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BIOMEDICAL ESSAY

Biological and clinical effects of ozone. Has ozone therapy a future in medicine?

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Abstract: Although ozone therapy has been used as an alternative medical approach for four decades, it has encountered scepticism, if not outright objection, by orthodox medicine. This prejudice is not unjustified because ozone therapy often has been used without rational basis or appropriate controls. With the advent of precise medical ozone generators, it is now possible to evaluate some mechanisms of action and possible toxicity. In contrast with the respiratory tract, human blood exposed to appropriate ozone concentrations is able to tame its strong oxidant properties and neither acute nor chronic side effects have ensued in millions of patients treated with ozonated autohaemotherapy. This paper summarises studies aimed at clarifying biological effects, defining any possible damage, the therapeutic window, and suitable doses able to express therapeutic activity. Although an unfashionable and unpopular approach, it is hoped that orthodox medicine will help to critically assess the validity of ozone therapy.

Key words: Antioxidants. Cytokines. 2,3-diphosphoglycerate. Oxidative stress. Ozone. Reactive oxygen species.

Introduction

Ozone (O_3) is a strong oxidant; however, after being used as a potent disinfectant for almost a century, its usefulness in medicine remains controversial.[1,2] The problem should not be neglected because every year many patients worldwide, including in the US, undergo some form of ozone therapy, and in 1995 the Office of Alternative Medicine of the National Institutes of Health (NIH, MD, USA) included ozone therapy among its pharmacological and biological approaches.

Unfortunately, there is no current constructive dialogue because proponents believe it represents a wonderful remedy and opponents say that O_3 is toxic and should not be used in medicine at all. It is obvious, however, that prejudice exists on both sides and this works against objective judgement. In fact, in my opinion, ozone therapy should only complement orthodox medicine, or substitute it in cases where no other effective therapy exists. On the other hand, although O_3 is very reactive,[3] it is not necessarily toxic, like any other drug, when used properly.

The purpose of this paper is, firstly, to evaluate objectively the clinical application of O_3, secondly, to show that its toxicity can be controlled — thus explaining the lack of adverse effects — and, finally, to point out that the efficacy of ozone therapy can be demonstrated only by randomised, double-blind clinical trials carried out in several medical centres.

The medical applications of ozone therapy

It appears correct to use ozone therapy when orthodox medicine fails to be effective, and the prejudicial view that this alternative therapy cures everything must be dispelled on the grounds that its versatility is simply due to blood cells having different biological functions.[2] Early work was carried out in Germany, and during the last six decades several methods for the application of O_3 in medical therapy have been developed on empirical bases.

The intravenous (IV) route was used mostly in the

past, and involved slow injection of a daily dose of up to 420 mL of O_2–O_3 over two weeks. This technique has been prohibited since 1984 because it caused lung embolism and other adverse effects, and produced doubtful therapeutic benefit. The rationale behind this technique was to consider the human body, composed of about 66% water, as a water sterilisation plant.

The intra-arterial route was used[4] for slow injection of up to 20 mL of O_2–O_3 (O_3 concentration: 30 μg/mL) into the femoral artery in cases of chronic limb ischaemia. Although it was not as dangerous as the IV route, it still presented problems and has been abandoned in favour of autohaemotherapy (AHT). Currently, the direct injection of O_2–O_3 is still practised: via the subcutaneous route, to treat lipodistrophy; via the intramuscular route in the paravertebral muscles, to treat low back pain; via the intradiscal–intraforaminal route, to treat a herniated disc; and via the intraperiarticular route, to treat acute and chronic arthrosis.[5]

Payr[6] and Aubourg[7] were the first to introduce rectal insufflation of O_2–O_3, now performed with a Teflon cannula (rubber is destroyed by O_3). This route has been used widely in human immunodeficiency virus (HIV) infection,[8] ulcerative colitis and Crohn's disease, with apparently satisfactory results,[9] using up to 800 mL of gas at a maximal O_3 concentration of 40 μg/mL, administered over a few minutes. In addition, low concentrations of O_3 (3–5 μg/mL) have been insufflated into the nasal, tubal, oral, vaginal, vesical, pleural and peritoneal cavities in cases of chronic bacterial and parasitic infection when they become resistant to conventional antibiotic therapy.[4]

All of these routes for O_3 administrations have been discussed extensively elsewhere,[2] but two brief observations should be emphasised. First, despite widely different applications, no toxic effects have been reported and only the respiratory tract appears extremely sensitive to O_3 (thus it should not be inhaled). Second, these procedures are empirical and difficult to standardise. Thus, the only approach amenable to scientific control is the exposure of a known amount of blood to a precise O_3 concentration in a known volume of gas.

The merit of having proposed ozonated autohaemotherapy (O_3AHT) goes to Wehrli and Steinbart,[10] and particularly to Wolff,[11] who applied it in the late 1960s. Millions of sessions have been carried out since then, without immediate or late side effects. Several years ago, I began to consider the value of ozonising blood, and what type of reactions would take place.

During the past few years, the autohaemotherapeutic procedure has been standardised, and now comprises *ex vivo* sterile exposure of a known weight of blood (ranging from 200–300 g) to a predetermined O_3 dose (gas volume × O_3 concentration), with O_3 concentration determined precisely in real time by ultraviolet photometry. In the autotransfusion glass bottle, the blood phase is allowed to equilibrate completely with the gas (O_2–O_3) phase for five minutes while it is gently and continuously mixed to avoid foaming. It is then reinfused into the donor.

Depending upon their solubility coefficients, both gases dissolve partially in the plasma water; however, while O_2 is practically stable, and the PO_2 reaches a plateau value well above 100 mmHg within five minutes, O_3 reacts immediately with a range of substrates, including polyunsaturated fatty acids (PUFAs), antioxidant compounds and carbohydrates, such that Henry's law does not apply to O_3. Using this procedure, most, if not all, of the O_3 dose reacts with plasma, and an effort is being made to inform practitioners that a shorter mixing time is suboptimal.

There are five main areas where O_3AHT can be useful — in infectious diseases, vascular disorders, immune depression, degenerative disease, and orthopaedic pathology.

Infectious diseases

Exploiting the disinfectant activity of O_3[1] and the activation of the immune system,[2] it is used as either ozonated bidistilled water or oil in the treatment of war wounds, anaerobic infection, trophic ulcers and burns.[12] Abscesses, anal fissures, bed sores, fistulae, fungal disease, furunculosis, gingivitis, inveterate osteomyelitis, peritonitis, sinusitis, stomatitis, vulvovaginitis and impaired wound healing improve because ozonated solutions have a cleansing effect and act as a powerful disinfectant to which even antibiotic-resistant or anaerobic bacteria succumb.[1,6,7,13–19] It would appear that O_3 not only reduces infection due to its bactericidal activity but also stimulates the metabolism by improving oxygenation and reducing local inflammation. With the current increase in medical costs and antibiotic-resistant infection, O_3 therapy deserves attention because it does not produce resistance and is extremely cheap.

Vascular disorders

Improved delivery of O_2 and release of growth factors appear beneficial in reducing ischaemia and enhancing wound healing. Several observations[20–24] have been reported for chronic lower limb ischaemia, severe Raynaud's syndrome, and cerebral and heart vascular disorders. In order to understand how vasodilation and increased O_2 delivery come about, a series of studies were undertaken to verify the biological effects induced by ozone in blood during the course of AHT.

It has been determined that erythrocytes increase their 2,3-diphosphoglycerate (2,3-DPG) content so

that the dissociation curve of oxyhaemoglobin (HbO_2) shifts to the right (to Hb + O_2) and enhances O_2 delivery to hypoxic tissues, and, more recently, a significant increase in intraerythrocytic adenosine triphosphate (ATP) and energy charge has been demonstrated (Bocci *et al.*, unpublished data). Another relevant line of research aims to evaluate the *in vitro* response of human endothelial cells after exposure to ozonated plasma, simulating the *in vivo* situation during reinfusion of ozonated blood.

Endothelial cells consistently release higher amounts of nitric oxide (NO), which stimulates vasodilation, thus explaining the rapid disappearance of spontaneous pain in patients with ischaemic limbs. However, as yet it is unknown whether or not O_3AHT is also able to induce neoangiogenesis in the ischaemic areas, a very important lead pursued at the same time by gene therapy.[25]

The recent finding[26] that platelets in heparinised plasma release huge amounts of platelet-derived growth factor (PDGF) and transforming growth factor β1 (TGF β1) after ozonation explains, at least in part, the enhanced healing of torpid ulcers in patients with limb ischaemia following O_3AHT.

In spite of these encouraging results, Kraft *et al.*[27] concluded that O_3AHT is not a useful alternative to conventional treatments in patients with mild hypertension. This report is important because rarely has a randomised double-blind, placebo-controlled crossover study been performed to evaluate the efficacy of O_3AHT. The treatment was able to reduce blood pressure significantly, but only for about four months — a result observed also in the treatment of age-related macular degeneration (ARMD). However, this is to be expected and can be minimised by extending the treatment, as happens with other forms of medication.

Another controversial issue is the treatment of retinitis pigmentosa with O_3AHT, electric stimulation and ocular surgery. Berson *et al.*[28] correctly criticised this simultaneous use of various approaches because they have not produced a significant improvement, and it remains unclear whether or not any one of them is effective. Similarly, mixing heat, O_3 and ultraviolet (UV) irradiation in the VasoCare™ therapy[24,29] makes the interpretation of biological and clinical studies very difficult. Moreover, retinitis pigmentosa is a genetic disorder and O_3AHT may only procure a temporary improvement, at best — hardly encouraging when observed in only one patient.[30,31]

Pathology linked to immune depression

Reactivation of a suppressed immune system represents a meaningful approach in various immunodeficiencies associated with chronic viral disease[32] and cancer,[33] particularly after high-intensity chemotherapy and radiotherapy. O_3AHT may activate several mechanisms, previously discussed at length,[32,33] leading to reactivation of immunological surveillance, with practically no side effects. While there are anecdotal reports[33] on the application of ozone therapy in cancer, no controlled clinical trials have been performed.

This approach is appealing in elderly patients where palliative monochemotherapy results in a poor quality of life and little therapeutic advantage. However, it is difficult to assess the number of autotransfusions needed to achieve reactivation of the immune system because it is unclear whether or not the mononuclear cells primed *ex vivo* trigger further activation of resting or suppressed immune cells once these reinfused leucocytes infiltrate lymphoid tissue. About 50 treatments (twice weekly for six months) may activate at least 3×10^{10} immune cells;[33] however, this is only a tentative estimate. Indeed, two clinical studies[34,35] failed to prove the efficacy of O_3 in HIV infection, although, in the study by Garber *et al.*,[34] blood was badly mistreated by heat, UV irradiation and O_3 in unknown concentrations.

Degenerative diseases

Surprisingly, a brief calculated oxidative stress, such as that achieved with O_3AHT, may correct a permanent imbalance caused by excessive and chronic oxidative injury. It has been shown that chronic exposure to increased O_2 tension and low O_3 levels induce tolerance in plants,[36,37] bacteria,[38] mammalian cells,[39] rats[40,41] and humans,[42] and this property is largely caused by upregulation of antioxidant enzymes. An improvement in antioxidant defence may be useful in conditions such as senile dementia, Parkinson's disease, optic nerve dysfunction, and maculopathies, in which the control of endogenous oxidation has gone awry owing to lifelong oxidative damage.[43–45]

It is becoming clear that modest, repeated ozone treatment increases the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH Px). Investigations of O_3AHT, carried out in patients with cardiac infarction,[22] neurodegenerative disease,[46] HIV infection,[42] and ARMD[47] have shown a marked increase in GSH Px, glucose-6-phosphate dehydrogenase (G-6-PD) and SOD in erythrocytes. These studies need to be extended because the possibility of inducing a state of oxidative stress adaptation is very interesting and has important therapeutic implications.

Orthopaedic pathology

Surprising results have been obtained in the treatment of acute and chronic arthropathies,[5] and discal hernia,[48,49] using small volumes of O_2–O_3 and peri-intra-

articular and intradiscal insufflation, respectively. It appears that the treatment for arthropathy, although somewhat painful for a few seconds, has no adverse effects and, in the majority of patients, produces pain relief, decongestion, reabsorption of oedema and increased mobility.

Thus far we can only hypothesise[50] that O_3 therapy induces over-expression of antioxidant enzymes able to neutralise excessive reactive oxygen species (ROS) formation. Ozone may also induce either the release of cytokine antagonists and/or soluble receptors or immunosuppressive cytokines such as interleukin-10 (IL-10) and TGFβ1. After intradiscal injection, O_3 can accelerate the degradation of proteoglycans in the degenerate nucleus pulposus, leading to its reabsorption and the consequent reduction of herniated material responsible for radicular pain.[50]

Finally, only experimental study will confirm the value of injecting O_2–O_3 (2×10 mL, O_3 concentration: 15–20 μg/mL) into the trigger points of the paravertebral muscles in patients with lower back pain syndrome.[51] The mode of action remains uncertain but a plausible explanation[50] is that it represents a type of 'chemical acupuncture' in which the needle and O_3 may inhibit amyelic nociceptor fibres and activate the antinociceptive system, via the stimulation of inhibitory interneurons, and the release of enkephalins.

The analgesia produced permits muscle relaxation and vasodilation, and, hence, a reactivation of muscle metabolism by favouring the oxidation of lactate and neutralisation of acidosis, the increased synthesis of ATP, Ca^{2+} reuptake and reabsorption of oedema. The procedure is very easy and practically free of risk,[51] and has become very popular in Italy. In those that show a response, approximately 10 treatments produce a marked improvement in 66% of patients.

Lower back pain syndrome affects approximately a third of the world's population, and this minimally invasive treatment is worth trying before surgical intervention.

Concern about ozone toxicity

Ozone is one of the most important components of photochemical smog[52] and its toxicity is potentiated by other compounds such as CO, NO_2 and H_2SO_4.[53] Both acute and chronic exposure to these pollutants is harmful to the lungs because the thin layer of respiratory tract lining fluid does not possess sufficient neutralising activity to correct the acid pH, thereby blocking oxidants and producing cell damage.[53–56] From a medical point of view, considerable effort is being made to minimise pulmonary toxicity,[57] but the best approach is to prevent O_3 inhalation.

An artificial situation was used to examine O_3 toxicity in human blood, when erythrocytes or other cells were resuspended in a saline medium and exposed to O_3.[58,59] In this nonphysiological environment, both the erythrocyte membrane and intracellular enzymes were oxidised; however, it would be wrong to conclude that O_3 is always toxic to blood. In contrast to the respiratory system,[60] blood is a fluid tissue, the components of which are in a highly dynamic state and have a considerable ability to rapidly renew antioxidants.[61,62] Furthermore, both plasma and blood cells are endowed with a powerful defence system comprising hydrophilic, lipophilic antioxidants and proteinaceous metal chelators, which limit ROS production.

The antioxidant system is normally effective because it is highly integrated, and oxidative processes can eventually be blocked.[66] Through the activation of biochemical pathways, this system also can rapidly regenerate depleted levels of antioxidants such as α-tocopherol, ascorbic acid and reduced glutathione (GSH).[61,62,65–67] Indeed, it is due to the antioxidant system that careful exposure of human blood to a gas mixture (approximately 97% O_2 and 3% O_3) is not harmful.

Pryor[3] has shown that one facet of the reaction between an olefin and O_3 is the generation of H_2O_2 and aldehydes. Ueno *et al.*,[68] using the electron spin resonance (ESR) technique, recently confirmed that whole blood exposed to O_3 generates two radical species, probably deriving from PUFAs which are abundantly present in blood, particularly in lipoproteins, albumin and cell membranes. On the basis of the data, testing O_3 concentrations in the range 20–80 μg/mL gas per gram of blood (0.42–1.66 mmol/L), a great deal of the oxidant power of O_3 is quenched by PUFAs, antioxidants and albumin rich in –SH groups.

It has been established[63,69] that, after oxidation, compounds such as uric acid and albumin act as 'sacrificial molecules' and are catabolised, while other compounds such as ascorbic acid, α-tocopherol and GSH are regenerated or resynthesised.[61,62,67] The fact that a great deal of O_3 reactivity is exhausted by plasma components and does not harm blood cells is consistent with the data: using up to 80 μg/mL O_3 per gram of blood, methaemoglobin is undetectable and haemolysis is no higher than 1.0% and 1.5% when blood is anticoagulated with citrate phosphate dextrose and heparin, respectively.

Comparatively, only slightly lower values are obtained when mixing blood with O_2 under the same conditions, probably because old erythrocytes are sensitive to mechanical stress.[70] However, when blood is exposed to O_3 concentrations between 100 and 250 μg/mL (2.1–5.2 mmol/L) per gram of blood, haemolysis increases progressively up to approximately 34%.[2] As an index of peroxidation, thiobarbituric acid reactive substances increase progressively with the O_3 dose, and at 80 μg/mL may become six- to eightfold

higher than base values. Interestingly, TBARS have been detected only in plasma and are unmeasurable in isolated erythrocytes after ozonation of whole blood, suggesting that these cells have not reacted with O_3 because they are shielded by albumin molecules.[71]

Evaluation of total antioxidant status[72,73] and protein thiol groups[73,74] in plasma showed, at worst, a mean decrease of 20% and 18%, respectively. Intra-erythrocytic GSH decreased by no more than 15% and 12% when blood was exposed to either O_2–O_3 (80 μg/mL) or O_2 alone.[75] Erythrocyte GSH reductase, GSH-Px, SOD, CAT and G-6-PD levels did not vary during blood ozonation, even when blood reinfusion was delayed for 35 min.[2] That an O_3 concentration as high as 80 μg/mL per gram of blood could exert such a modest and reversible depletion of antioxidants without cell damage was an unexpected finding.

The evaluation of biochemical parameters in blood exposed, almost stoichiometrically, to increasing O_3 concentration (μg/mL per gram of blood) has been instrumental in determining biological effect and/or possible toxicity, and it is evident that for decades ozone therapy failed to progress because it was used by untrained practitioners who were unable to critically analyse the results.

Three interesting points have emerged with ozonation of blood: (i) H_2O_2 is generated and although not a free radical it has been included among ROS and has a relatively long half-life;[2,3] (ii) H_2O_2 levels depend upon O_3 concentration and result from a dynamic equilibrium during its formation, diffusion into intracellular water and degradation; and (iii) intracytoplasmic H_2O_2, although transient, can activate biochemical and immunological pathways.[2,76–78] After ozonation (80 μg/mL) of human plasma, levels of H_2O_2 up to 28 μmol/L have been measured consistently that decline with an average half-life of 2.5 min.[79] In contrast, H_2O_2 disappears very rapidly in whole blood, even in the presence of CAT inhibitors, owing to a powerful combination of antioxidants.

These considerations help to put the problem of O_3 toxicity into the right perspective. We must bear in mind that endogenous ROS are produced throughout life, in several different cell types, during mitochondrial electron transport, metabolism of peroxisomal fatty acids, cytochrome P-450 reactions in the presence of xenobiotics, and in the respiratory burst activity of phagocytes. Therefore, vital cell structures are literally besieged by the continual production of ROS, and sooner or later, despite the presence of the antioxidant system, a shift in favour of oxidation appears unavoidable. Therefore, as O_3 is one of the strongest oxidants, it seems foolish to propose its use as a therapeutic modality. However, the concept that the drug has an intrinsic toxicity is accepted and the beneficial effects must be carefully weighed against toxicity. Moreover, it is now clear that ROS from O_3 are generated and mostly quenched in the plasma; therefore, this exogenous oxidant is not as dangerous as endogenous ones. A second substantial difference is that in using an O_3 dose no higher than 80 μg/mL per gram of blood, the calculated oxidative stress induced is transient. During a whole therapeutic cycle that may last up to six months (two AHT sessions weekly), up to 14.4 kg of blood are exposed briefly to approximately 0.9 g O_3, and Halliwell[80] has estimated that a 70 kg human at rest produces no less than 5 g $^{\bullet}O_2$ per day.

The problem of mutagenicity has been discussed extensively elsewhere.[2] With regard to ozonated blood, no risks have been demonstrated, provided that O_3 concentration is no higher than 80 μg/mL per gram of blood. There is no evidence that O_3AHT produces acute or chronic side effects, even after 60 sessions, and most patients report a feeling of well-being.[2,42] As yet, we do not know if this is because of improved oxygenation and metabolism, real hormonal responses evoked during the reinfusion of ozonated blood, or a psychological factor.

In Germany, Jacobs[81] analysed side effects occurring in over five million ozone therapy sessions in 384 775 patients. Technical errors accounted for minor problems (blood extravasation from the venous access, transient tremor of the lips, occasional nausea) in a minute percentage (0.0007%) of patients — one of the lowest in alternative medicine. However, four deaths occurred as a result of lung embolism following direct intravenous injection of O_2–O_3 — a technique now prohibited. Because of blatant malpractice, two deaths due to lung embolism were registered in Italy in 1997 and 1998.

Which are the effector molecules and which are the targets?

Theories that O_3 is capable of transferring a vital energy to the blood remain in the realm of fiction. Fig. 1 indicates how O_3 works. The sudden increase of intracytoplasmic H_2O_2 appears crucial for the activation of the hexose monophosphate shunt, with important implication for the function of erythrocytes and O_2 delivery.[2] Moreover, in line with current thinking,[82–84] a sudden surge of intracytoplasmic H_2O_2 is responsible for the activation of the nuclear transcription factor (NFκB), first identified by Sen and Baltimore.[85]

In resting lymphocytes, the heterodimer (comprising one 50 kDa [p50] and one 65 kDa [p65] polypeptide) is complexed with the inhibitor protein moieties I-κB. In simple terms, a protein kinase, activated in the presence of H_2O_2, phosphorylates the I-κB subunit and releases the heterodimer to move into the nucleus where, after binding to DNA control elements, it activates gene expression and cytokine synthesis. The

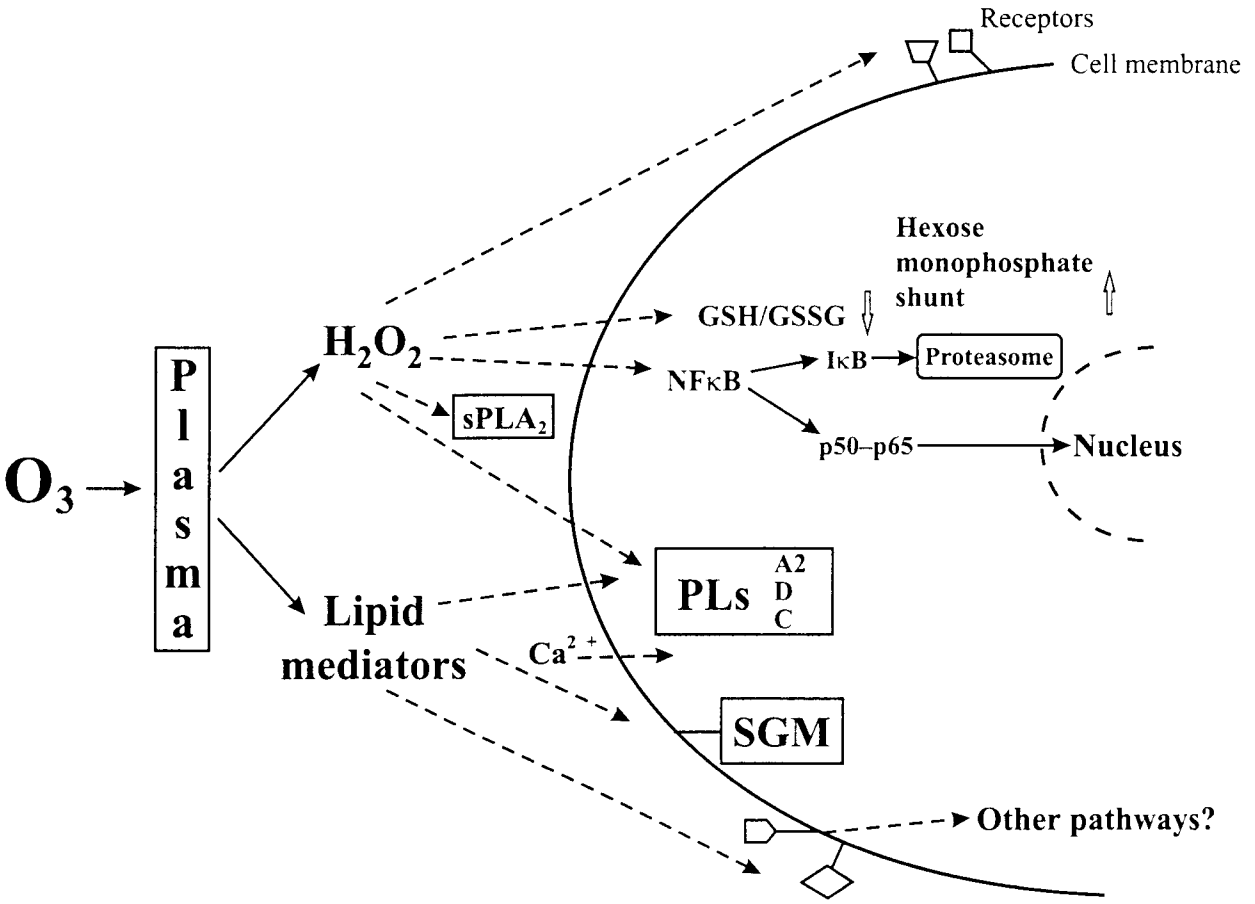

Fig. 1. Hypothetical sites and mechanisms of action of mediators generated from the reaction between O_3 and plasma. The aldehyde 4-hydroxynonenal favours the influx of Ca^{2+}, which in turn enhances intracellular events. GSSG, oxidised glutathione; NFκB: nuclear factor κB; PLs, phospholipases; SGM, sphyngomyelinase; $sPLA_2$, soluble phospholipase A_2.

transient rise of intracytoplasmic H_2O_2 prompts a few considerations. First, O_3 concentration must be adequate to allow sufficient H_2O_2 generation to activate transducer molecules and to counteract the simultaneous degradation. Second, H_2O_2 concentration must reach a critical threshold; below this no stimulation will occur, but, if the concentration is excessive, oxidative damage may result. Therefore, a therapeutic window must be identified.

In the European working population, the mean total antioxidant status is between 1.28 and 1.83 mmol/L plasma,[86] and, owing to the individual variability of the antioxidant system, useful O_3 concentrations range from 20–80 μg/mL (0.42–1.66 mmol/L) per gram of blood. This is in perfect agreement with Ueno *et al.*,[68] who, using another method, found that 'less than 100 μg/mL O_3 was completely consumed by 1 mL of blood by 30 sec mixing.' Although not a practical proposition, it would be ideal to evaluate the optimal O_3 concentration for each patient. Below 20 μg/mL most of the oxidant power of O_3 is quenched by natural antioxidants and, therefore, precise measurement of O_3 concentration is crucially important in avoiding either a placebo or toxic effect.

In reacting with PUFAs,[3,87] O_3 generates an array of lipid oxidation products (LOPs) including hydroperoxides, isoprostanes,[88] and terminal products such as malondialdehyde and 4-hydroxynonenal. These terminal products are of particular interest because, depending upon final concentration (>10 μmol/L or <1 μmol/L), they may be either harmful[89] or act as a physiological messenger.[90,91] Owing to the wealth and heterogeneity of PUFAs, several types of LOPs may be generated and their biological activities, including potential toxicity, are unknown areas that need to be explored *in vivo*.

Phospholipases and sphyngomyelinase (Fig. 1) are likely to be activated by LOPs, and this may lead to an amplification of some biological processes such as enhanced Ca^{2+} flux and further production of active

LOPs. Furthermore, LOPs have a short half-life but, upon reinfusion of ozonated blood, may reach specific sensors situated in critical organs such as bone marrow, spleen, liver and other sectors of the immune system. If true, LOPs may be responsible for transmitting[92] peroxidative stress information and possibly inducing up-regulation of antioxidant enzymes, hence the tolerance to O_3. This interesting phenomenon, termed oxidative stress adaptation,[2,42] has an important practical implication in the sense that judicious use of O_3AHT may reverse chronic oxidative stress syndrome in patients with chronic viral infections,[93] cancer[43,94] and neurodegenerative diseases.[95]

Administration of various antioxidants may only partly correct chronic oxidative stress, and the exciting possibility is to reverse it by inducing O_3 tolerance. This is a complex phenomenon, including a number of events represented by changes in gene expression,[38] followed by synthesis of heat shock proteins (HSPs) involved in adaptation to stress. When exposed to low levels of a toxic agent, living organisms may respond by apoptosis or become resistant by activating inducible genes. The latter is carried out by synthesising a variety of HSPs, several antioxidant enzymes, haem oxygenase and DNA repair enzymes. In this way, organisms may readjust the redox balance.[42] Obviously, the calculated therapeutic stress of ozone therapy must continue, but its length of effectiveness remains to be determined during long follow-up of patients.

Leucocytes appear to be primed and able to release small amounts of cytokines, such as interferon β and γ, and several ILs.[73,75–79,96,97] On reaching the lymphoid microenvironments, cytokines and LOPs may modulate immune cells and possibly correct an immune deficiency. However, a great deal of experimental study remains to be done to assess the full extent of immunological activation and the role of O_3AHT in the treatment of chronic viral infection.

It would appear that O_3AHT simultaneously triggers a range of biological effects that work together in hypoxic tissues, where, for example, vasodilation, enhanced O_2 delivery and release of growth factors accelerate healing of chronic ulcers.[98]

Finally, the critical problem of the O_3 dose in different pathological conditions needs to be addressed.

The problem of ozone dose and the application of the 'start low, go slow' principle

The question of O_3 dose is one of the most frequently debated and it appears useful to give a guideline. On the basis of present knowledge, the therapeutic window range is 20–80 μg/mL of O_3 per gram of blood. Within this range, toxicity is minimal or absent, even if the total antioxidant status of plasma is as low as 1.2 mmol/L. As yet, it is impossible to provide specific doses for individual pathologies because ample and controlled clinical studies have yet to be performed. However, on the basis of biochemical and empirical results, it is possible to make suggestions (Table 1).

Table 1. Ozone doses used in autohaemotherapy

Pathology	O_3 doses (μg/mL per gram of blood)	
	Initial	Final
Vascular disease	20	40
Degenerative disease	20	40
Infectious disease	25	70
Respiratory disease	20	40
Autoimmune disease	20	?
Metastatic cancer	25	80

In order to avoid toxicity and allow oxidative stress adaptation, the safest strategy starts with very low doses, increasing in single steps of 5 μg/mL per gram of blood to the highest level. As treatments are performed on a day-hospital basis, O_3AHT twice weekly is practical and sufficient to achieve a clinical response. If necessary, this can be increased up to four times weekly, allowing adaptation during the first three weeks.

In elderly patients who are undernourished or not on a proper diet, a multivitamin complex can be administered orally on the day before O_3AHT. Normally, a daily dose of vitamin C (0.5 g), supplemented with *N*-acetylcysteine (0.6 g) as a precursor of GSH is optimal. Larger amounts may prove useless or even have a detrimental effect.[99]

Conclusions

The complementary approach of ozone therapy has been used for at least four decades, but objective discussion between those for and against it has yet to begin. After previous attempts,[2,100,101] it is hoped that this review will succeed in opening constructive dialogue.

There is no doubt about the potential toxicity of O_3 and the harmful effects of acute and chronic exposure on the respiratory tract. Moreover, direct IV administration of O_3 should *never* be performed, as it is both irrational and dangerous. On the other hand, experimental data have shown that transient *ex vivo* exposure of blood to controlled and appropriate O_3 concentrations is not toxic to humans.

Recent advances in the understanding of signal transduction have shown that minute amounts of oxidants, gaseous regulators and even 4-hydroxynonenal

act as cell messengers, and therefore the appropriate use of O_3 in medicine no longer appears irrational.

I have come to regard O_3 as a real drug and, as such, it must be used with the utmost care — too little may be useless and too much can be harmful. The relevance of a placebo effect, either due to O_2 or too little O_3, also needs to be clarified. Equally important is the definition of its clinical efficacy and when ozone therapy can be used. A typical example is the treatment of chronic hepatitis in elderly patients who are intolerant of interferon α — a costly substance which often produces side effects. This last point needs to be considered in relation to other pathological conditions, notwithstanding the necessity for careful monitoring of possible, as yet undetected, problems.

Although much remains to be done, ozone therapy is now amenable to scientific scrutiny and attention should be concentrated on the following points:

- accurate dosimetry of O_3 concentration;
- standardisation of the procedure for O_3AHT;
- further understanding of biological effects, particularly oxidative stress adaptation;
- evaluation of any possible long-term toxicity;
- definition of optimal O_3 dose in different pathological conditions;
- randomised, double-blind clinical studies using either O_2–O_3 or O_2 alone versus conventional treatments, with assessment of long-term follow up; and
- evaluation of novel approaches and routes of O_3 administration.

In the age of molecular medicine it is a real 'act of faith' to believe that ozone therapy might be a valid therapeutic option, but the history of medicine teaches us that we should not disregard any possibility. While strongly disapproving of the misuse of O_3 and the deplorable exploitation of patients, to remain sceptical and inert does nothing to help solve this problem.

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References

1 Viebahn R. *The use of ozone in medicine*, vol. 2. Heidelberg: Karl F Haug Publishers, 1994.
2 Bocci V. Ozone as a bioregulator. Pharmacology and toxicology of ozonetherapy today. *J Biol Regul Homeost Agents* 1996; 10:31–53.
3 Pryor WA. Mechanisms of radical formation from reactions of ozone with target molecules in the lung. *Free Radical Biol Med* 1994; 17:451–65.
4 Mattassi R. *Ozonoterapia*. Milano: Organizzazione Editoriale Medico Farmaceutica, 1985.
5 Siemsen C-H. Ozon-Anwendung bei akuten und chronischen Gelenkerkrankungen. In: Beck EG, Viebahn-Hänsler R, eds. *Ozon-Handbuch. Grundlagen. Prävention. Therapie.* Landsberg/Lech: Ecomed, 1995: V9.2 1–V9.2 14.
6 Payr E. Über ozonbehandlung in der chirurgie. *Münch med Wochenschr* 1935; 82:220–91.
7 Aubourg P. Ozon in der chirurgie. *Mem Acad Chir* 1940; 65:1183–92.
8 Carpendale MT, Freeberg J, McLeod Griffiss J. Does ozone alleviate AIDS diarrhoea? *J Clin Gastroenterol* 1993; 17:142–5.
9 Knoch H-G, Klug W. Ozon-sauerstoff-therapie der proktitis. *Med Welt* 1990; 41:371–4.
10 Wehrli F, Steinbart H. Erfahrungen mit der haematogenen oxydations — therapie (HOT). *Ars Medici* 1954; 10:44–51.
11 Wolff HH. *Das medizinische ozon. Theoretische grundlagen, therapeutische anwedungen.* Heidelberg: Verlag für Medizin, 1979.
12 Miroshin SJ, Kontorshikova CN. The use of ozonetherapy technology in the treatment of modern war surgical trauma. In: *The ozone in biology and medicine.* 2nd All Russian Scientific–Practical Conference, September 6–8, 1995. Russian Association of Ozonetherapy, Reshetnikovskaya Street 2, Nizhni Novgorod, 603006 Russia. 1995:16.
13 Shaschova NM, Kachalina TS, Nevmjatullin AL. Application of ozonotherapy in complex treatment of inner female genital inflammatory diseases. In: International Ozone Association, ed. *Proceedings: ozone in medicine.* 12th World Congress of the International Ozone Association, 15–18 May 1995, Lille France. Tours: Instaprint SA, 1995:145–55.
14 Filippi A, Kirschner H. Ozoniertes wasser zur desinfektion und prophylaxe in der zahn-mund-kieferheilkunde. In: Beck EG, Viebahn-Hänsler R, eds. *Ozon-handbuch. Grundlagen. Prävention. Therapie.* Landsberg/Lech: Ecomed, 1995: V12.2 1–V12.2 12.
15 Wasser GH. Behandlung von verletzungen mit ozoniertem wasser. In: Beck EG, Viebahn-Hänsler R, eds. *Ozon-handbuch. Grundlagen. Prävention. Therapie.* Landsberg/Lech: Ecomed, 1995: V7.4 1–V7.4 8.
16 Bulinin VI, Solod NV, Moshurov IP. The first experience of chronic abscesses and pleura emphyema treatment by the method of ozonization. In: *The ozone in biology and medicine.* 2nd All Russian Scientific–Practical Conference, September 6–8, 1995. Russian Association of Ozonetherapy, Reshetnikovskaya Street 2, Nizhni Novgorod, 603006 Russia. 1995:20.
17 Kudravcev BP, Miroshin SJ, Semyonov SV. The use of ozonized solutions in complex treatment of peritonitis. In: *The ozone in biology and medicine.* 2nd All Russian Scientific–Practical Conference, September 6–8, 1995, Russian Association of Ozonetherapy, Reshetnikovskaya Street 2, Nizhni Novgorod, 603006 Russia. Nizhni Novgorod: 1995:20.
18 Kasumjan SA, Lelyanov AD, Guseva ED, Alexeev BP. The ozonetherapy of the acute suppurative infection. In: *The ozone in biology and medicine.* 2nd All Russian Scientific–Practical Conference, September 6–8, 1995, Russian Association of Ozonetherapy, Reshetnikovskaya Street 2, Nizhni Novgorod, 603006 Russia. Nizhni Novgorod: 1995:16
19 Schultz S. The role of ozone/oxygen in clindamycin-associated enterocolitis in the Djungarian hamster (*Phodopus sungorus sungorus*). *Lab Anim* 1986; 20:41–8.
20 Rokitansky O, Rokitansky A, Steiner I, Trubel W, Viebahn R, Washüttl J. Die Ozontherapie bei peripheren, arteriellen durchblutungsstörungen: klinik, biochemische und blutgasanalytische untersuchungen. In: *Wassar.* Berlin: Ozon-Weltkongress, 1981:53–75.

21 Werkmeister H. Dekubitalgeschwüre und die Behandlung mit der Ozon-Unterdruckbegasung. In: Beck EG, Viebahn-Hänsler R, eds. *Ozon-handbuck. Grundlagen. Prävention. Therapie.* Landsberg/Lech: Ecomed, 1995:V7.1 1–V7.1 22.
22 Hernàndez F, Menéndez S, Wong R. Decrease of blood cholesterol and stimulation of antioxidative response in cardiopathy patients treated with endovenous ozone therapy. *Free Radical Biol Med* 1995; 19:115–9.
23 Wasser GH. Zerebrale durchblutungsstörungen. In: Beck EG, Viebahn-Hänsler R, eds. *Ozon-Handbuch. Grundlagen. Prävention. Therapie.* Landsberg/Lech: Ecomed, 1995:V6.3 1–V6.3 12.
24 Cooke ED, Pockley AG, Tucker AT, Kirby JDT, Bolton AE. Treatment of severe Raynaud's syndrome by injection of autologous blood pretreated by heating, ozonation and exposure to ultraviolet light (H-O-U) therapy. *Int Angiol* 1997; 16:250–4.
25 Laitinen M, Mäkinen K, Manninen H *et al.* Adenovirus-mediated gene transfer to lower limb artery of patients with chronic critical leg ischemia. *Hum Gene Ther* 1998; 9:1481–6.
26 Valacchi G, Bocci V. Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets. *Mediat Inflamm* 1999; 8:In press.
27 Kraft K, Stenkamp E, Sachinidis A, Seewald S, Vetter H. Effect of autohemotherapy with ozone on cardiovascular risk factors in patients with mild hypertension. *Perfusion* 1998; 11:216–9.
28 Berson EL, Remulla JFC, Rosner B, Sandberg MA, Weigel-DiFranco C. Evaluation of patients with retinitis pigmentosa receiving electric stimulation, ozonated blood, and ocular surgery in Cuba. *Arch Ophthalmol* 1996; 114:560–3.
29 Bulmer J, Bolton AE, Pockley AG. Effect of combined heat, ozonation and ultraviolet irradiation (VasoCare™) on heat shock protein expression by peripheral blood leukocyte populations. *J Biol Regul Homeost Agents* 1997; 11:104–10.
30 Marmer RH, Parks S. Ozone treatment in retinitis pigmentosa: effect on color perception and blood gases. *Ann Ophthalmol* 1998; 30:161–3.
31 Marmer RH, Parks S. Ozone treatment in retinitis pigmentosa: effect on photo stress and contrast sensitivity. *Ann Ophthalmol* 1998; 30:164–6.
32 Bocci V. A reasonable approach for the treatment of HIV infection in the early phase with ozonetherapy (autohemotherapy). How inflammatory cytokines may have a therapeutic role. *Mediat Inflamm* 1994; 3:315–21.
33 Bocci V. Ozonetherapy as a possible biological response modifier in cancer. *Forsch Komplementärmed* 1998; 5:54–60.
34 Garber GE, Cameron DW, Hawley-Foss N, Greenway D, Shannon ME. The use of ozone-treated blood in the therapy of HIV infection and immune disease: a pilot study of safety and efficacy. *AIDS* 1991; 5:981–4.
35 Bocci V, Venturi G, Catucci M, Valensin PE, Zazzi M. Lack of efficacy of ozonetherapy in HIV infection. *Clin Microbiol Infect* 1998; 4:667–9.
36 Sharma YK, León J, Raskin I, Davis KR. Ozone-induced responses in *Arabidopsis thaliana*: the role of salicyclic acid in the accumulation of defense-related transcripts and induced resistance. *Proc Natl Acad Sci USA* 1996; 93:5099–104.
37 Sandermann Jr H, Ernst D, Heller W, Langebartels C. Ozone: an abiotic elicitor of plant defence reactions. *Trends Plant Sci* 1998; 3:47–50.
38 Christman MF, Morgan RW, Jacobson FS, Ames BN. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* 1985; 41:753–62.
39 Spitz DR, Dewey WC, Li GC. Hydrogen peroxide or heat shock induces resistance to hydrogen peroxide in chinese hamster fibroblasts. *J Cell Physiol* 1987; 131:364–73.
40 Rahman I, Clerch LB, Massaro D. Rat lung antioxidant enzyme induction by ozone. *Am J Physiol* 1991; 260:L412–8.
41 León OS, Menéndez S, Merino N *et al.* Ozone oxidative preconditioning: a protection against cellular damage by free radicals. *Mediat Inflamm* 1998; 7:289–94.
42 Bocci V. Does ozone therapy normalize the cellular redox balance? *Med Hypotheses* 1996; 46:150–4.
43 Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants and the degenerative diseases of aging. *Proc Natl Acad Sci USA* 1993; 90:7915–22.
44 Bondy SC. The relation of oxidative stress and hyperexcitation to neurological disease. *Proc Soc Exp Biol Med* 1995; 208:337–45.
45 Spector A. Oxidative stress-induced cataract: mechanism of action. *FASEB J* 1995; 9:1173–82.
46 Gomez Moraleda MA. Ozone therapy in the functional recovery from diseases involving damage to central nervous system cells. In: International Ozone Association, ed. *Proceedings: ozone in medicine.* 12th World Congress of the International Ozone Association, 15–18 May 1995, Lille, France. Tours: Instaprint SA, 1995:111–23.
47 Bocci V. Die senile makulopathie und verwandte erkrankungen. In: Beck EG, Viebahn-Hänsler R, eds. *Ozon-handbuch. Grundlagen. Prävention. Therapie.* Landsberg: Ecomed, 1997:1–25.
48 Fabris G, Tommasini G, Lavaroni A *et al.* Percutaneous treatment of lumbar herniated disk. *Riv Neuroradiol* 1997; 10:523–32.
49 Alexandre A, Fumo G. Discolisi percutanea mediante O_2O_3 nell'ernia discale lombare. In: Ceccherelli F, Ricciardi A, eds. *Lombalgie e lombosciatalgie. Criteri di diagnosi e cura.* Torino: Edizioni Libreria Cortina, 1998:367–77.
50 Bocci V. Ipotetici meccanismi di azione dell'ozono nel trattamento del conflitto discoradicolare. In: Ceccherelli F, Ricciardi A, eds. *Lombalgie e lombosciatalgie. Criteri di diagnosi e cura.* Torino: Edizioni Libreria Cortina, 1998:331–40.
51 Brayda-Bruno M, Cinnella P. Il trattamento dell'ernia discale con infiltrazioni di ossigeno-ozono in paravertebrale. In: Ceccherelli F, Ricciardi A, eds. *Lombalgie e lombosciatalgie. Criteri di diagnosi e cura.* Torino: Edizioni Libreria Cortina, 1998:361–5.
52 US Environmental Protection Agency. *Air quality criteria for ozone and other photochemical oxidants. Environmental criteria and assessment office.* Research Triangle Park, NC: US Environmental Protection Agency. Publication no. EPA-600/8–84–020aF, 1986.
53 Warren DL, Last JA. Synergistic interaction of ozone and respirable aerosols on rat lungs. III. Ozone and sulfuric acid aerosol. *Toxicol Appl Pharmacol* 1987; 88:203–16.
54 O'Neill CA, van der Vliet A, Hu M-L *et al.* Oxidation of biologic molecules by ozone: the effect of pH. *J Lab Clin Med* 1993; 122:497–505.
55 Cross CE, van der Vliet A, O'Neill CA, Louie S, Halliwell B. Oxidants, antioxidants and respiratory tract lining fluids. *Environ Health Perspect* 1994; 102:185–91.
56 Kelly FJ, Mudway IS. Sensitivity to ozone: could it be related to an individual's complement of antioxidants in lung epithelium lining fluid? *Redox Report* 1997; 3:199–206.
57 Heffner JE, Repine JE. Pulmonary strategies of antioxidant defense. *Am Rev Respir Dis* 1989; 140:531–54.
58 Goldstein BD, Balchum OJ. Effect of ozone on lipid peroxidation in the red blood cell. *Proc Soc Exp Biol Med* 1967; 126:356–9.

59 Freeman BA, Mudd JB. Reaction of ozone with sulfhydryls of human erythrocytes. *Arch Biochem Biophys* 1981; **208**:212–20.

60 Pryor WA. How far does ozone penetrate into the pulmonary air/tissue boundary before it reacts? *Free Radical Biol Med* 1992; **12**:83–8.

61 Meister A. Glutathione–ascorbic acid antioxidant system in animals. *J Biol Chem* 1994; **269**:9397–400.

62 May JM, Qu Z-C, Whitesell RR, Cobb CE. Ascorbate recycling in human erythrocytes: role of GSH in reducing dehydroascorbate. *Free Radical Biol Med* 1996; **20**:543–51.

63 van der Vliet A, O'Neill CA, Eiserich JP, Cross CE. Oxidative damage to extracellular fluids by ozone and possible protective effects of thiols. *Arch Biochem Biophys* 1995; **321**:43–50.

64 Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol Rev* 1994; **74**:139–62.

65 Galleano M, Puntarulo S. Role of antioxidants on the erythrocytes resistance to lipid peroxidation after acute iron overload in rats. *Biochim Biophys Acta* 1995; **1271**:321–6.

66 Halliwell B. Antioxidants in human health and disease. *Annu Rev Nutr* 1996; **16**:33–50.

67 Mendiratta S, Qu Z-C, May JM. Erythrocyte ascorbate recycling: antioxidant effects in blood. *Free Radical Biol Med* 1998; **24**:789–97.

68 Ueno I, Hoshino M, Miura T, Shinriki N. Ozone exposure generates free radicals in the blood samples *in vitro*. Detection by the ESR spin-trapping technique. *Free Radical Res* 1998; **29**:127–35.

69 Cross CE, Reznick AZ, Packer L, Davis PA, Suzuki YJ, Halliwell B. Oxidative damage to human plasma proteins by ozone. *Free Radical Res Commun* 1992; **15**:347–52.

70 Bocci V. Determinants of erythrocyte ageing: a reappraisal. *Br J Haematol* 1981; **48**:515–22.

71 Shinriki N, Suzuki T, Takama K *et al.* Susceptibilities of plasma antioxidants and erythrocyte constituents to low levels of ozone. *Haematologia* 1998; **29**:229–39.

72 Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci* 1993; **84**:407–12.

73 Bocci V, Valacchi G, Corradeschi F, Fanetti G. Studies on the biological effects of ozone: 8. Effects on the total antioxidant status and on interleukin-8 production. *Mediat Inflamm* 1998; **7**:313–7.

74 Hu M-L. Measurement of protein thiol groups and glutathione in plasma. *Methods Enzymol* 1994; **233**:380–5.

75 Bocci V, Luzzi E, Corradeschi F *et al.* Studies on the biological effects of ozone: 4. Cytokine production and glutathione levels in human erythrocytes. *J Biol Regul Homeost Agents* 1993; **7**:133–8.

76 Bocci V, Paulesu L. Studies on the biological effects of ozone. 1. Induction of interferon gamma on human leucocytes. *Haematologica* 1990; **75**:510–5.

77 Paulesu L, Luzzi E, Bocci V. Studies on the biological effects of ozone: 2. Induction of tumor necrosis factor (TNF-α) on human leucocytes. *Lymphokine Cytokine Res* 1991; **10**:409–12.

78 Bocci V, Luzzi E, Corradeschi F, Paulesu L, Di Stefano A. Studies on the biological effects of ozone: 3. An attempt to define conditions for optimal induction of cytokines. *Lymphokine Cytokine Res* 1993; **12**:121–6.

79 Bocci V, Valacchi G, Corradeschi F *et al.* Studies on the biological effects of ozone: 7. Generation of reactive oxygen species (ROS) after exposure of human blood to ozone. *J Biol Regul Homeost Agents* 1998; **12**:67–75.

80 Halliwell B. Free radicals and antioxidants: a personal view. *Nutr Rev* 1994; **53**:253–65.

81 Jacobs M-T. Untersuchung uber zwischenfalle und typische komplikationen in der ozon-sauerstoff-therapie. *Ozo Nachrichten* 1982; **1**:5.

82 Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J* 1991; **10**:2247–58.

83 Los M, Dröge W, Stricker K, Baeuerle PA, Schulze-Osthoff K. Hydrogen peroxide as a potent activator of T-lymphocyte functions. *Eur J Immunol* 1995; **25**:159–65.

84 Suzuki YJ, Forman HJ, Sevanian A. Oxidants as stimulators of signal transduction. *Free Radical Biol Med* 1997; **22**:269–85.

85 Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1986; **46**:705–16.

86 Rice-Evans C, Miller NJ. Total antioxidant status in plasma and body fluids. In: *Methods in enzymology*. New York: Academic Press, Inc, 1994:279–93.

87 Pryor WA, Squadrito GL, Friedman M. The cascade mechanism to explain ozone toxicity: the role of lipid ozonation products. *Free Radical Biol Med* 1995; **19**:935–41.

88 Morrow JD, Roberts LJ. The isoprostanes: unique bioactive products of lipid peroxidation. *Prog Lipid Res* 1997; **36**:1–21.

89 Del Corso A, Dal Monte M, Vilardo PG *et al.* Site-specific inactivation of aldose reductase by 4-hydroxynonenal. *Arch Biochem Biophys* 1998; **350**:245–8.

90 Leonarduzzi G, Scavazza A, Biasi F *et al.* The lipid peroxidation end product 4-hydroxy-2,3-nonenal up-regulates transforming growth factor β1 expression in the macrophage lineage: a link between oxidative injury and fibrosclerosis. *FASEB J* 1997; **11**:851–7.

91 Dianzani MU. 4-Hydroxynonenal and cell signalling. *Free Radical Res* 1998; **28**:553–60.

92 Serhan CN, Haeggström JZ, Leslie CC. Lipid mediator networks in cell signalling: update and impact of cytokines. *FASEB J* 1996; **10**:1147–58.

93 Schwarz KB. Oxidative stress during viral infection: a review. *Free Radical Biol Med* 1996; **21**:641–9.

94 Toyokuni S, Okamoto K, Yodoi J, Hiai H. Persistent oxidative stress in cancer. *FEBS Lett* 1995; **358**:1–3.

95 Simonian NA, Coyle JT. Oxidative stress in neurodegenerative diseases. *Annu Rev Pharmacol Toxicol* 1996; **36**:83–106.

96 Bocci V, Luzzi E, Corradeschi F, Paulesu L. Studies on the biological effects of ozone: 5. Evaluation of immunological parameters and tolerability in normal volunteers receiving ambulatory autohaemotherapy. *Biotherapy* 1994; **7**:83–90.

97 Bocci V, Luzzi E, Corradeschi F, Silvestri S. Studies on the biological effects of ozone: 6. Production of transforming growth factor b1 by human blood after ozone treatment. *J Biol Regul Homeost Agents* 1994; **8**:108–12.

98 Martin P. Wound healing — aiming for perfect skin regeneration. *Science* 1997; **276**:75–81.

99 Levine M, Conry-Cantilena C, Wang Y *et al.* Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci USA* 1996; **93**:3704–9.

100 Bocci V. Autohaemotherapy after treatment of blood with ozone. A reappraisal. *J Int Med Res* 1994; **22**:131–44.

101 Bocci V. Ozone: a mixed blessing. New mechanisms of the action of ozone on blood cells make ozonated major autohaemotherapy (MAH) a rational approach. *Forsch Komplementärmed* 1996; **3**:25–33.