of the Society of Interventional Radiology in San Diego, CA (March 2009), reported a study performed in more than 8000 patients with herniated disk injected with ozone intradiscally injected (unpublished data). Results have been so good (about 80% success rate) to predict that the procedure so simple, atraumatic, safe, and atoxic will become standard in the US within the next five years. Surprisingly some patients have no longer pain 30 min after the injection suggesting the rapid effect of ozone in releasing the pressure and inflammation on the nerve root. He acknowledged that the procedure had been discovered in Italy, where more than 20,000 patients have been already treated [110], not to count many thousands in China, India and Spain. Patients treated with ozone fare much better than those treated with steroids [111,112].

7) THE INTRACAVITARY AND INTRALESIONAL ADMINISTRATION OF OZONE

Although the intraperitoneal (IP) administration has been advocated in several pathologies, such as chronic hepatitis B and C in nephropathic patients under peritoneal dialysis [10,11], and in ovarian carcinosis with peritoneal involvement, so far it has not been possible to perform a study in patients. During peritoneal dialysis, when a patient has already a medical grade silastic catheter, would be simple to try the concept. However, clinicians are skeptical and are afraid of a potential ozone toxicity. Schulz *et al.* [113] have recently shown that 6 rabbits over 14 implanted with VXZcarcinoma HNSCC tumor cells can be “cured” after the IP injection for 5 consecutive days of

12 mg of ozone. The results are interesting, but also in the past the “cure” had been observed in experimental mice with IL-2, tissue-infiltrating lymphocytes (TIL) and angiogenesis inhibitors, drugs that in clinical trials have failed to solve the cancer problem. Indeed, in a letter the question posed was: “Does ozone really cure cancer?” [114]. In fact, after performing prolonged cycles of major AHT in preterminal metastatic cancer patients, results have been disappointing [115]. However, peritoneal carcinosis and mesothelioma, besides being almost refractory to chemo-radiotherapy remains to be evaluated, by using ozonated water and oil as well as gas well retained in the peritoneal and pleural cavities. In preliminary experiments in rabbits, an intraperitoneal injection of 250 ml of gas, with an ozone concentration of 20 μg/ml has not procured any noxious effect. Also a localized melanoma could be destroyed by direct administration of ozone but unfortunately ozone, intended as a direct cytotoxic agent, can never reach metastatic cancer cells. The cytotoxic effect of ozone in GBM remains to be ascertained.

8) THE ADMINISTRATION OF OXYGEN-OZONE VIA THE COLON-RECTAL ROUTE

This problem has been extensively discussed [116] because it is an easy and rapid way of administering ozone. It has the additional advantage that the patient could self-administer ozone at home under the physician’s advice. There are however several caveats to bear in mind. Firstly, while oxygen is partly absorbed, it has a negligible value. Ozone cannot be absorbed by the colon-rectal mucosa because it immediately reacts with the heterogeneous luminal content (e.g. feces, mucoproteins, immunoglobulins). A variety of peroxidation compounds detected in portal blood were absorbed as it was shown in rabbits after preventing the loss of the administered gas [117]. This route is widely used at Cuba and in Russia: clinical data have been published [118-121], but results need to be confirmed by other institutions. The insufflated dose of 6-12 mg ozone (200-400 ml of gas with an ozone concentration below 30 μg/ml) is known but it always uncertain the quantity of ozone effectively acting in the gut. Ozone concentration higher than 30 μg/ml can be mutagenic on the mucosal cells especially if the patient has made a clysma before the treatment. Thus the rectal route remains unreliable and it was found less effective than major AHT [119].

CONCLUSION AND PERSPECTIVES

The evaluation of the mechanism of action of ozone in biological fluids has allowed to precisely clarify how ozone works and why it is not toxic when used within the therapeutic range. The versatility of ozone applications is impressive and today ozonetherapy can be performed using several different modalities. Besides the old but still quite valid methods of major and minor autohemotherapy, other options such as the quasi-total body exposure to oxygen-ozone and the EBOO have been developed and evaluated. In non-diabetic patients with precarious venous access, as a blood substitute, the glucose-peroxide solution, which represents a form of biooxidative therapy with a clear rationale and the advantage of being inexpensive and potentially useful to millions of people without medical assistance can be profitably used. Although all of these procedures must be controlled and supervised by physicians expert in ozonetherapy, a few of them are amenable to be used at home by the patient. Ozone must never be breathed but, if the dose is adapted to the potent antioxidant capacity of body fluids, the above described methods offer flexible and useful therapeutic advantages. Whenever necessary, it is possible to combine these different approaches. The central aim of ozonetherapy is to give a precise, atoxic shock to an organism which for various reasons has gone astray. In agreement with the "hormesis" concept, repeated, small shocks will readjust several biological functions by means of many messengers (ROS, LOPs and autacoids generated by ozone) delivered by circulating blood to the whole body. The term "therapeutic shock" has been coined to symbolize the possibility of reactivating the natural positive capabilities to restore health or, in better words, to stimulate the "vis medicatrix naturae".

The simultaneous induction of an acute and precisely calculated oxidative stress on different areas such as blood, the skin and gut mucosal system can result in a more comprehensive and perhaps synergistic response of the body defense system. Indeed, chronic diseases must be attacked from different angles and we have evidence that the stimulation of several biochemical pathways in different organs can be therapeutically beneficial.

Some Nations in the world still do not allow the practice of ozonetherapy either for negligence, prejudice or because it is dangerously performed by incompetent quacks without medical qualifications. As a consequence it is necessary to establish precise regulations, the first of which is that only physicians, after an appropriate university qualification, can perform it. A further significant step forward will be done by performing randomized clinical trials definitively proving the advantage of ozonetherapy over orthodox medications in vascular disorders. Much remains to explore in other pathologies where ozonetherapy today can only used as a supporting therapy. An obvious remark is that, against the minimal cost of ozone, the expenses of medical assistance and drug development increases every day and normally a decade is necessary before a drug becomes of practical use. The review aims to clarify to inexpert clinicians not only the specific medical advantages of this approach but also the possibility of extending its use in poor countries. It remains deplorable that Health Authorities do not sponsor further studies and an application of this approach in every hospital.

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