Validity of Oxygen-Ozone Therapy as Integrated Medication Form in Chronic Inflammatory Diseases

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VALIDITY OF OXYGEN-OZONE THERAPY AS INTEGRATED MEDICATION FORM IN CHRONIC INFLAMMATORY DISEASES

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ABSTRACT

The state-of-the-art of oxygen-ozone therapy is now clarified and all the mechanisms of action of medical ozone are within classical biochemistry and molecular biology. The outcomes of standard treatments in peripheral arterial occlusive disease (PAOD) and dry-form of age-related macular degeneration (AMD) have been compared with the documented therapeutic results achieved with ozonated autohemotherapy (O-AHT). On the other hand, the clinical data of O-AHT on stroke remain indicative. As the cost of O-AHT is almost irrelevant, its application in all public hospitals, especially those of poor Countries, would allow two advantages: the first is for the patient, who will improve her/his conditions, and the second is for Health Authorities burdened with increasing costs. The aim of this paper is to report to clinical scientists that O-AHT is a scientific-based therapeutic approach without side effects. The integration of O-AHT with effective approved drugs is likely to yield the best clinical results in several chronic inflammatory diseases.

KEYWORDS: Age-related macular degeneration, dry form; Peripheral arterial occlusive disease; Stroke; Chronic obstructive pulmonary disease; Reactive oxygen species; Oxygen-ozone therapy; Integrative medicine

INTRODUCTION

Peripheral arterial occlusive disease (PAOD), chronic heart failure (CHF) and stroke, not only are the first cause of death but, owing to morbidity and disability, represent one of the most debilitating and expensive social problems [1]. A retinal degenerative disorder, especially the dry-form of age-related macular degeneration (AMD) can be also cited because retinal ischaemia and photoreceptor degeneration compromise the central vision and deeply affects the quality of life [2]. Factors as the progressive ageing of the world population associated with diabetes and obesity contribute to worsen atherosclerosis [3]. These pathologies have much in common and they are due to abnormalities of the cholesterol metabolism, the β-adrenergic and the renin-angiotensin systems which, at a different extent, cause a chronic inflammatory situation characterized by the excessive release of the main reactive oxygen species (ROS), as $^{\cdot}O_2^-$, $^{\cdot}OH$, H_2O_2, HClO, proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNFα) [4]. Consequently hypertension, enhanced platelet aggregation, tissue ischaemia, retinal pigment epithelium (RPE) and photoreceptors degeneration associated to a chronic oxidative stress follow and present the typical symptoms of PAOD, CHF, stroke, and AMD. Moreover, chronic obstructive pulmonary disease (COPD) although it is a lung disease, it is associated with systemic manifestations and ischaemic heart disease [5].

I. How mainstream medicine has attempted to treat chronic inflammatory diseases?

Results will be separately discussed for each pathology.

1a) Peripheral arterial occlusive disease (PAOD)

Four stages indicating the worsening of chronic limbs ischaemia (CLI) have been classified by Fontaine-Leriche indicating the progressive relevance of the disease [6]. PAOD patients have the same relative risk of death from cardiovascular causes as do patients with a cerebrovascular or coronary disease. Patients with the ankle-brachial index (ABI below 0.40; normal values ranging between 0.91-1.30) are at high risk and present an annual mortality of about 25%. Medical

treatment should be performed at the earliest possible time but even at stage IV, it is possible to avoid or delay amputation [7,8].

When any possible surgical revascularisation is no longer possible, the actual golden standard is represented by the infusion of a stable prostacyclin analogue in patients with PAOD frequently complicated with necrosis. The drug improves vasodilatation but frequently has side effects such as headache, palpitation and dizziness. It is most frequently associated with statins, platelet aggregation inhibitors, anti-hypertensive and antidiabetic drugs [9].

1b) Chronic Heart Failure (CHF).

Great attention has been paid to block the progression of CHF in patients not susceptible to receive a transplant. Large randomized trials have been performed with poor clinical outcomes. The lack of results has been surprising because it was hypothesized that either neutralizing or reducing several inflammatory mediators such as TNFα [10], IL-1 and IL-6 [11], autoantibodies and complement activation would have solved the problem. However, the employment of either the antibody against TNFα (infliximab) [12] or of etanercept [13] have been disappointing [14]. With the benefit of hindsight, this therapy cannot work because it does not block the synthesis of inflammatory cytokines. Two initial trials with rosuvastatin have been unable to show clinical benefits [15,16] although the final study by Mc Murray et al. [17] has shown a reduction of C-reactive protein (CRP), suggesting that the drug may also reduce inflammation.

Moreover, a failure has not been restricted to the SIMPADICO trial performed in 533 patients with symptomatic PAOD [18] but it has included the successive ACCLAIM multicenter, double-blind placebo controlled study based on using an extremely ozone-oxidized blood (107.5 mg ozone per ml of blood) plus an undetermined UV irradiation and heat exposure at 42.5 °C which was thought to display an immune suppressive activity able to abolish the chronic heart inflammation [19]. This rationale has been severely criticized [20-24] and it is hoped that physicians will not privately use this approach.

Intravenous immunoglobulin therapy (IVIG) has been tested on the hope that it would neutralize autoantibodies, inhibit complement activation and enhance the release of IL-10, an immunosuppressive cytokine [25]. Moreover, on the basis that therapeutic apheresis helps autoimmune patients, a new therapeutic approach, based on therapeutic plasma exchange, has been proposed. The removal of large amount of plasma from CHF patients, which thereafter is substituted with 5% albumin and intravenous immunoglobulin may represent a new strategy [26]. However this procedure appears to be practically cumbersome and expensive and needs to be demonstrated useful for the prognosis of CHF.

1c) Stroke.

It is the second most common cause of death and, owing to the limitation of present therapies, is the major cause of long term physical, psychological and social disability. In 2009 in England direct care costs for stroke amounted to 2.8 billion of pounds. Moreover indirect costs were of 1.8 billion of pounds due to lost productivity and disability and of 2.4 billion for informal care costs [27-31]. About 80% of strokes are due to an occlusion of a major cerebral artery due either to a local thrombosis or to an embolism successive to either atrial fibrillation or carotid stenosis. Owing to the lack of a collateral circulation, the blockage of a cerebral artery rapidly causes brain ischemia with a central zone (the core) of cell death bordered by a peripheral more resilient area called the *penumbra*. The anoxic nervous tissue undergoes a very rapid damage due to lack of oxygen and glucose: indeed, in the core, about 1.9 million neurons dye for each minute in which stroke is untreated [32]. Moreover the loss of ionic gradients results in the depolarisation of neurons and glia, followed by the activation of voltage-dependent Ca^{2+} channels and Ca^{2+} overload. The consequent accumulation of glutamate, cause the excitotoxicity increasing the concentration of both the intracellular Ca^{2+} and Na^{+}, with consequent brain edema [33,34].

The next event is the release of proteolytic enzymes, phospholipases, cyclooxygenases and activation of NO synthase. The formation of ROS such as superoxide favours the formation of peroxynitrite, the activation of NFkB and the successive release of proinflammatory cytokines.

Thus, 24 hours after the initial insult, a progressive inflammation spreads from the core into the ischemic *penumbra* which ultimately, after neuronal death, worsens the prognosis [35]. It is obvious that an acute stroke requires an urgent therapy that is delayed due to practical problems. Consequently only about 5-10% of patients can reach the stroke unit and be treated within 3-4.5 hours from the first symptoms with the thrombolysis performed with the intravenous infusion of recombinant tissue plasminogen activator (Alteplase rTPA 0.9 mg/kg I.V., maximum 90 mg over 1 h) [36-39]. However *the main problem is to find the most effective treatment for about 90% of patients, who reach the hospital after 4.5 hours.* In such case which are the drug treatments most used? Aspirin at the dosage of 75-150 mg per day is the current therapy [37]. Clopidogrel may be better and it is used for patients aspirin-intolerant. Intravenous heparin carries unacceptable risk of haemorragic side effects. Oral anticoagulants are necessary in patients with atrial fibrillation. Blood pressure should be controlled and possibly lowered to 130/70 mmHg. New nonpeptidic small-molecule drugs may prove to be therapeutically effective [40]. Integrated medicine [41] is evaluating the value of either infusion of albumin [42,43] or magnesium sulphate [44,45] other than hypothermia [46-48].

1d. Age-related macular degeneration (AMD), dry-form

Some degenerative disorders of the retina and optic nerve are progressive and, for most of them, there is no therapy. By affecting people in fairly good health, AMD compromises the central vision and deeply affects the quality of life [49,50].

AMD affects 20-30% of people over the age of 65 and is a serious and expensive public health problem [51]. In the UK there are about 240,000 partially sighted or blind patients [52] and in the US, almost eight million Americans are blind [53]. AMD's aetiology is linked to ageing, smoking [54], obesity, oxidative damage–induced inflammation [55], excessive exposure to sunlight causing a photo-oxidative stress [56], and, in some patients, to a mutation of the ABCR protein which favours the accumulation of degraded material able to interfere with the retinal metabolism [57]. The retinal outer face is in contact with the Bruch's membrane, which separates the vascular

choroids from the retinal pigment epithelium (RPE), which represents both the histological and functional connection with the photoreceptors situated in the outer layer, while the axons of the ganglion cells are placed on the inner layer to form the optic nerve. The RPE is vital to the integrity of the photoreceptors because it exerts the daily phagocytosis of about 10% of the tips of the outer segments of the photoreceptors, it recycles vitamin A and transfer oxygen and nutrients from the choroids to the photoreceptors and outer retina. The *foveola*, located in the center of the *macula lutea*, is avascular. It has the highest concentration of cones and it is responsible for the visual acuity [58]. For its metabolic requirements the *foveola* depends on the choriocapillaris circulation because there are no retinal vessels and it has the highest consumption of oxygen of all body tissues. Atherosclerosis and then ischemia and hypoxia lead to central and peripheral loss of vision by degeneration of the neurosensorial cells [59]. Having detected a good correlation between CRP serum level and AMD, Seddon *et al* have postulated the role of a chronic inflammatory process in the pathogenesis of the disease [60]. Consequently, an excessive formation of free radicals, among which peroxinitrite, Ca^{2+}-induced damage and glutamate toxicity lead to a progressive death of the photoreceptors. Two forms of AMD have been described:

- "dry" (non exudative, or atrophic) form, characterized by the presence of drusen (as pale yellow spots) and areolar atrophy of the RPE. This form occurs in about 90% of patients and the visual deterioration is slow becoming serious in about 20% of patients, who become blind. For the dry form there are no useful therapies at all. Administration of antioxidants such as vitamin A, C and E, and zinc [61-63], lutein and zeoxanthin is not harmful but it neither improve vision, nor delay the pathological progress [64].

- "wet" (exudative-neovascular) form, characterized by choroidal neovascularisation, detachment of the RPE and fibrovascular disciform scarring. It is fairly rare (about 10% of patients) but it is associated with poor visual prognosis owing to the loss of central vision in about 75% of patients. In the "wet" form, ischemia induces the production of the vascular endothelial growth factor (VEGF) which, by stimulating an imperfect neo-angiogenesis from the choriocapillaris through the

RPE, is deleterious because this abnormal vascular network favours the leakage of an exudate, which causes a retinal detachment able to exclude the photoreceptors from the light stimuli. Currently, the intravitreal injections of an anti-VEGF antibody (ranibizumab, Lucentis®), has been selected [65].

Ie- Chronic obstructive pulmonary disease (COPD)

COPD is the fourth cause of death and it will become one of the major health challenges of the next decades. The bronchial damage is mostly due to cigarette smoking and it is aggravated by a chronic oxidative stress with successive lung fibrosis and loss of function. The most common comorbidities are, among others, ischaemic heart disease, diabetes, cachexia, lung cancer and they suggest common risk factors and mechanistic pathways. The therapy at the different stage of chronic obstructive pulmonary disease is represented by the so-called GOLD. It is a complex series of intervention, starting from smoking cessation up to long-term oxygen if chronic respiratory failure occurs [5].

II. Why oxygen-ozone therapy appears to be advantageous when associated with the described medical interventions?

Historically, the fact that " we know next to nothing about this branch of the subject" was published in 1869 [66]. About a century later a physician, Hans Wolff proposed the method of exposing 100-200 ml of human blood collected in a closed gas bottle under vacuum to a gas mixture composed of about 96% medical oxygen and 4% ozone to be infused into the donor patient within 3-4 minutes [67]. Thus the O-AHT, had been invented but for two decades it remained an empirical procedure performed only by private physicians in Germany. In 1994, Pryor defined the chemical reaction occurring between ozone and polyunsaturated fatty acids (PUFA) present in the alveolar lining fluid (ALF) [68,69]. During the following few years two relevant events occurred: the first consisted in perfecting the ozone generator able to deliver precise ozone concentrations photometrically detected at 253.7 nm and the second was the demonstration of the pharmacokinetic relative to the formation

of reactive oxygen species (ROS) after exposure of human blood to ozone concentrations ranging from 5 up to 100 µg/ml of gaseous ozone per ml of blood [70]. While oxygen simply saturates haemoglobin (Hb_4O_8), the ozone dose is partially reduced by the plasma antioxidants (uric acid, ascorbic acid), while the bulk, reacting with PUFA bound to albumin, induces a very rapid formation of progressively increasing amounts of H_2O_2 and of lipid oxidation products (LOPs) mainly represented by 4-hydroxy-2,3-trans-nonenal (4-HNE). The gentle mixing of blood with the gas mixture exhausts the reaction in a few minutes and while ozone is totally consumed, it generates the just mentioned crucial messengers (Figure 1). It must be emphasized that the potent antioxidant capacity of the plasma decreases no more than 30% even by using the higher ozone dose (80 µg/ml gas per ml of blood, corresponding to ozone concentration of 1.68 mM) and returns to the normal value during the following 15 min due to the rapid reduction of the oxidized antioxidants operated by erythrocytes [71-73]. The sudden formation of H_2O_2 in the plasma produces a chemical gradient between plasma and the blood cell cytoplasm but, within the preferred therapeutic window (10-40 µg/ml gaseous oxygen-ozone mixture per ml of blood or 0.21-0.84 µmol/ml), H_2O_2 is not toxic because inside the cells is never higher than 4-5 µmol, corresponding to 10% of its concentration in the plasma because it is promptly reduced by GSH and antioxidant enzymes (GSH-Px, thioredoxins and catalase) [74-76]. Although its half life is less than 30 seconds, this concentration is effective to trigger a number of biochemical pathways in all blood cells (Figure 2).

Alkenals as 4-HNE and 4-HHE [77-79] bind to either Cys34 of albumin or to GSH and are promptly infused into the donor patient. At first the infused ozonated blood interacts with the vascular bed and alkenals stimulate NO synthase supporting a modest vasodilatation [80] useful in ischaemic areas. Alkenals undergo either detoxification by at least five enzymes, or elimination via bile and urine, or enter into parenchymal cells [81]. The crucial function of alkenals is to inform a great variety of cells of a transient acute oxidative stress. *This is their most important activity, qualifying ozonetherapy as a treatment able to enhance the antioxidant capacity of the body.* Recent papers [82-84] have reported that alkenals can activate a nuclear transcriptional factor, called

nuclear factor-erythroid 2-related (Nrf2) present in the cell cytoplasm bound to Keap-1 protein. Such a protein has -NH_2 and, mainly, -SH groups (Cys273 and Cys288) which, by binding alkenals at picomolar levels, causes a conformational change favoring the dissociation of Nrf2, which is then imported into the nucleus where, after forming a heterodimer with Maf protein, interacts with the antioxidant response element (ARE) on DNA (Figure 3). The Nrf2, which activates about 230 genes, is the master regulator of a great number of antioxidant and phase-2 enzymes [85-87].

Consequently, the synthesis of several antioxidative enzymes (SOD, catalase, GSH-reductase, GSH-S-transferases, NADPH-quinone oxidoreductase, heat shock protein 70, phase II enzymes and Haem-oxygenase-1) is upregulated in various organs. HO-1, by reacting with haem generates CO, bilirubin and Fe^{2+} promptly bound by the system transferrin-ferritin [88,89]. *Cell internalisation of alkenals with the consequent increase of antioxidant capacity is the crucial step to counteract the chronic inflammation typical of diseases aggravated by a chronic oxidative stress*. Today, no other known treatment and, certainly, no administration of antioxidants, is sufficiently able to normalize the oxidant/antioxidant balance. Although alkenals are intrinsically toxic at micromolar level, the combined process of dilution, detoxification, excretion and final internalisation make them atoxic and invaluable as real therapeutic agents. *In this sense ozone therapy is the only integrative treatment where the biochemical and molecular mechanisms of action have been clarified.* Moreover, within the therapeutic range of ozone used, there is none of the otherwise typical offensive damages due to ozone chronically acting on the lungs [90].

In conclusion, depending upon the type and the stage of chronic oxidative diseases, the therapeutic use of ozone improves:

i) the glycolytic pathway on erythrocytes and significantly increases their ATP content [91];

ii) the hexose-monophosphate shunt, owing to the activation of 2,3-DPG mutase. In detail, the 1,3-DPG changes in 2,3-DPG (Rapoport-Luebering shunt) which, by binding to the β–chain of haemoglobin, causes a shift to the right of the Hb dissociation curve enhancing the release of

oxygen in the hypoxic tissues. Normally, at sea level the 2,3-DPG is about 4 µmol/g erythrocytes but it can increase up to five-fold [81];

iii) vasodilation of ischaemic areas due to enhanced release of NO and CO, possibly intensified by prostacyclin (PGI2) COX-2 dependent [92,93];

iv) the antioxidant status on the basis of Nrf2 interaction with ARE on DNA. This is the most important step for counteracting the chronic oxidative stress. Also the activation of HO-1 by increasing the release of CO and bilirubin contributes to reduce inflammation [70,89];

v) a feeling of wellness observed in most of the patients throughout the therapeutic sessions, due to stimulation of the neuro-endocrine system, after a few treatments [83,94].

In summary, the positive aspect of ozone therapy is the ability of activating a number of defence mechanisms which cooperates for recovering a normal redox system. Today, ozone therapy represents the most practical approach for integrating standard therapies and for achieving homeostasis. For comprehensiveness' sake, citation of publications in Italian, German, and Spanish languages as well as abstracts and reports from meetings have been taken into account. As regards the negative effects attributable to the medical use of ozone, they can be summarized as very rare single case reports and however not related to the reinfusion of autologous blood after treatment with ozone [95-97].

III. Oxygen-ozone therapy. What has been done and what is urgent to perform.

3a) PAOD. The pioneering work of Rokitansky et al. [98] is revered as the best because it was the first breakthrough. Between 1974 and 1980 he treated 152 patients with O-AHT and his results are reported in Table I.

In 1987, Mattassi et al. compared O-AHT with the direct intraarterial (IA) injection of 20 mL O_3 at a concentration of 40 µg/mL at least three times weekly in 113 patients. O-AHT was performed in 106 patients for five weeks with an O_3 concentration of 30-40 µg/ml. The best results were achieved

with O-AHT particularly in patients at the II and III stages and the femoral injection approach has been abandoned [99]. Subsequently a number of small (20-30 patients) trials have been reported [100-111].

All of these studies have shown that O-AHT can result in:

a) a significant improvement in blood flow and oxygenation of ischemic tissues due to NO, S-nitrosothiols cooperating with CO and released prostacyclin;

b) release of growth factors (PDGF-AB, TGF-beta) from ozone-activated platelets enhances healing of ulcers, especially when O-AHT is combined with topical therapy (either gaseous O_3 in a closed bag or ozonated water and oil. Healing of leg ulcers has been improved and the rate of amputation has been reduced

c) induction of antioxidant enzymes and HO-1;

d) O-AHT is practically free of adverse effects;

e) it is far less expensive than iloprost.

However, attention should be paid to the number of treatments and the schedule. Indeed these parameters depends upon the PAOD stage as it is indicated in Table II.

In patients not taking oral anticoagulants, Ca^{2+} heparin can be used but any possible dyscoagulation must be checked. With heparin, the range of ozone concentrations is from 20 up to 40 µg/ml per ml of blood. After the initial, less or more aggressive treatment (Table II) it is necessary to continue with a maintenance treatment (4 treatments monthly) for life in order to preserve the regained relative state of health. The compliance of patients has been shown to be excellent. The remarkable and very prolonged improvement leads to postulate the possible release of staminal cells which may be released from the bone marrow and implanted in the ischaemic tissues [91].

3b) CHF. It is unfortunate that our experience for the first cause of death is very limited because so far it has not been possible to find a collaboration from official medicine. A clinical trial in CHF patients by using O-AHT has been started in a Havana's Hospital by Dr. V. Borroto and conclusive results have been just submitted for publication. Moreover, for this complex and multiform

pathology the extracorporeal blood circulation against oxygen-ozone (EBOO) gaseous mixture was developed with the enthusiastic collaboration of Prof. Nicola Di Paolo [112]. After several years of treatments, it can be stated that this new procedure works well but it has the disadvantages to be more invasive and far more complex and expensive than O-AHT and therefore it will not be discussed [113].

3c. Stroke. On the basis that "time is brain" [37,38], the reperfusion of the ischemic *penumbra* surrounding the core of a cerebral infarction should be performed as soon as possible in about 90% of patients, who either reach the stroke unit after 4.5 h from the prodromic symptoms or are older than 80. The current Guideline rules out IV heparin but recommends oral aspirin (at 325 mg/day as the initial dose). 75-150 mg as a daily dose seems sufficient thereafter. Besides this basic therapy, it appears critical to begin the treatment at the earliest possible time (i.e. after 5 hours from the stroke symptoms) for reducing the irreversible damage due to reperfusion. Moreover, the ability of oxygenating the *penumbra* and an up-regulation induction of the antioxidant protective enzymes synthesis are very important factors. The following results, although anecdotal, lead to believe that O-AHT can be more proficient than either hypothermia, or hyperbaric oxygen treatment, or an albumin infusion. It is unfortunate that data are anecdotal. Wasser [114] presented the results achieved in several patients, who had undergone an acute stroke 1-3 days before. In spite of this limitation, only one daily O-AHT treatment when repeated for a week, improved the outcome in the sense that no patients died and they rapidly recovered. At Cuba, all stroke patients are currently treated with ozone therapy. Dr. Rodriguez et al [115] have treated 150 patients with rectal insufflations of ozone (200 ml of the O_2-O_3 mixture with an ozone concentration of 50 µg/ml (dose 10 mg) and have observed "an astonishing improvement" in 86% of the patients. In another Cuban trial in 100 patients, 200 mL of ozone were daily administered with the same approach at a concentration of 40 µg/mL (8 mg) of ozone for a total of 15 sessions with antiaggregant medication. The clinical stage improved in 80% of patients with an increase of their QoL [116]. Preliminary study on overall 8 subjects have shown a marked improvement of cerebral blood flow after O-AHT

[117,118]. Dr. Peng Kairun, Director of the Neurological Department of the General Hospital of Guangzhou (China) wrote a letter (to VB) stating that, since 2009, they had practiced O-AHT in over 1000 patients with different cerebrovascular diseases with "good results" even in diabetics. All of these results do not get any credit in official medicine because published in unknown journals. The only exception is a recent preclinical study [119] carried out by using rat hippocampal and cortical brain slices subjected to O_2-glucose deprivation (OGD). The results demonstrate that ozone present in the reperfusion medium with a trace of human albumin was very effective in reverting OGD damages. Thus, on the whole, they compel to perform a randomized trial to clarify if O-AHT is indeed a valid therapy. An online-available protocol [120] has elicited interest, but it is stringent to find a "stroke unit" willing to evaluate O-AHT and no rectal insufflations of ozone. We need to evaluate at least 100 stroke patients arrived at the stroke unit after 5-12 hours after the initial symptoms: 50 patients will receive only the official medicine, while the other 50 patients will receive also O-AHT daily for at least three weeks.

3d) AMD, dry form. The first documented clinical trial was performed in the Department of Ophthalmology of the University of Siena and was completed in 1999 [121]. A total of 54 patients were evaluated with O-AHT and only 23 patients with oxygenated blood. All patients presented the dry form and age ranged between 63-81 years old. Mean baseline visual acuity (logMar equivalent) was either 1.27 ± 0.49 or 0.95 ± 0.50 for the O-AHT treated or control group, respectively. An average of 14 sessions (twice weekly) were performed. Best corrected distance visual acuity (Snellen chart), and a complete biomicroscopic and ophthalmoscopic examination with intraocular pressure measured by applanation tonometry were recorded before the first treatment (baseline), after the last one (post-treatment) and then, when possible, every 3 months for up to 18 months; in addition, in order to check the safety of prolonged O-AHT, general haematochemical parameters were recorded at the baseline time and after the end of the cycle of treatments. With regard to ophthalmological results, change in visual acuity from baseline at each follow-up examination was the primary parameter used in order to verify the response, if any, to O-AHT, compared with the

other group. Mean distance best corrected visual acuity (logMar equivalents) was significantly improved in the treatment group of dry AMD's patients, while in the control group only a modest improvement in mean distance visual acuity was observed, which was not statistically significant. In the treatment group an improvement in visual acuity more than 2 ETDRS lines in 36 patients (66.6%), equal or less than 2 ETDRS lines in 18 patients (33.3%) was observed. In the control group an improvement in visual acuity more than 2 ETDRS lines was observed in 7 patients (30.4%), equal or less than 2 ETDRS lines in 16 patients (68.5%). These differences were statistically significant. In the treatment group the improvement remained stable during the first semester, and then slowly declined, but after 18 months only a minimal visual improvement remained in comparison to the acuity values assessed at the start of the study. In the group treated only with oxygen, after 6 months visual acuity had returned to the pre-treatment values and the natural course of dry AMD progressed, with its continuous and rapid visual loss. Haematological data did not show modifications of critical parameters measured before and at the end of the treatment [121]. No side effects either during or after the O-AHT treatment have been observed and actually most of the patients reported a feeling of wellness as well as an improved mental concentration and memory. The compliance remained excellent throughout the study and the maintenance period. Moreover, a second clinical trial on the dry form of AMD by using O-AHT has been published by Borrelli et al. with excellent results [122]. *At the present time there is no other effective medical therapy for the atrophic form of AMD* [50,62,123-125]. Most of these patients, still being physically and mentally active, are very concerned about the lack of a standard treatment. On this basis it is ethically correct to use ozone therapy, not only because patients appreciate it, but because this approach is based on precise biochemical reactions, it is not toxic and it prevents a further photoreceptors death. It is deeply regretted that ophthalmologists, having been informed of this possibility and without knowing anything about ozone therapy, remain uninterested. From 2000 to date, one of us (VB), in a charity clinic, has continued to treat about 80 patients, most of them for several years. Two lessons have been learnt: the first is that O-AHT treatment should be started as

soon as the AMD has been diagnosed to prevent further photoreceptors death and the second is that, after the initial intensive sessions (at least 20 treatments, twice weekly), optimal maintenance therapy is of three treatments/month. Nonetheless, in almost blind patients, even a small gain cannot be disregarded because they report an improved QoL and are less depressed. Finally, retinitis pigmentosa in young patients can be treated with O-AHT for compassionate reasons but only a tenuous and transitory improvement has been observed.

3e) COPD. On March 2014 the therapeutic results achieved in 50 COPD patients have been published [126]. Besides using effective drugs, half of the patients received also O-AHT, twice a week for the first five weeks and then a single treatment every week for another ten weeks. The results have shown that the integration of ozone therapy has been very effective because they markedly improved the six-minute walking test as well as the Saint George Respiratory Questionnaire total score. All these patients reported a striking improvement of their quality of life.

CONCLUDING REMARKS

The aim of this paper is to attract the attention of clinicians on either a neglected or unknown complementary approach, such as the O-AHT. The golden standard of medical therapies for PAOD, CHF, stroke, AMD and COPD have been discussed and, particularly for CHF, they are only partly satisfactory. Their limitations are based on wrong postulations and compel to evaluate a more effective advancement based on the *integration* of standard treatments with O-AHT. Both the basic and molecular mechanisms of O-AHT, not only remain unknown to most clinicians but at least some are sceptical because they cannot believe that small, but well determined doses of ozone, can act as an effective drug. However, they will learn that now several gases such as NO, CO, H_2S, H_2 have become important medical drugs, as shown by recent authoritative reviews and journals dedicated to this topic [127,128], with particular interest on the NO-releasing and the H_2S releasing NSAIDs in inflammatory, cardiovascular and cancer therapy [129,130]. It is therefore regretful that, after having clarified the basic and molecular mechanisms of action of ozone, clinicians and above all patients cannot take advantage of this therapy. *Another vexing problem is that ozone therapy has neither sponsors, nor funds* and valid clinical trials cannot be produced by worldwide private ozone therapists who, too frequently, combine the use of several approaches [131]. It remains to be clarified that the ACCLAIM trial, although supported by a huge investment, showed the failure of the Celacade approach in blocking the progress of CHF [19]. Except than a few criticisms [20,132-134], the irrationality of the approach oxidizing the blood with an enormous ozone dose has not been noticed. Such a clarification must be said because the usual therapeutic dosage of ozone ranges between 10-60 µg (0.21-1.26 µmol)/ml of gas (pure $O_2 \geq 95\%$ and $O_3 \leq 5\%$) per ml of human blood, i.e., at least 140-fold lower than the Celacade technique [19]. As a matter of fact, a previous trial (SIMPADICO) with this technique was suspended owing to the risk of increasing tumour development, though further investigation in 2005 concluded that no safety concerns were related to the trial [135]. The great value of the activation of Nrf2 in patients with AMD and COPD has been

already evaluated. It remains of doubtful value in metastatic cancer because of the cancer cell resilience. This paper may seem provocative but in reality its scope is to present the O-AHT in terms of: i) state-of-the-art; ii) validity in PAOD, AMD, and COPD; and iii) lack of toxicity. In fact, besides the excellent compliance of patients, ozone is not toxic during O-AHT because its dose is minimal, very transitory and far below the antioxidant capacity of plasma [81]. On the other hand, chronic inhalation of ozone, even at low level, is toxic for the pulmonary system, because its prolonged duration and the extremely low level of antioxidant in the respiratory tract [90,136]. The ozone messengers, H_2O_2 and alkenals, are regularly produced by eukaryotic cells and, at the elicited concentrations, they only activate critical biochemical pathways gone astray. O-AHT, if performed as suggested in the protocols, by enhancing the antioxidant defence can indeed restore homeostasis and yield a feeling of wellness. The combination of the best drug treatments (statin, hyperglycemia and hypertension inhibitors) integrated by ozone therapy has the potential to defeat the chronic oxidative stress of cardiovascular pathologies. It is very much hoped that clinical scientists will read this paper and may get an interest to verify the therapeutic validity of the proposed integrated approach, in large, randomized, placebo-controlled, international trials of thousands of patients.

AUTHOR CONTRIBUTIONS

VB conceived, planned, drafted the paper, collated and analysed the data, gathered references and approved the final manuscript. VT contributed to the critical revision of the draft, refined the search for information, analysed the data, assisted with the editing of the paper, gathered references and approved the final manuscript. IZ gathered references and generated the tables as well as the figures. GV contributed with part of the biochemistry. EB helped to manuscript editing. EB, GV, IZ contributed to the approval of the final manuscript.

LIST of ABBREVIATIONS

4-hydroxy-2,3-trans-nonenal (4-HNE); age-related macular degeneration (AMD); alveolar lining fluid (ALF); ankle-brachial index (ABI); antioxidant response element (ARE); chronic heart failure (CHF); chronic limbs ischaemia (CLI); C-reactive protein (CRP); interleukin (IL); intravenous immunoglobulin (IVIG); lipid oxidation products (LOPs); nuclear factor-erythroid 2-related (Nrf2); nuclear factor kB (NFkB); ozonated autohemotherapy (O-AHT); peripheral arterial occlusive disease (PAOD); quality of life (QoL); reactive oxygen species (ROS); retinal pigment epithelium (RPE); recombinant tissue plasminogen activator (rTPA); tumor necrosis factor (TNF); vascular endothelial growth factor (VEGF).

CONFLICT OF INTEREST

The Authors report no conflicts of interest. No funds have been received for this paper. The Authors alone are responsible for the content and writing of the paper.

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FIGURE LEGENDS

Figure 1 – The reaction of ozone with polyunsaturated fatty acids in the presence of water generates 1 mol of hydrogen peroxide and two moles of aldehydes. The actual stereochemistry has not been considered.

Figure 2 – A scheme showing the reaction of ozone with plasma. The generated hydrogen peroxide triggers biochemical pathways in blood cells, while alkenals, after the infusion of ozonated blood into the donor, act on a variety of cells, upregulating the synthesis of many antioxidant proteins.

Figure 3 – The transcription factor Nrf2 bound to Keap-1 activated by alkenals. The released Nrf2 translocates into the nucleus and, after binding to Maf, docks on ARE and activates a number of genes leading to the synthesis of antioxidant proteins.

Table I - Results in PAOD patients treated with O-AHT after Rokitansky et al. [98]

	Leriche-Fontaine stages		
	II	III	IV
Number of Patients	62	51	39
Very good improvement (%)	87	71	54
Walking distance (m)	>1000	>800 (no pain at rest)	>500 (gangrene healed)
Modest improvement (%)	10	22	26[a]
Walking distance (m)	400-500	300-400 (occasional pain)	n.d.[b]
No improvement (%)	3	7	20

[a]In about half patient amputation of toes was necessary but the stump healed; [b]not determined.

Table II - Number and frequency of O-AHT sessions suggested for a PAOD trial

	Frequency of treatments	**Total blood volume (L) treated with O_3**	**Ozone concentration**
Leriche-Fontaine classification			
Stage I	Once weekly for twelve weeks	2.16	From initial 10 µg/mL up to 40 µg/mL of blood. With a slow increase of 5 µg/mL step, when possible
Stage II	Twice weekly for twelve weeks	4.32	From initial 15-20 µg/mL up to 50 µg/mL of blood. With a slow increase of 5 µg/mL step, when possible
Stage III	Thrice weekly for fifteen weeks	8.1	idem
Stage IV	Five times weekly for twenty weeks	18	idem

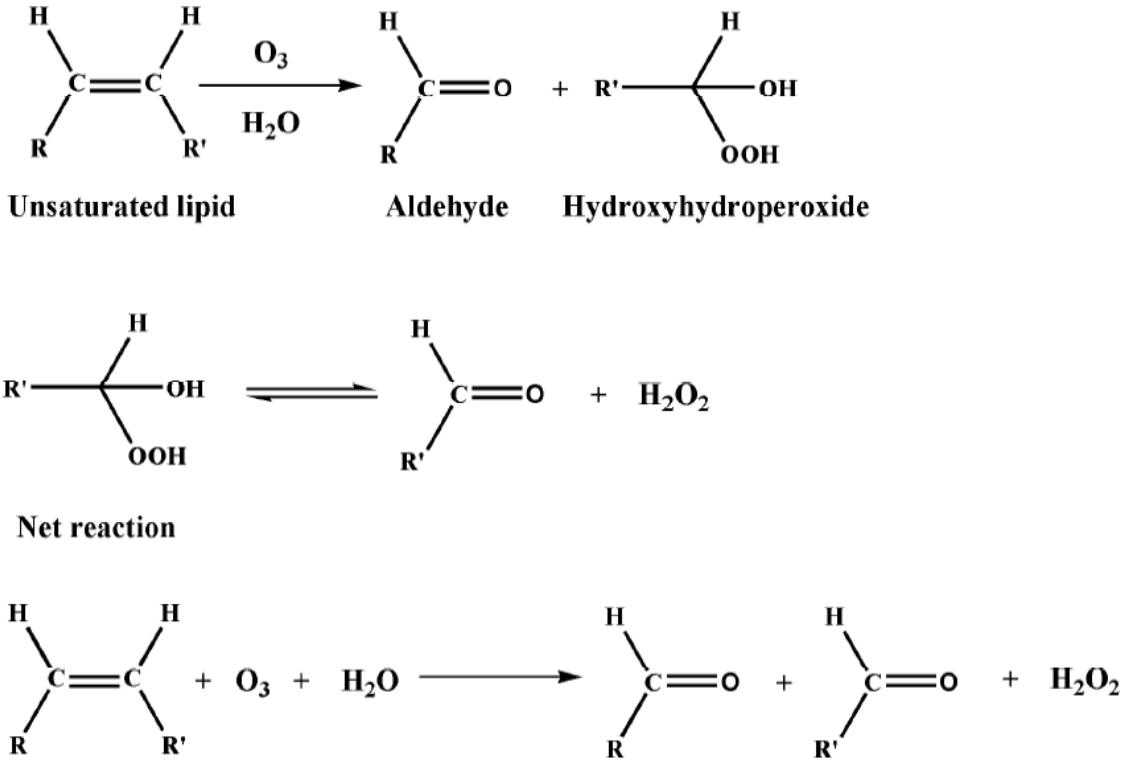
H
H
C
C
R
R'
O3
H2O
H
C
O
R
+
R'
H
OH
OOH
Unsaturated lipid
Aldehyde
Hydroxyhydroperoxide
R'
H
OH
OOH
H
C
O
R'
+
H2O2
Net reaction
+
O3
+
H2O
+
+
H2O2

Figure 1

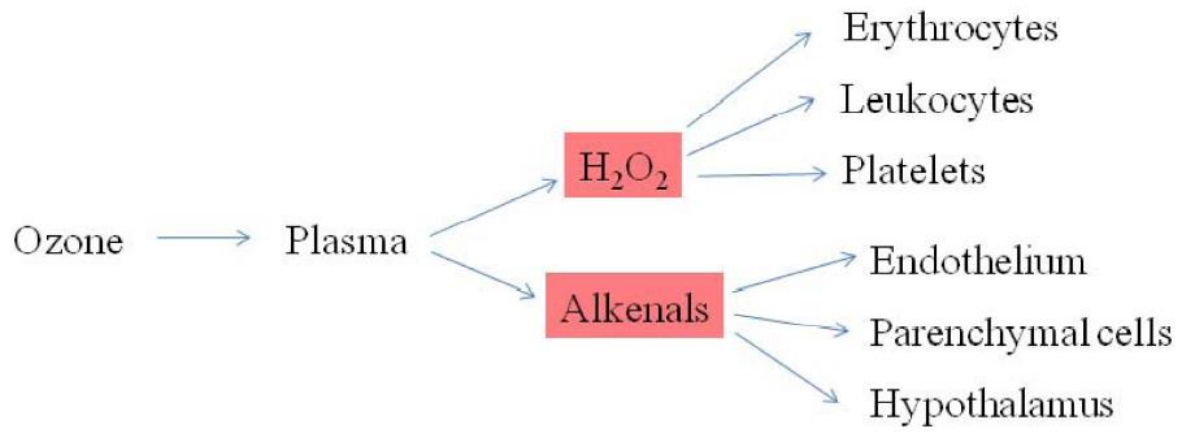
Ozone
Plasma
H_2O_2
Alkenals
Erythrocytes
Leukocytes
Platelets
Endothelium
Parenchymal cells
Hypothalamus

Figure 2

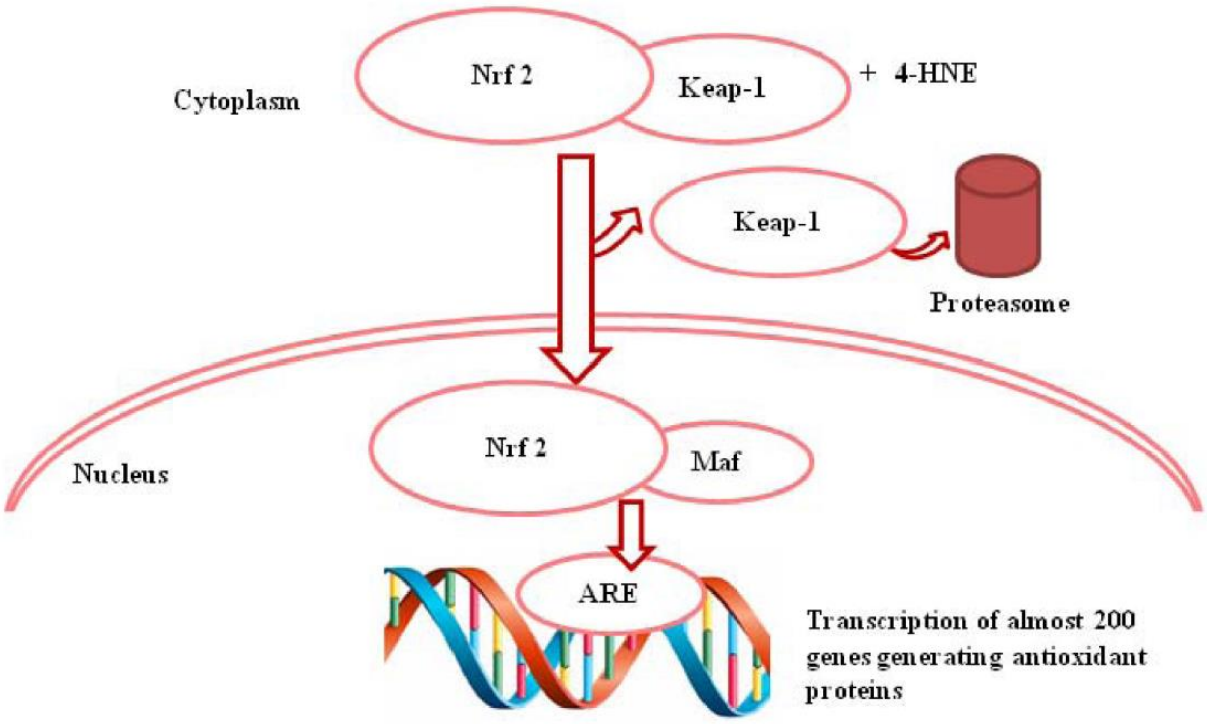
Cytoplasm
Nrf 2
Keap-1
+ 4-HNE
Keap-1
Proteasome
Nucleus
Nrf 2
Maf
ARE
Transcription of almost 200 genes generating antioxidant proteins

Figure 3