

# Ozone as a bioregulator. Pharmacology and toxicology of ozonotherapy today

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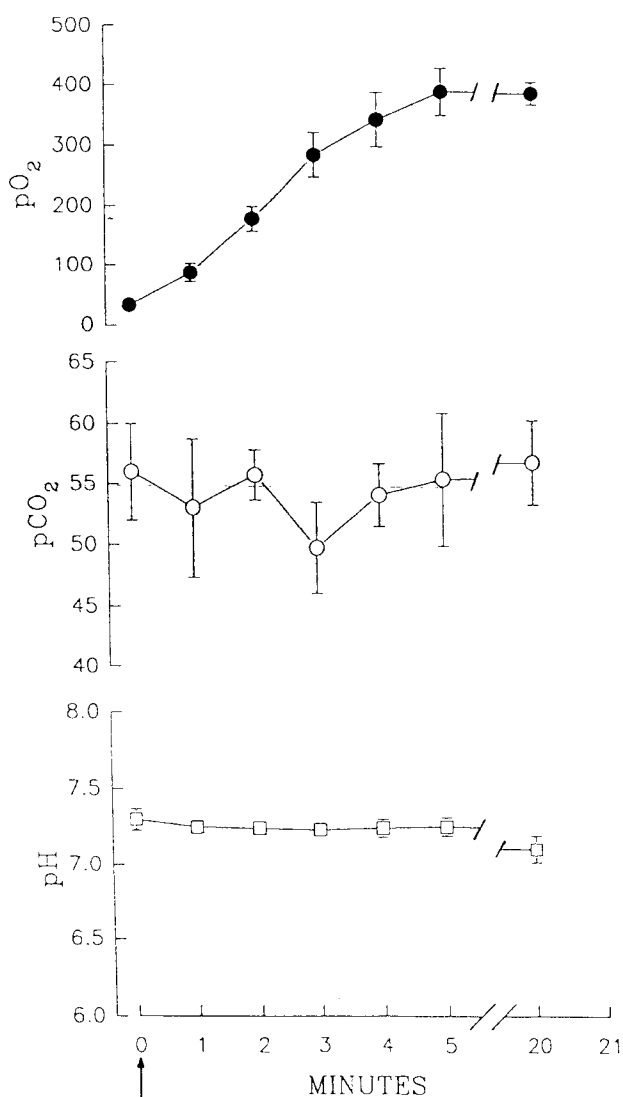
*ABSTRACT: The disinfectant activity of ozone is well recognized and ozone is used worldwide for sterilization of water. The use of ozone as a complementary medical approach is less known, because it has mostly been used in an empirical fashion without a rational basis and appropriate controls. In spite of this drawback, the use of judicious and standardized ozone dosages can elicit the formation of ROS acting as natural physiological activators of several biological functions. There is now a reasonable understanding of a few mechanisms of action and, using classical pharmacological concepts, it appears possible to formulate a rationale for optimizing clinical applications. A further exciting development is that ozone, being an oxidizer, can upregulate the intracellular anti-oxidant enzymes eventually inhibiting the constant, life-long oxidative stress responsible for degenerative diseases and aging. Among various routes for the administration of ozone, the autohemotransfusion procedure, consisting in exposing blood to ozone, i.e. to a calculated and brief oxidative stress, appears safe, simple, inexpensive and amenable to be adjusted to different pathological states. It is hoped that this review will help to dispel prejudices, to clarify that ozone toxicity can be tamed, to show that ozone can act as a bioregulator and to encourage controlled clinical investigations to evaluate definitively the validity of ozonotherapy. (J Biol Regul Homeost Agents, 1996; 10: 31-53)*

*KEY WORDS: Antioxidants, Cytokines, Hydrogen peroxide, Ozone, Peroxidation, Reactive oxygen species*

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## 1. INTRODUCTION

In June 1995, in order to facilitate the grant review process, the advisory panel to the office of alternative medicine of the National Institutes of Health (Bethesda MD, USA) classified up to 61 approaches of alternative medicine (Office of Alternative Medicine, Public Information Center, 1995). For several reasons the term "complementary" appears more appropriate than "alternative" and it will be used in this review. Among several subgroups, the pharmacological and biological treatments included the use of oxidizing agents (ozone and hydrogen peroxide).

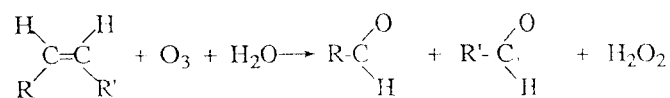


**Fig. 1** - Kinetic of pO<sub>2</sub>, pCO<sub>2</sub> (mm Hg) and of pH after exposure of blood to 70 µg/ml. Insufflation of O<sub>2</sub>-O<sub>3</sub> into the blood bag was carried out at the time indicated by the arrow. Mean values of five samples ± SD.

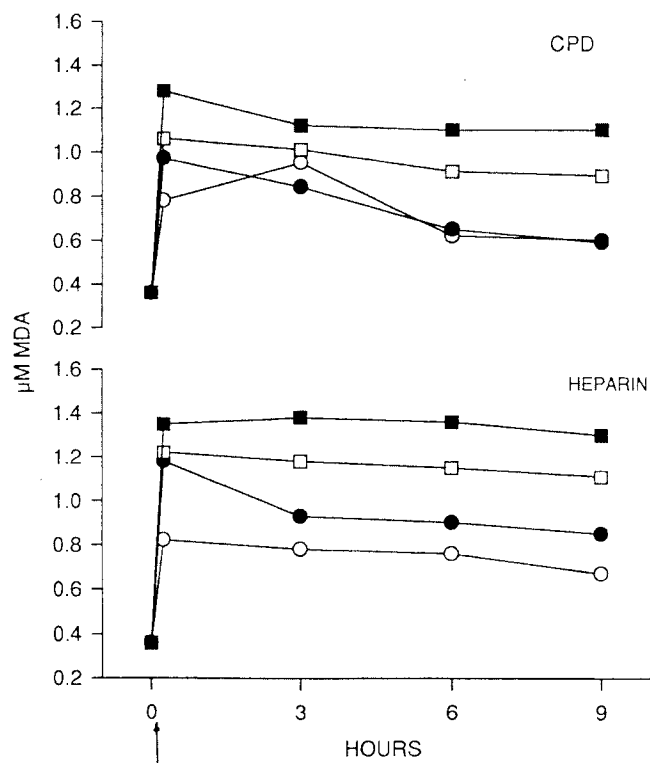
During the last decades there has been a growing interest (1, 2) in expanding medical horizons, but unfortunately several complementary medical practices have not yet a rational background and have been performed on an empirical basis, without a standardized protocol (3-6). This problem applies also to ozonotherapy in spite of the fact that ozone was used as a potent disinfectant since the first world war and saved the lives of wounded soldiers with gas gangrene (7, 8). There is a general consensus (9) in regard to the virucidal, bactericidal and fungicidal properties of ozone, which is probably the best agent for the disinfection of drinking and wastewater (10). On the other hand ozone has become a controversial gas and is almost a daily argument: the ozone layer, by blocking ultraviolet irradiation in the stratosphere, prevents skin cancer while an increased ozone concentration in the troposphere, particularly in large cities during Summertime, is responsible for pulmonary toxicity (11-15).

Thus ozone toxicity has become a matter of debate (13,16,17) and is a motif of anxiety for patients undergoing ozonotherapy. There are now two schools of thought: one states that ozone is definitively toxic (14,17-20) and another believes that ozone can be used as a therapeutic agent (7,8,21-24). Eigenblutbehandlung or major ozonated autohemotherapy (O<sub>3</sub>-AHT) was first described in 1954 by Wehrli and Steinbart (25) and consists of exposing *ex vivo* human blood to a gas mixture composed of therapeutic oxygen and ozone (O<sub>2</sub>-O<sub>3</sub>) for a short time followed by reinfusion in the donor. This procedure, as well as the administration of O<sub>2</sub>-O<sub>3</sub> by several routes, has been used in millions of patients during the last four decades particularly in Germany, Austria, Switzerland and Italy with practically no side-effects (26). However four deaths have been recorded due to pulmonary embolism which occurred during direct intravenous administration of O<sub>2</sub>-O<sub>3</sub>, an application since 1983 prohibited by the European Society of Ozonotherapy. It has been claimed (reviewed in 7 and 8) that skin and mucosal infections, chronic viral diseases, ischemic diseases and cancer benefit from the use of ozone but unfortunately clinical data, although encouraging, are largely anecdotal (27-30). Similarly, biological research has been scarce and not well documented.

During the last eight years we have attempted first to understand the mechanisms of action (23,31-34) and more recently to evaluate in a controlled fashion possible clinical effects. The aim of this paper is to critically review the following topics: firstly, I will describe the ozone targets and how biological actions are elicited. Secondly, I will try to demonstrate that ozone, as



**Fig. 2** - The reaction shows that when ozone reacts with an unsaturated fatty acid containing a *cis* double bond gives two moles of aldehyde and one mole of hydrogen peroxide per mole of ozone and olefin used (according to Pryor et al, 1995).



**Fig. 3** - Kinetics of thiobarbituric acid material (estimated by the fluorometric dosage of malondialdehyde) present in human plasma before and after exposure to ozone (arrow) at concentrations of 45 (○), 72 (●), 90 (□) and 156 (■) µg/ml per g of blood. Mean value of two plasma samples incubated at 37°C after ozonation for 9 hrs. The arrow indicates the ozonation time.

ny other drug, can be toxic and must be used with caution. Thirdly, pros and cons of an incredible number of administration routes of ozone will be critically evaluated. As far as major O<sub>3</sub>-AHT is concerned, it will be shown that, by selecting appropriate parameters, it has been possible to suggest a range of ozone concentrations able to trigger useful biological effects with minimal toxicity. Fourthly, so far available rational bases for attempting the treatment of some human diseases will be discussed. On the whole, I feel that it will be rewarding to open a dialogue between the opposite schools of thought showing that both are partly right and wrong. Obviously the most important goal is to stimulate extensive and controlled clinical research so that we can decide, once and for all, whether ozonotherapy can be useful either when conventional therapies fail to be effective or as a complementary approach.

## 2. MULTIPLICITY OF OZONE ACTION AND GENERATION OF SIGNALING MOLECULES

While ozone having a  $t_{1/2}$  of 40 min at 20°C, is relatively stable in gaseous form (8), it dissolves in water more abun-

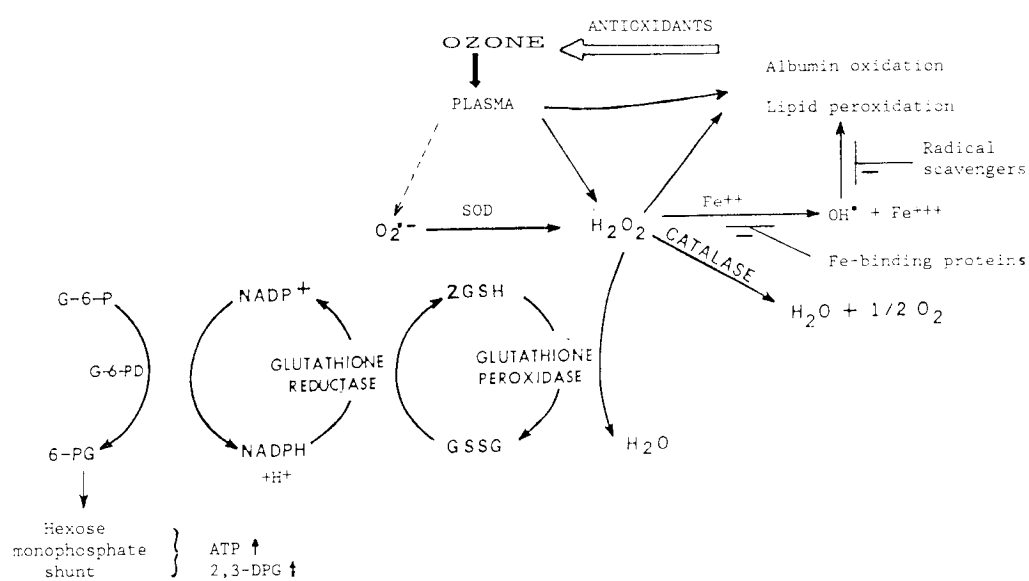
dantly than oxygen and decomposes rapidly. Therefore when the gas mixture composed of 95% oxygen and of no more than 5% ozone, is mixed with blood *ex vivo*, a complex series of physical and chemical processes occur: oxygen, dissolved in plasmatic water, almost instantaneously saturates hemoglobin (Hb) to form oxyhemoglobin (Hb<sub>4</sub>-O<sub>8</sub>) and the pO<sub>2</sub> increases far higher (Fig. 1) than physiological level (normal arterial pO<sub>2</sub> is about 98 mm Hg) while the pCO<sub>2</sub> and the pH's value remain fairly constant.

### 2.1. Plasma

Ozone reacts with a variety of substrates represented by mono- and polyunsaturated fatty acids and cholesterol, both present in lipoproteins and cellular membranes. Moreover free and protein bound cysteine, methionine, tyrosine, tryptophane and histidine as well as free and protein-bound carbohydrates are potential targets. It must be pointed out at once that during evolution, aerobic organisms have developed a powerful and multiform defence system against reactive oxygen species (ROS), which are generated continuously during mitochondrial respiration (35,36). The antioxidant system has been extensively reviewed (37-39) and is represented by either water-soluble reducing compounds such as ascorbic acid, uric acid, creatinine, taurine, etc or of proteins such as albumin, transferrin, ceruloplasmin able to chelate free iron and copper, or lipophylic compounds such as  $\alpha$ -tocopherol,  $\beta$ -carotene, lycopene and bilirubin. Owing to the wealth of compounds it is versatile and capable of neutralizing ROS (40,41). Cross et al (16) have clearly shown that particularly uric acid, ascorbic acid and albumin may act as "sacrificial targets" of an oxidative attack implying that the bulk of the ozone dose is usually neutralized by the antioxidant system. Ozonation of these various substrates, at physiological pH, leads to the formation of an extremely complex and heterogenous cascade of compounds (17). Most of them have an extremely short half-life but some products such as lipid-soluble peroxy radicals and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Fig. 2) are fairly stable (42,43), can be measured in plasma (44) and their concentration is ozone-dose-dependent (Fig. 3). Although H<sub>2</sub>O<sub>2</sub> is not a free radical, it can be included among ROS and the interesting novelty is that a suitable amount of ROS is capable of activating biochemical mechanisms and gene expression (45-48). This can be achieved by either the direct action of ROS onto cell membrane receptors or/and by diffusion into the cytosol where trans-activating factors and enzymes are located. It can be immediately perceived that ROS, to be effective, must reach a cytoplasmic concentration above a critical threshold. This concept will be expanded in subsection 2.2., 2.3. and 4.7.

### 2.2. Erythrocytes

The enormous erythrocyte surface (about 70 m<sup>2</sup> for 100 ml blood) may act as a sponge and limit the damaging proper-



**Fig. 4** - Biochemical events occurring during blood exposure to ozone. Ozone dissolves in the plasmatic water and decomposes immediately generating ROS and organic compounds. Extracellular (particularly uric and ascorbic acid) and intracellular antioxidants quench most of the ozone dose. However a slight excess of the diffusible  $H_2O_2$  is crucial for activating biological pathways. Formation of  $OH^{\bullet}$  is unlikely either because  $Fe^{++}$  is unavailable and because CAT and GSH promptly neutralize  $H_2O_2$ . The hexose monophosphate shunt is stimulated either directly or via the GSH-dependent peroxidative pathway.

ties of ROS.  $H_2O_2$ , generated in plasma after ozonation, is very diffusible within and between cells (35) but its concentration in the cytoplasm rises depending upon the efficiency of the intracellular antioxidant system. Besides the hydro- and liposoluble antioxidants just mentioned, there is a coordinated system of proteins mainly composed of superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R), glutathione S-transferase (GSH-S-tr) capable of forming and disposing of hydrogen peroxide and aldehyde products of lipid peroxidation by several pathways, one of which is the oxidation of reduced glutathione (GSH) to the oxidized form (GSSG) (Fig. 4). The GSH redox cycle keeps the GSH/GSSG ratio as high as possible because GSH is one of the most valuable intracellular antioxidants (49,50) able to scavenge  $H_2O_2$  as well as hydroperoxides. We have shown (51) that during exposure of normal human blood to non-toxic concentration of ozone (70  $\mu g/ml$  per g of blood), intraerythrocytic GSH concentration decreases by 20-25% but, after blood reinfusion, GSH level returns to normal level. This result confirms previous data (52,53) and shows that as soon as the GSH content undergoes a depletion, erythrocytes activate the hexose monophosphate shunt (Fig.4) generating more reduced nicotinamide adenine dinucleotide phosphate (NADPH) necessary for reducing GSSG to GSH by GSH-RX (54). Moreover, they can synthesize GSH *ex novo* fairly rapidly.

We are evaluating the role and levels of glucose-6-phosphate-dehydrogenase (G6-PD), 6-phosphogluconate dehydrogenase (6-PGD) and glyceraldehyde 3 P dehydrogenase (G3-PD) in relation to different ozonation levels to verify exactly how the increase of 2-3 diphosphoglycerate (2-3 DPG) is brought about because in patients, it has observed (55) an increased level of 2-3 DPG and adenosine triphosphate (ATP) during the course of ozonotherapy, usually 2-3

weeks after starting the treatment. 2-3 DPG is known typically to increase with hypoxia at high altitude (56): during ozonotherapy the arterial  $pO_2$  does not change but, owing to increased oxygen release, the venous  $pO_2$  decreases and therefore the 2-3 DPG increase becomes understandable and can be related to the activation of both the glycolytic pathway and the pentose cycle. If this assumption is correct, we should be able to correlate the increase of G6-PD, 6-PGD, GSH-Px and GSH-R activity with 2-3 DPG levels depending upon the weight (hence the number of erythrocytes) of ozonated blood for each treatment, the frequency of autohemotherapy and the erythrocyte turnover. Moreover, it has been shown that oxidative damage to erythrocytes is prevented by  $\alpha$ -tocopherol (57) and that both GSH and ascorbic acid complement its antioxidant functions by providing reducing equivalents indispensable for its recycling (50,58-60).

I cannot omit to mention a new and intriguing development due to a dynamic relationship existing between Hb and nitric oxide ( $NO^{\bullet}$ ) (see also subsection 2.5.).  $NO^{\bullet}$  plus eicosanoids on the one hand (61,62) and anion superoxide ( $O_2^{\bullet -}$ ) plus endothelins and thromboxane A2 on the other hand have been recognized as the crucial factors regulating either relaxation or contraction of blood vessels, respectively. *In vivo*,  $NO^{\bullet}$  has a half-life of a few seconds and therefore even a slight increased production of  $O_2^{\bullet -}$  may cause vasoconstriction and hypertension (63). Jia et al. (64) has now clarified that  $NO^{\bullet}$  becomes bound to both the haem iron and the reactive sulphhydryl groups of cysteine 93- $\beta$  of hemoglobin. When, during deoxygenation,  $NO^{\bullet}$  is liberated from this site, it is transferred to small thiols forming S-nitroso (SNO) thiols with GSH or cysteine which, in contrast to free  $NO^{\bullet}$ , have a half-life of several hrs and can slowly return  $NO^{\bullet}$  to endothelial receptors. In this way, not only  $NO^{\bullet}$  toxicity is reduced but a steady relaxation of vascular tension is ensu-

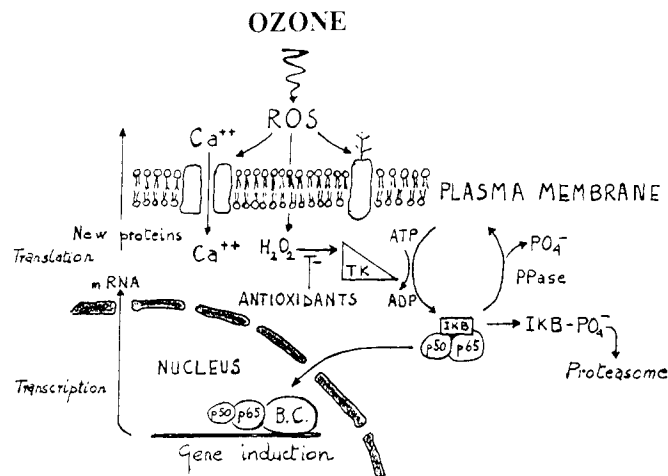
red. The exciting possibility is that reinfusion of properly ozonated blood enhances  $\text{NO}^\bullet$  release and the increased formation of S-nitrosothiols may explain the persistent vasodilation in ischemic tissues following autohemotherapy.

To date it appears that, after exposing a known weight of human blood to a precise ozone concentration, a transient increase of  $\text{H}_2\text{O}_2$  and other ROS act as triggers of multiple biological functions. A great deal of ROS are probably neutralized by plasmatic scavengers but, using an appropriate ozone concentration, some of them pass the cell membrane and reach the cytoplasm. This is a crucial stage because levels of  $\text{H}_2\text{O}_2$  are critical in the sense that too low levels will not elicit any biological response (subsection 4.7). On the other hand, very high levels may be effective but, by overwhelming antioxidant defenses, can be toxic. Two questions must be answered: the first regards the type and validity of biological responses and the second aims to define which is the optimal ozone concentration for achieving therapeutic effects without toxicity. So far biological modifications observed in human erythrocytes after repeated blood ozonation can be summarized as follows: there is already evidence that G-6-PD, 6-PGD, pyruvate kinase (PK) and lactate dehydrogenase (LDH) increase with time (52,54,65). The enhanced glycolysis and the activation of the pentose shunt appear responsible for the increased erythrocytic content of ATP and 2-3 DPG (27,55). An intriguing possibility is the microrelease of ATP from ozonated erythrocytes: while pharmacological infusion of ATP is known to cause hypotension actually depriving the hypoxic tissue of an already critical blood supply (66), the microrelease of ATP in the ischemic microenvironment may cause vasodilation thereby improving local blood flow. At the same time, the shift to the right of the  $\text{Hb}_4\text{O}_8$  dissociation curve due to a higher 2-3 DPG content improves oxygen availability. Ozone, at low concentrations, may also improve the rheology of erythrocytes by increasing their electric charge and flexibility (7,8,27).

There is no final data to answer the second question because a systematic and precise investigation has never been performed. However blood collected in citrate-phosphate-dextrose (CPD) has been exposed only to very low ozone concentrations ranging from 10 to 30  $\mu\text{g}/\text{ml}$  (7,8,27,30) and it is important to explore whether heparin as well as higher ozone concentration (in the safe range from 30 to 70  $\mu\text{g}/\text{ml}$ ) are more effective.

### 2.3. Leukocytes

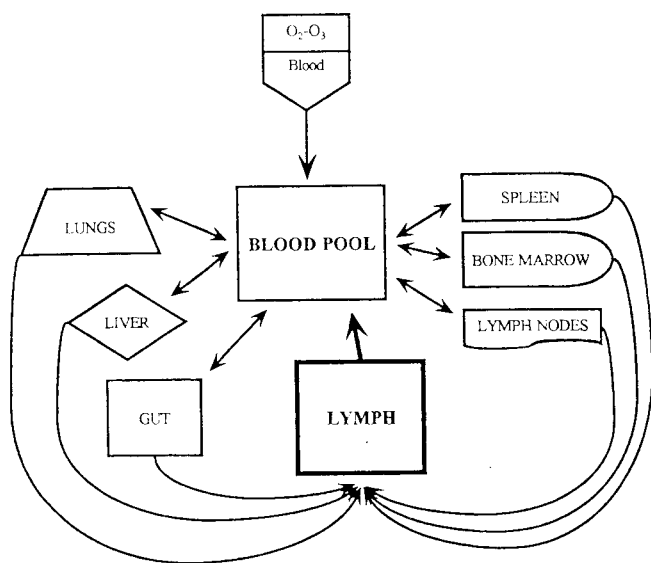
Besides the disinfectant properties, ozone appears to stimulate the phagocytic activity of neutrophils and to modify immunoglobulin levels (67,68). In response to an array of endogenous and exogenous factors, the activation of leukocytes is paralleled by an abrupt rise in oxygen consumption, a metabolic event termed the "respiratory burst" during which ROS, including also oxidized halogens, are generated (69). These products kill infectious agents, which



**Fig. 5** - A schematic view of signal transduction in lymphocytes due to oxidative stress. The nuclear transcription factor NF- $\kappa$ B is a heterodimer of p65 and p50 subunits. In resting T lymphocytes, it exists in an inactive form complexed with the inhibitor I $\kappa$ B. Ozone decomposes in plasma and generate ROS. These may act either onto lectins situated in the plasma membrane, possibly opening  $\text{Ca}^{++}$  channels or/and activating protein kinases. Phosphorylation of I $\kappa$ B by a I $\kappa$ B kinase removes the inhibition and while I $\kappa$ B- $\text{PO}_4^-$  is being degraded in the proteasome, the heterodimer will move promptly from the cytosol into the nucleus where it regulates gene expression. Activation of a phosphatase (PPase) or an excess of intracytoplasmic antioxidants (GSH, NAC, CAT, thioredoxin,  $\alpha$ -lipoic acid etc) inhibits the process.

are then taken up by phagocytosis and it is almost needless to say that an excess of ROS can injure host cells as well. However from Washüttl's reports (67,68), it was not clear how ozone stimulated professional phagocytes and an exciting development of this field has been to discover that ozone, used in judicious concentrations, can act upon human peripheral blood mononuclear cells (PBMC), as a mild cytokine's inducer. This finding (31) has been enlightening because it has clarified that ozone does not act directly as an antiviral or an antineoplastic agent, but rather stimulates the production of cytokines which then activate immune cells. After detecting interferon ( $\text{IFN } \gamma$ ), we have subsequently measured the production of other cytokines namely tumor necrosis factor ( $\text{TNF } \alpha$ ) (70), IL-2, 6, 8, and granulocyte-monocyte colony stimulating factor (GM-CSF) (32,51). Particularly the release of IL-8 is known to be regulated by reactive oxygen metabolites such as  $\text{H}_2\text{O}_2$  (71).

Moreover, in line with our findings, other groups have recently shown that ozone induces on alveolar macrophages and airway epithelium the expression of adhesion molecules and pro-inflammatory cytokines which, in this case, may be deleterious to the respiratory microenvironment (72-75). The important discovery that ROS can act as messengers in the activation of the nuclear factor  $\kappa$ B (NF $\kappa$ B) (76,77) allowed to postulate that after blood exposure to ozone, a sudden and



**Fig. 6** - After reinfusion of ozonated blood, erythrocytes circulate in the vascular compartment, while leukocytes preferentially home in some organs where they release cytokines in the microenvironment. Platelets may also release growth factors. The complexity of the biological response may be understood by the overlapping of these phenomena. The life-span of ozonated erythrocytes is under study.

brief increase of  $H_2O_2$  in the cytosol could be the critical activator of the transcription factor (24). There is now good evidence (45,78-80) that  $H_2O_2$  activates a protein kinase that, by phosphorylating the subunit I-KB, removes the brake that keeps the heterodimer composed of p50 and p65 subunits in a latent form. After translocation to the nucleus (Fig. 5), the heterodimer allows the transcription of several cellular and viral genes including cytokines, acute phase proteins, hematopoietins, cell adhesion molecules and last but not least, proteins of the human immunodeficiency virus (HIV). Activation of NF $\kappa$ B is not necessarily a good thing because in HIV infection (76), as well as in systemic inflammatory syndromes and autoimmune diseases should be avoided and actually counteracted by the administration of thiol compounds. The possibility that  $O_3$ -AHT could be detrimental has been discussed elsewhere (81).

We have analyzed the process of induction and shown that it is  $Ca^{++}$  and ozone-concentration dependent (32,51). Particularly for blood anticoagulated with CPD, ozone concentrations ranging from 10 to 40  $\mu$ g/ml/ are practically ineffective. On the other hand, blood anticoagulated with heparin (which preserves a  $Ca^{++}$  concentration of about 1 mM) can be exposed to ozone concentrations ranging from 40 to 80  $\mu$ g/ml for 10 min without any adverse effects. Higher ozone concentrations (up to 200  $\mu$ g/ml) appear progressively detrimental. Upon 4-8 hrs incubation, PBMC release in plasma small amounts of cytokines that for GM-CSF can be 10-fold higher than control value. As it could be expected, the

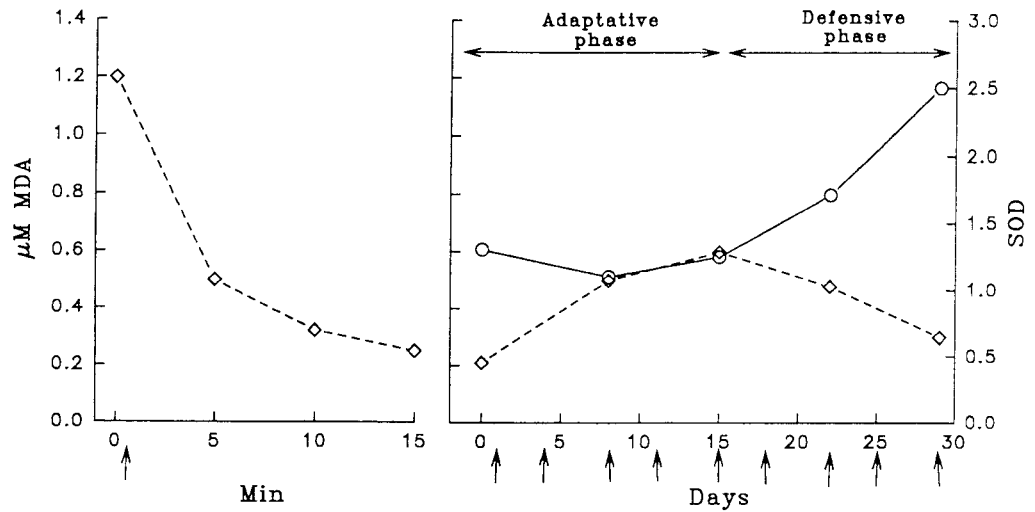
response of PBMC to ozone is quite different depending upon whether they had been exposed in whole blood or have been isolated, washed and resuspended in culture media with the addition of only 10% fetal calf serum. In the latter condition, the substantial deprivation of the antioxidant system (erythrocyte mass and plasma) makes PBMC very sensitive to ozone and a slight stimulation is occasionally obtained only after exposure to as little as 3  $\mu$ g/ml for no longer than 3 sec. In agreement with the work of Becker et al (82) we have found that longer periods, or even slightly higher ozone concentration progressively depress cell proliferation and inhibit cytokine production. Let me comment by saying that these studies have been very instructive and have taught us that the ozone boundary between its vices and virtues must well be kept in mind by scrupulously checking ozone concentration, gas volume, time of exposure as well as volume or weight of substrates, particularly of reducing compounds.

At the first sight the small release of cytokines from ozonated blood may seem a drawback but in practice it is advantageous because, after blood reinfusion, both human volunteers (33,81) and patients with chronic viral infections (7,8) have never been affected by the typical flu-like syndrome due to exogenous administration of either IFN $\alpha$  or TNF $\beta$  or IL-1, 2 and 6 (83). The lack of side-effects has been interpreted as due to a minimal release of lymphokines, once the reinfused PBMC have homed into various microenvironments (Fig. 6). Indeed in healthy volunteers, we have never detected any modification of the cytokine levels in the plasma after autohemotherapy (33) and cytokine's toxicity is known to be linked to their unphysiological high levels (83). The exclusive microenvironmental release of cytokines (84) may have the advantage of priming or/and activating resting or suppressed immune cells with the possible consequence of stimulating phagocytosis, natural killer and many other T-cell cytotoxic activities possibly depressed during viral infections or following surgery, radio- and chemotherapy.

#### 2.4. Platelets

So far very little is known about the effect of ozone on platelets. Our preliminary observations show that platelets tend to aggregate more easily when ozonated blood is collected in heparin than in CPD. In order to prevent coagulation 25-30 Units of heparin must be used for each g of blood exposed to 70  $\mu$ g/ml of ozone. We have observed (34) that release of transforming growth factor (TGF $\beta$ 1), at least in part stored in  $\alpha$ -granules of platelets, is higher in blood anticoagulated with heparin than CPD. It appears reasonable to postulate that, above a certain threshold, ROS, in the presence of the physiological  $Ca^{++}$  level (about 1 mM), can stimulate platelet degranulation more intensely than after  $Ca^{++}$  chelation. This observation deserves a thorough investigation because release of platelet-derived growth factor (PDGF) and TGF $\beta$ 1 may have an important role in wound healing (subsection 5.1.), as it has been shown that human chronic

**Fig. 7** - A patient's (age-related macular degeneration) response to a single (left hand side) or intermittent (right hand side) infusion of  $O_3$ -AHT (300 g blood treated with an ozone dose of 21 mg per session). Thiobarbituric acid reactive substances ( $\diamond$ ) and Mn-SOD ( $\circ$ ) are reported in the ordinate. Arrows indicate the time of blood reinfusion.



ulcers treated with recombinant PDGF show increased healing (85). Furthermore it appears important to investigate how the wounded or altered skin reacts to topical application of ozone either as a tightly contained gas or as ozonated water. Several cytokines such as TGF- $\beta$ , basic fibroblast growth factor (bFGF), monocyte chemotactic protein 1 (MCP-1/JE) and keratinocyte growth factor (KGF) may be released by fibroblast and epithelial cells and stimulate the repair of the dermal-epidermal layers (86-88).

### 2.5. Endothelial cells

ROS, generated in blood exposed to ozone *ex vivo*, are likely to act on the vast surface of endothelial cells. As it was discussed in subsection 2.2., S-nitrosothiols are likely to be formed in the pulmonary bed particularly during the reinfusion period (64). Human endothelial cells are under study in our laboratory because they are a potentially important target and do not represent a mere interface between the blood and tissues but the source of crucial factors such as NO $^*$ , eicosanoids, vascular endothelial growth factors (VEGF), endothelins able to modulate the vascular tone, platelet aggregation, transendothelial leukocyte migration and vascular remodeling (63,89). Although much work remains to be done, we now begin to have some reasonable ideas for exploring mechanisms of actions.

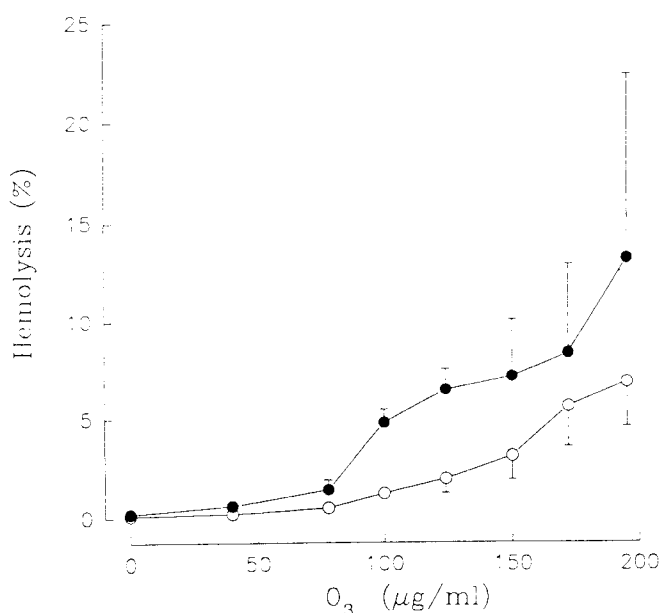
## 3. OZONE AND ROS CAN BE DOUBLE-EDGED SWORDS

In order to justify the use of ozonotherapy one must bear in mind that life on Earth is possible only because cells are normally able to release O $_2^*$ , H $_2$ O $_2$ , NO $^*$ , hypochlorous acid (HOCl) and that these oxidizing compounds, after solving their scope, are readily quenched even though an excessive production may end up with some cellular damage. By using

a fairly precise stoichiometric relationship for a definite time between a known weight of blood on the one hand, and the dose (volume and gas concentration) of ozone on the other hand, and by correlating biological activities versus toxicity, it has been possible to find a useful range of ozone concentrations. This approach is helpful for avoiding either a placebo or a toxic effect upon blood components. Nonetheless it must be said that the relation between blood weight and ozone concentration is relatively precise because antioxidant levels in blood vary considerably among individuals depending upon sex, age, diet, season and metabolism. This variability implies that a certain ozone concentration may fluctuate within an effective range for each blood sample.

### 3.1. Plasma

Endogenous reducing compounds such as ascorbic acid and uric acid progressively decline in relation to ozone concentration and time of exposure (90). Neither electrophoretic changes of lipoproteins, nor activity of several enzymes have been detected probably because ozone oxidizes preferentially sulfhydryl groups present in albumin (16) which afterwards may become more susceptible to be catabolized but rapidly replaced by hepatic biosynthesis. The lack of oxidation of low density lipoproteins (LDL) is interesting on the ground that its irreversible damage may favour atherosclerosis (91,92); in our experimental condition (1 g of blood exposed up to 70-80  $\mu\text{g/ml}$  ozone for 10 min) we have shown the formation of TBA reactive substances (44) that decay slowly with time (Fig. 3) if blood is incubated *in vitro*. An important question, as yet unanswered, is how relevant are lipid mediators as chemical communicators of oxidative stress. Fatty acids such as linoleic and arachidonic acid present in plasma undergo peroxidation during ozone exposure and generate a cascade of compounds some of which may recognize particular cell receptors able to initiate signal transduction events.



**Fig. 8** - Relationship between the rate of hemolysis (expressed as a percentage) and ozone concentrations of five samples of human blood collected in CPD (○) and in heparin (●) (30 U/g blood). Mean value  $\pm$  SD.

The identification of biologically active molecules and of their site of action is needed for understanding how O<sub>3</sub>-AHT works *in vivo*. In fact after reinfusion of ozonated blood the behaviour of lipid peroxides in plasma is very complex: after the first treatment, they disappear within 15 min owing to hemodilution and metabolism (Fig. 7) but, on continuing the therapy, they tend to increase in most of the patients particularly during the first 2-3 weeks (4-6 treatments) and then they return slowly to pretreatment values. Their decline approximately coincides with the time of overexpression of antioxidant enzymes namely SOD, (subsection 3.6.). As schematically shown in Fig. 7, plasma lipid peroxides and SOD levels cross over after about 7 treatments between the adaptive and the defensive phase.

Comparison of protidograms before and after ozonation do not evidentiate significant changes of the serum protein pattern. A more sensitive marker could be represented by the evaluation of the inhibitory activity of  $\alpha$ 1- proteinase inhibitor (ex  $\alpha$ 1 - antitrypsin) in plasma if methionine 358 residue undergoes oxidation (93).

### 3.2. Erythrocytes

Human erythrocytes are cells with varying age up to about 120 days and it appears likely that old erythrocytes are more sensitive to peroxidation (94). Measurement of hemolysis is the quickest method for detecting damage and release of Hb. As it is shown in Figure 8, ozone concentration higher than 100  $\mu$ g/ml becomes prohibitive and excessive levels of Hb, beyond the binding capacity of haptoglobin, could induce

tubular damage. Even low ozone concentrations in the presence of physiological Ca<sup>++</sup> and particularly after the experimental addition of 5-10-25-50 mM Ca<sup>++</sup> enhances hemolysis (32). Within the ozone therapeutic range, we have never detected methemoglobin.

Washed erythrocytes or ghosts resuspended in buffered saline, without any natural antioxidants are very sensitive to ozone (53,95) but these data cannot be compared to our experimental condition. In line with our results, a recent study (59) has shown that erythrocytes have a high resistance to oxidative stress *in vivo*. The increased level of enzymes of the pentose phosphate shunt suggests that erythrocyte metabolism is usefully stimulated by ozone (52,54,65) (subsection 2.2.)

### 3.3. Leukocytes and platelets

Some leukocytic activities have been assessed after ozone inhalation on the premise that damage to defensive mechanisms in the respiratory tract induced by air pollutants may increase the susceptibility to bronchopneumonitis and asthma. Phagocytic, bactericidal activities and antibody responses were transiently reduced after prolonged inhalation of either ozone or ozone and nitrogen dioxide supporting the thesis that respiratory defense mechanisms can be impaired by oxidant challenge (96-98). On the other hand Dzedzic and White (99,100) have shown that in mice exposed to ozone inhalation, lymphocyte hyperplasia is ozone dose-dependent and that lymphocytes appeared "primed" and quite reactive.

O<sub>3</sub>-AHT may increase phagocytic activity (8) and induction and release of cytokines (subsection 2.3.) is instrumental in activating the immune system. Leukocytes are far less sensitive to ROS toxicity in whole blood than after isolation and resuspension in culture medium supplemented with 10% fetal calf serum. Cardile et al. (101) have determined that human PBMC treated with 20  $\mu$ g/ml ozone remain viable and synthesize heat shock proteins 70 KDa, but doses of 40 and 100  $\mu$ g/ml reduce the biosynthesis progressively. Similarly murine macrophages were partly unable to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), after treatment with an ozone dose of 100  $\mu$ g/ml. In a study in progress, intended to evaluate the proliferation index of human PBMC, we have found that isolated human leukocytes resuspended in medium (with 10% fetal calf serum) responded as control samples to phytohemagglutinin after being exposed to ozone (3  $\mu$ g/ml) for no longer than 3 sec. However, prolonging the exposure for 6-12-24-96 sec caused a progressive inhibition of proliferation. These findings tend to exclude the possibility of treating with ozone *ex vivo* a concentrate of PBMC separated by leukapheresis in patients with cancer either for the risk of bacterial contamination or/and ozone toxicity.

The problem of the sensitivity of platelets to ozone is of great relevance and will be examined in relation to the relea-



se of active biological components. At present, data are not yet available.

### 3.4. Side effects and possible sequelae

In subsection 2.3. it has been already alluded to the lack of adverse effect even when intensive O<sub>3</sub>-AHT (ozone concentration 70 µg/ml) is carried out 2-3 times weekly for achieving immune stimulation. The same applies by performing two successive treatments with an overall ozone dose of about 42 mg with an interval of only 10 min. One problem, we and others (7,8,26) have encountered is the maintenance of a good venous access particularly in women and elderly patients after having undergone chemotherapy. In ongoing clinical trials, patients with good venous access have received regularly (twice weekly) up to 70 treatments within 8.5 months without any discomfort. During the whole period about 21 L of blood underwent ozonation. If necessary, autohemotherapy can be continued, possibly once a week, or biweekly. Routinary hematologic, biochemical and immunological parameters were checked every two weeks and practically remained within pretreatment values.

One surprising aspect is that the majority of patients report a feeling of well-being and euphoria. This is usually noticed 1-2 days after the initial treatment and can last for the following 4-5 days or remains fairly stable during a prolonged treatment. Reasons for this subjective improvement remain speculative and it appears worth while to evaluate the intensity and pattern of hormonal responses, focusing the attention upon corticotropin-releasing factor, adrenocorticotrophic hormone, cortisol, growth hormone, dehydroepiandrosterone and melatonin. Keeping in mind that we are dealing with uncontrolled clinical studies we cannot exclude a placebo effect (102), now renamed "remembered wellness" (103). Ozonation of blood in our hands is a "calculated and transient oxidative stress" but even a minimal stress could lead to undesirable sequelae with time. Development of cataract, hemolytic crisis, leukemia, atherosclerosis, neurodegenerative diseases are among the disorders that must be watched most carefully. So far I am not aware of any of these complications and I have proposed that the European Society of Ozonotherapy send an appropriate questionnaire to all practicing ozonotherapists to carry out an adequate follow-up of the patients. This sort of investigation is overdue for ascertaining that ozonotherapy does not procure late side-effects.

### 3.5. Is ozone mutagenic?

Every biomolecule including nucleic acids is a potential target for reaction with ozone. Owing to the importance of oxidative DNA lesions in aging and cancer, it does not surprise me to be often asked: is ozone mutagenic? and does ozonotherapy accelerate aging?

To my knowledge, Fetner (104) was the first to study this problem and demonstrate (105) that KB human cells exposed

to ozone showed chromatid breakages similar to those produced by x-rays. While this report caused much concern, it must be said that experimental conditions were unphysiological because "cells were washed once in a protein-free saline solution to remove extraneous reducing materials. Ozone is very reactive, and small amounts of reducing materials provide considerable protection". Subsequent investigations have been conducted in somewhat more realistic conditions: Gooch et al (106) exposed human leukocytes to ozone in culture medium without antioxidants and expectedly observed a slight increase in the frequency of chromatid-type aberrations. However leukocytes from mice exposed to ozone inhalation did not show any chromosome damage. At about the same time there were two discordant reports: one by Merz et al (107) showing an increase in chromatid aberrations and the other by McKenzie et al (108) unable to demonstrate any damage in lymphocytes collected from volunteers exposed to 0.4-0.5 ppm<sup>3</sup> ozone for several hours. In order to overcome this inconsistency, Guerrero et al (109) carried out a careful study using the sensitive analysis of sister chromatid exchange (SCE), and concluded that there was no difference in the SCE values of the lymphocytes collected from students exposed to either 0.05 ppm ozone or air for 2 hrs. Once again however, WI-38 cells incubated with serum-free medium showed a linear dose-related increase in SCEs per chromosome spread over an ozone concentration range from 0 to 1.0 ppm for 1 hr, implying that the lack of serum allowed chromosomal damage. The study by Sarto and Viola (110), although suggestive of a cytogenetic damage on chronic ozone-exposed workers must be regarded as inconclusive because smoking or other confounding factors were not taken into consideration. Subsequently, Victorin (111) in extensively reviewing this topic, stated that "no cytogenetic effects have been reported for bone marrow cells or spermatocytes and the few experimental and epidemiological studies with human subjects do not allow a conclusion on the cytogenetic effects of ozone in human lymphocytes". The latest study by Diaz et al (112) is relevant because it has been specifically carried out in lymphocytes of 8 *Retinitis pigmentosa* patients before and after 15 treatments of O<sub>3</sub>-AHT. Results have not shown significant differences in SCEs, micronuclei frequencies and proliferation index between control and ozone-treated lymphocytes. As far as induction of tumors is concerned, lung adenomas were induced in the sensitive strain A/J but not in Swiss-Webster male mice after 4.5 months of inhalation exposure to 0.8 ppm ozone (113). Finally Kozumbo et al. (114) have shown that in cultured human lung cells, incubated without catalase, the formation of DNA single strand breaks could be due to either hydrogen peroxide or/and ozonized arachidonate.

Trying to sum up this important topic, it appears that the lack of natural antioxidants is critical in allowing mutagenic changes in cells exposed to ozone *in vitro* for a length of time. Conversely, lymphocytes isolated from blood exposed to ozone *ex vivo* for a brief time before reinfusion have not shown any genetic damage.

Thus, regarding the first question the answer is: yes, ozone is potentially mutagenic (like all cytotoxic drugs!) but so far experimental evidence has not shown any risk with regard to ozonated blood. Whether this is due to the low and precise ozone concentration, the short-time exposure and the efficiency of the detoxification system possibly in conjunction with the DNA repair enzymes (115), is difficult to say but it is likely that ROS, particularly  $H_2O_2$ , transiently rising in the cytoplasm, is metabolized before reaching the mitochondrial and nuclear membranes.

As far as the problem of aging is concerned, the answer is reassuring in the sense that  $O_3$ -AHT, by reactivating the metabolism (subsection 2.2.), the immune system (subsection 2.3.) and inducing the overexpression of antioxidant enzymes (subsection 3.6.), it may actually delay aging. It has not yet been clearly ascertained whether ozonotherapy can induce hypersecretion of some hormones that could justify the feeling of well-being noted by elderly patients. Owing to the pulsatile-type hormonal secretion, the presence of binding proteins and the rapid turnover, it may be difficult to quantify significant differences. It will be also very awkward to determine if the mood-elevating effect of  $O_3$ -AHT is due to an increased production and availability of neurotransmitters (serotonin, endorphins?) in the central nervous system.

It is of interest to note that healthy, elderly patients often ask to undergo 3-4 sessions of  $O_3$ -AHT in the Spring and Fall because they report to feel better and to be less prone to common respiratory infections. However, once again it is not yet possible to exclude placebo effects (102,116) due to a psychoneuroimmunological response.

### 3.6. Endogenous ROS versus ozone toxicity

One reason of the unpopularity of ozonotherapy in the medical field is that the toxicity of free radicals (ROS) is equalized to that of ozone. In fact there are substantial differences because ozonotherapy is occasional and can be controlled whereas endogenous ROS formation goes on unperturbed throughout life (115,117).

The topography of formation of ROS is also quite different: mitochondria, that convert 90-95% of the inhaled oxygen to harmless  $H_2O$ , are the main source of ROS since at least 1% of oxygen is converted to  $O_2^{\bullet}$ . Dismutation of  $O_2^{\bullet}$  by SODs (118, 119) is the source of  $H_2O_2$ , whose reduction generates the fearsome, non-specific oxidant hydroxyl radical ( $OH^{\bullet}$ ). Halliwell (35) has estimated that a 70 Kg human produces not less than 0.147 moles or 5 g/day of  $O_2^{\bullet}$  and Esterbauer (44) has calculated a production of 0.8-1.7 tons of ROS during the human life-span of 70 years! Each large autohemotherapy (300 g blood) uses less than 20 mg of ozone that is equivalent to less than 0.4% of the minimal daily production of  $O_2^{\bullet}$ !

The huge formation of endogenous ROS in mitochondria, deeply immersed in the cell, explains the damage to DNA (120), particularly of mitochondrial DNA, that is oxidized

about 10 times more than nuclear DNA (121). Conversely, ozone acts from the outside mainly onto the plasma where an extensive detoxification quenches its bulk (section 2.1.). That is why we must use an ozone dose capable of generating enough  $H_2O_2$ , just above the threshold level in the cytoplasm, where probably it triggers an all-or-none-type biological response. At the same time antioxidant enzymes will promptly reduce the residual  $H_2O_2$  so that formation of  $OH^{\bullet}$  is highly unlikely and so is any oxidation on DNA (subsection 3.5.). It may also be useful to recall that  $O_3$ -AHT is usually carried out 2-3 times weekly, usually for short periods and that blood is exposed for no longer than 10 min to the minimally sufficient ozone dosing.

However the most striking difference between endogenous ROS and ozone toxicity is that ozonotherapy is capable of upregulating the antioxidant enzymatic system, a peculiar property that may constitute a pillar of this medical approach. An improvement of the antioxidant defence is bound to be particularly useful in diseases in which the control of endogenous oxidation has gone awry and cell damage is intensified (115,122-125). Since the 40's, it has been known that, particularly in premature babies, prolonged exposure to high concentration of oxygen, is toxic and in retrospect it is now easy to understand why cells exposed to an oxidant stress have either the solution to programme their own death, or to fight back by increasing their detoxification capacity. It is becoming clear that giving a modest ozone stress and time, both plants (126-128) and humans respond positively and increase the activity of SOD, CAT and peroxidases. Inhalation of oxygen, as currently done in critical patients, is unable to induce enzymatic changes but treatment with hyperbaric oxygen (129,130) capable of rising as much as 20-fold of the solubilized oxygen in plasma (131), favours the increase of SOD in tissues but the change is transient and the procedure cumbersome and expensive. The small percentage of ozone given with oxygen during autohemotherapy seems to be a critical stimulus and the initial data in rats (54,132) regarding increased activities of peroxidases, CAT and SOD have been correctly interpreted as a development of adaptation to ozone inhalation. For the time being, investigations using  $O_3$ -AHT, carried out in patients with cardiac infarction (65), with age-related macular degeneration and with HIV infection (133) have already detected in erythrocytes a marked increase of GSH Px, G-6-PD and SOD. These observations need to be extended because the possibility of inducing a state of oxidative stress adaptation or preconditioning is very interesting and has practical implications.

Cell death induced by elevation of intracellular  $Ca^{++}$  or of ROS can be prevented by the expression of the proto-oncogene bcl-2 and it would be of interest to investigate if the bcl-2 protein is overexpressed during ozonotherapy. It remains controversial (134,135) whether this protein acts as an antioxidant, or paradoxically as a pro-oxidant which, by creating oxidative stress, in turn induces the CAT gene.

**TABLE I - ROUTES FOR THE ADMINISTRATION OF OZONE**

Parenteral	Topic Application (gaz, ozonated water, saline and oil)
Intravenous	Nasal
Intra-arterial	Tubal
Intramuscular	Oral
Subcutaneous	Vaginal
Intra-articular	Vesical
Intraperitoneal	Gastro-intestinal
Intrapleural	Rectal Cutaneous

Experimental and clinical work (115,122,123,125,136-139) has suggested that degenerative diseases and cancer (140) are associated with an oxidative stress, at least in part caused by an imbalance between ROS production and the antioxidant defence system. The progressive decay of this system may be traced to genetic defects (141), aging, exogenous pollutants, chronic infections, dietary deficiencies, etc., and therefore an increased uptake of dietary antioxidants as suggested by Ames et al (115), Halliwell (35,142) may, in some cases, be helpful. However, this does not seem enough (143) and provided that cell degeneration is not too advanced, there is now the bright possibility of inducing an overexpression of critical antioxidant enzymes, potentially able to re-adjust the redox balance and ideally stop the progression of the disease. The possibility to correct the excessive oxidative stress by O<sub>3</sub>-AHT appears as a potential breakthrough and it would be paradoxical that ozone “*in all its reactive splendor*” (20), if used judiciously, could eventually enhance the antioxidant defences. The promising results so far obtained must be followed by a thorough investigation aiming to define optimal blood-ozone dosing, schedule of administration, kinetic of enzymatic induction and, most important of all, to ascertain if the increase of antioxidant enzymes is persistent and occurs in neuronal cells as well as in newborn erythrocytes. Whether a dietary support with antioxidant vitamins and N-acetylcysteine (NAC) simultaneous with autohemotherapy somewhat depress the overexpression of antioxidant enzymes remains to be carefully examined for possibly correcting the questionable fashion of administering megadoses of vitamin C.

The problem of ozone toxicity for the respiratory tract deserves a final comment because ozone is correctly considered one of the most hazardous pollutants worldwide (13, 144) and therefore one wonders how it can be therapeutically useful.

On the basis of the axiom that any drug can be toxic, we have carefully selected the range of ozone concentration (40-70 µg/ml) that offers a “calculated oxidative stress”. In other words, autohemotherapy, as currently done, does not instaurate a constant oxidative stress but only a very transient one during a brief exposure of blood to O<sub>2</sub>-O<sub>3</sub> *ex vivo*. In contrast, exposure of the respiratory tract to ozone is chronic (months or even years) and even more important ozone acts with other acidic pollutants such as nitrogen oxides (145) on the lung lining fluid layer (LLFL), notoriously less capable than plasma to neutralize acidic and oxidizing compounds (146-150). However the question of time is not perhaps as relevant as the fact that ozone action is constantly displayed on a very thin compartment represented by the LLFL and alveolar cells (13). One can envisage the formation of a steep gradient between the tracheo-broncho-alveolar layer (with the highest concentration of ROS) and the pulmonary capillary vessels, where ROS are diluted and metabolized in the circulation. In contrast, exposure of about 300 g blood to ozone last only 10 min (that is the optimal mixing time) and afterwards the reinfused blood mixes rapidly with both the blood (about 5 L) and the extracellular fluid (about 14 L), hence, with an enormous antioxidant reservoir.

It may be useful to recall that for inhalation studies, ozone concentration is measured in terms of part per million (ppm). 0.1 ppm is equivalent to 200 µg/m<sup>3</sup> or 0.2 µg/l. The World Health Organization (WHO) in 1987 established air quality guidelines for Europe: 1 hr and 8 hr exposures correspond to 0.1 ppm and 0.05 ppm, respectively.

#### 4. ROUTES FOR THE ADMINISTRATION OF OZONE

During the last six decades, several methods have been developed for the application of ozone in medical therapy (Table I). Owing to ozone instability, it must be generated when needed by therapeutical oxygen that remains the bulk of the gaseous mixture.

##### 4.1. Direct intra-arterial and intravenous (i.v.) administration

Using particularly the intravenous (i.v.) route a volume of up to 420 ml of O<sub>2</sub>-O<sub>3</sub> (concentration 70 µg/ml) has been injected daily, slowly for several hours, for two weeks. This technique is prohibited in Europe but to my knowledge is unfortunately still used in the Americas mostly by non-physicians. By procuring lung embolism, it can be deadly and in any case procures serious adverse effects and no proved therapeutic benefit. It has been mostly used in AIDS patients with the foolish idea that the human body is composed of about 66% water and therefore the principal used for purifying the drinking water by bubbling ozone through it to kill bacteria and viruses, should be applied to the water of

the human body. There are not severe enough words to condemn this practice.

The intra-arterial route was used to slowly inject 20 ml O<sub>2</sub>-O<sub>3</sub> (30 µg/ml) into the femoral artery in advanced cases of limb ischemia (7). It was not as dangerous as the IV route but it should not be used now because O<sub>3</sub>-AHT is far more effective and safe.

#### 4.2. Intramuscular (i.m.) and subcutaneous (s.c.) injection

A volume of 10-30 ml of O<sub>2</sub>-O<sub>3</sub> mixture is injected either intramuscularly or subcutaneously. It does not seem to be harmful but for a short time it is certainly painful. There is no rational basis for using these routes for treating infectious diseases or cancer.

#### 4.3. Rectal insufflation

This method was introduced by Payr (21) and Aubourg (22) and has been used extensively since then. A mixture of O<sub>2</sub>-O<sub>3</sub> with a concentration no higher than 40 µg/ml is insufflated into the rectal canal as high as possible with a Teflon cannula (rubber is destroyed by ozone). By using a measuring device, up to 750 ml of gas can be tolerated daily for several months. However one should start with daily application of 150 ml gas at a concentration of 10µg/ml, slowly scaling up ozone concentration and volume. Even higher volumes have been used by Carpendale et al (151) for alleviating diarrhea in AIDS patients with cryptosporidiosis (152). Knock and Klug (28) have used this method for treating ulcerative colitis, proctitis, Crohn's disease, acute and chronic hepatitis with apparently satisfactory results. While the rectal route has a poor patient compliance in some cultures, it is extensively used in Germany, Russia and Cuba under medical supervision but, because the use of ozone is prohibited in most of the USA, usually HIV patients use it as a domiciliary self-treatment. It is reasonably safe and no serious side-effects except occasional intestinal cramps have been reported.

It is certainly not my preferred method because the ozone dosing cannot be precise, reactions with targets are unpredictable and, unless we define some reliable markers, it is difficult to establish a reproducible dosage. There is experimental evidence (28) that there is some gas absorption through the gut lumen and portal blood is more oxygenated than normal. Presumably there are local effects such as hyperoxygenation, activation of metabolism and induction of cytokines that may favour healing of ulcers. Conceptually, it is also possible that a local and generalized immunomodulatory effect either due to the ozone as a cytokine's inducer or to an increased absorption of muramylpeptides and lipopolysaccharides derived from the gut flora, that notoriously have an important immunoadjuvant activity (84,153). It may be worth while to clarify in rabbits whether there is some ozone absorption via gut lymphatics with the possibility of activating immune

**TABLE II - APPROXIMATE AMOUNT OF BLOOD TO BE DRAWN IN RELATION TO THE PATIENT'S BODY WEIGHT**

Body weight (kg)	Collected blood (g)
50	200
52	210
54	215
56	220
58	225
60	235
62	240
64	250
66	255
68	260
70	270
72	275
74	280
76	285
78	295
80	300

cells in the lymph and in the vast retroperitoneal lymph node network. Rectal insufflation of ozone can be carried out as one option when O<sub>3</sub>-AHT is not feasible or is refused by the patient (subsection 4.7.).

#### 4.4. Insufflation in other body's cavities or topic treatment

Ozone at low concentration (3-5 µg/ml) has been insufflated for periods of 2-5 min into the nasal, tubal, oral cavities as well as in the vagina and the bladder in the case of chronic bacterial or fungine infections. Also cutaneous infections (decubitus, ulcers of the limb and foot), insect bites, fungal skin infections, burns, after humidification and airtight bagging have been treated with an ozone flow at concentrations as high as 80 µg/ml for about 20 min. However, because the lungs are most sensitive to ozone (subsection 3.6.), inhalation of the gas must be avoided and, except rectal insufflation, all the above mentioned organs are now more easily treated with ozonated bidistilled water, or saline, or oil. In Russia and Cuba there is now a trend for using extensively either ozonated water or sunflower oil either for irrigating infected body cavities, or for drinking to treat gastritis, gastro-duodenal ulcers, enterocolitis due to bacteria, Giardia, Cryptosporidium and perhaps Helycobacter piloris.

#### 4.5. Total body exposure

Exposure of the body (excluding the neck and head for avoiding respiratory toxicity) in a thermostatically-controlled cabinet to humidified O<sub>2</sub>-O<sub>3</sub> mixture may represent the last

option for patients who refuse rectal insufflation. However, one should first evaluate transcutaneous gas absorption and other parameters such as plasma levels of TBA reactive substances before, during and after ozone exposure.

#### 4.6. Intra-articular and intradiscal injection

A volume of 2-10 ml humidified ozone (concentration 5-10  $\mu\text{g/ml}$ ) can be injected by an experienced orthopedician directly into the intra-articular cavity. Pain is tolerable and recedes rapidly. Apparently acute and chronic inflammatory joint diseases seem to improve with this treatment. In the case of hernial disc, injection of 1-2 ml gas directly into the degenerated pulp seems to yield better results than other methods (aspiration, chymopapain injection) used so far.

#### 4.7. Minor and major ozonated autohemotherapy ( $\text{O}_3$ -AHT)

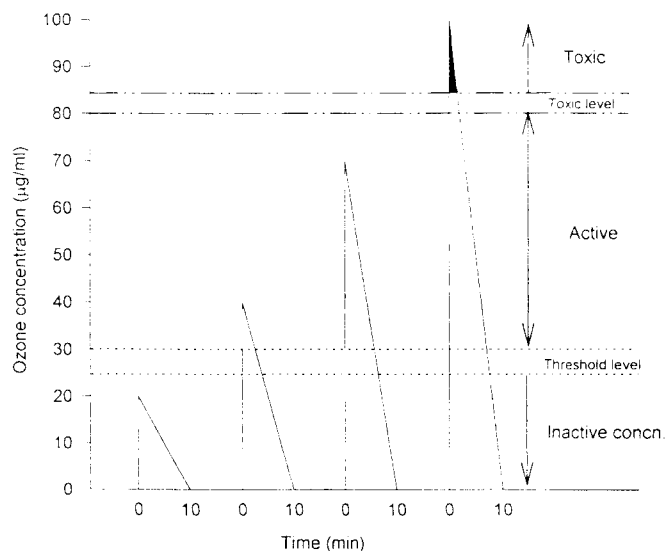
In the minor technique 5-10 ml of venous blood, drawn from the patient, is immediately mixed with an equal volume of ozone and injected intramuscularly. It is being used for the treatment of herpetic infections, asthma and cancer (7,8). Although it may exert some immunomodulatory activity, this has never been convincingly demonstrated and it may well have only a placebo effect.

The so-called major  $\text{O}_3$ -AHT has been mostly carried out 2-3 times weekly, or even daily, with 60-100 ml of blood collected in CPD, slightly ozonated (ozone concentration: 10-30  $\mu\text{g/ml}$ ) mixed and rapidly reinfused. This protocol, still widely used in Germany, is based on the premises that either low (10-20  $\mu\text{g}$ ) or high (30-40  $\mu\text{g/ml}$ ) doses of ozone are either stimulatory or suppressive, respectively. Unfortunately this statement has no experimental basis although ozonetherapists claim clinical benefits in ischemic and chronic infectious diseases (8,29). In actual fact there is no objective clinical data to support this contention that has lent to believe that ozone acts almost as a magic entity transferring its energy to the body. The homeopathic concept may have influenced this type of formulation.

That is why we have changed the protocol. For each treatment we withdraw from 200 up to 300 g of blood depending upon the body weight of the patient (Table II). Attention to this detail minimizes the risk of hypovolemic shock although the physician must check the arterial pressure and reactivity of the patient. There is no need to carry out any previous hydration with saline. Ozone concentration is always given in  $\mu\text{g/ml}$  of gas ( $\text{O}_2$ - $\text{O}_3$ ) per g of blood or medium. It is assumed that practically all the ozone dose reacts with blood. Recommended concentrations range between 40 and 70  $\mu\text{g/ml}$  ozone equivalent to  $\sim 0.8$ -1.5mM.

The following comments must be kept in mind:

a) at this stage optimal dosages have not yet been defined and it appears likely that each type of disease, if not different stages of the disease, may need a different ozone dosage. As an example, ischemic diseases may be best treated with a



**Fig. 9** - A schematic representation of the therapeutic window of ozone. Concentrations of ozone below 30  $\mu\text{g/ml}$  produce ROS largely quenched by the antioxidant reservoir of blood. If ROS levels do not reach the intracellular threshold level, no biological effects will ensue (placebo effect). On the other hand, concentrations of ozone higher than 80  $\mu\text{g/ml}$  are likely to produce an excess of ROS, thus leading to some cellular damage.

dosage of 40  $\mu\text{g/ml}$ , while some chronic viral infections may need an average dosage of 70  $\mu\text{g/ml}$ , although in the late phase of HIV infection, the latter concentration may be detrimental. At this time flexibility and common sense must guide the selection of the dose. In Figure 9, the evaluation of several parameters (rate of hemolysis, GSH content, cytokine expression, etc) has allowed to approximately define the therapeutic window. The scheme suggests that ozone dosing is critical in the sense that if ROS are too low, they may be unable to activate a biological response (placebo effect), while, if their concentration is very high, activation as well as intracellular oxidative damage may ensue. As a further complication both threshold and toxic levels may oscillate up and down for each blood sample depending upon the individual blood antioxidant level. In order to avoid a brisk oxidative stress during the first two weeks, we can use low dosages and, after adaptation to the stress, the dosage can be scaled up. Obviously, we need to monitor this change by following appropriate markers as plasma levels of lipoperoxides, erythrocytic levels of SOD, GSH Px, 2,3-DPG and GSH. It must be added that our studies (24,32,51) performed on human healthy volunteers, were primarily interested to evaluate the induction of cytokines likely produced by  $\text{CD4}^+$  lymphocytes with a Th1 phenotype. As a consequence, by using the same ozone concentration, the pattern of cytokine production could be quite variable depending upon the individual  $\text{CD4}^+/\text{CD8}^+$  and Th1/Th2 ratios. If this reasoning is correct, it implies that endogenous production of cytokines induced by

**TABLE III - MEDICAL APPLICATION OF OZONETHERAPY**

Intensive care	Angiology	Neurology	Oncology
Surgery	Dermatology	Gerontology	Hepatogastroenterology
Urology		Rheumatology	
Gynecology			
Proctology			
Odontology			
Orthopedics			

**TABLE IV - AN OVERVIEW OF POSSIBLE BIOLOGICAL EFFECTS ELICITED BY O<sub>3</sub>-AHT AND TOPICAL OZONE APPLICATION IN HIND-LIMB CIRCULATORY DISTURBANCES**

	OH <sub>3</sub> -AHT				Topical O <sub>3</sub>
Plasma	Erythrocytes	Platelets	Leukocytes	Endothelial cells	Fibroblasts keratinocytes
Fibrinogenemia ↓	Glycolysis ↑	TGF β ↑	PGE 2 ↑	NO?	KGF?
Cholesterol ↓	ATP ↑	PDGF ↑	Histamine?	VEGF?	bFGF?
Angiostatin?	2-3 DPG ↑		Bradykinin?	Fibronectin?	Hyaluronic acid?
	O <sub>2</sub> -availability ↑				Sulfate dermatan?
	Filterability ↑				Collagen I/III?
	Membrane charge ↑				
	Sedimentation rate ↓				
	Arterial pO <sub>2</sub> →				
	Venous pO <sub>2</sub> ↓				

↓ reduced; ↑ increased; → unchanged; ? doubtful

ozone *ex vivo* may not always be the most idoneous for correcting the imbalance observed either in autoimmune diseases (Th1>>Th2) or, on the contrary in allergy, AIDS and parasitic diseases (Th2>>Th1).

b) The ozone dosage (up to a maximum of 80 µg/ml) and a time of 10 min of exposure before reinfusion has never yielded an hemolysis higher than 2%. This neither led to anemia, nor tubular damage, side effects, and adverse sequelae even after 70 consecutive treatments.

c) Mixing of blood with the gas in the bag must be gentle to avoid foaming but owing to blood viscosity, continuous rotation must be applied for 5-10 min to ensure a homogeneous interaction (Fig. 1). Gas should never be bubbled into the blood because flushing increases hemolysis and protein denaturation. This procedure, accompanied by heating and ultraviolet treatment, makes autohemotherapy useless (154).

d) Reinfusion into the donor can be fairly rapid (20-30 min) and has neither caused transfusion-related acute lung injury (155) nor other complications typical of allogenic transfusion. Utmost care must be exercised to preserve the viability of the venous access.

e) It is not yet known which is the critical mass of ozonated blood for achieving a therapeutic effect. Obviously, this

cannot simply be the sum of blood volumes for each session because we do not yet know the turnover of the ozonated cells, and the duration of the biochemical or/and immunological effect, although surprisingly long, is definite. Several questions have still to be answered: is O<sub>3</sub>-AHT able to influence enzymatic synthesis already in the bone marrow so that newly-born erythrocytes are already adapted to the new metabolic environment? Which is the role and relevance of the reinfused ROS, particularly lipid mediators, in stimulating other biological activities? Does the priming of PBMC, *ex vivo*, trigger further activation of resting or suppressed immune cells, once the reinfused leukocytes home into lymphoid tissues? It is, therefore, almost impossible to foresee how many autotransfusions are needed to achieve either an improvement of ischemic conditions, or a minimal reactivation of the immune system. In the latter case the system comprises about 10<sup>12</sup> cells dispersed in the gut, spleen, lymph nodes, bone marrow and thymus and about 1% of these cells are replaced every day (156). Ozonation of 300 g blood stimulates at best about 6 x 10<sup>8</sup> cells i.e., only 0.06% of the total cell number. Thus, after fifty treatments (twice weekly for 6 months) we may barely have activated only 3 x 10<sup>10</sup> cells equivalent to a mere 3% of the cell pool! However, rein-

fused ROS and microenvironmental release of cytokines may unpredictably amplify the process several fold and therefore it is necessary to monitor clinical conditions as well as biochemical and immunological parameters to evidentiate if the patient responds to the treatment.

f) Particularly for treating viral diseases, it is not yet known whether, O<sub>3</sub>-AHT should be carried out in the morning or late afternoon because it is likely to modify hormonal and immunological responses. Unfortunately, so far only the physician and patient's schedules have been considered, completely disregarding the potentially important chronopharmacological aspect.

g) In spite of being the most rational, safe and simple way of ozone administration, O<sub>3</sub>-AHT has a few drawbacks. The disposable material costs about 15 US dollars and each treatment is time-consuming (at least 40 min). Unless one uses a portable ozone generator or, unadvisably, carries an ozone-filled up container, cannot be done at home. Occasionally, I have noticed some reluctance by the medical personnel to handle blood of infectious patients. Utmost care must be exercised to prevent exchange of blood bags when autotransfusion is carried out on several patients simultaneously. Finally, particularly elderly women have such a poor venous access to prevent the treatment. In this eventuality, parenteral administration of ozone can be carried out by either rectal insufflation, or by infusion of slightly ozonated physiological solution. Both options are far from ideal and the latter, even though very easy and rapid to perform, for several reasons does not guarantee a beneficial effect. For the sake of simplicity and negligible cost, infusion of ozonated saline is vastly employed in Russian hospitals but comparative studies between O<sub>3</sub>-AHT and O<sub>3</sub>-saline were not presented at the 2nd Russian Conference of Ozonotherapy in 1995.

h) Finally extracorporeal exposure of up to 7 L of blood to O<sub>2</sub>-O<sub>3</sub>, within 70-90 min, is being carried out in terminal cancer patients trying to evaluate pros and cons of this approach. In comparison to conventional O<sub>3</sub>-AHT, clinical advantages will have to prove highly significant because the procedure is fairly expensive and need of specialized equipment and personnel. Nonetheless, not even this intensive treatment gives side effects and is actually well tolerated.

## 5. CAN OZONETHERAPY BE USEFUL IN SOME HUMAN DISEASES ?

There are several areas where O<sub>3</sub>-AHT has been used and examining Table III it seems that ozonotherapy is almost a panacea but actually it is not: the fact that it can be useful in unrelated pathologies is simply due to the fact that ozone can activate distinct blood cells which express different functions. There are five main pathologic areas, namely: 1) infectious states, 2) immune depression, 3) ischemic conditions, 4) neurodegenerative diseases and 5) acute and chronic articular diseases including discal hernias, where, in spite of striking advances, conventional medicine is still unable to

provide a definitive improvement. It seems reasonable and ethically correct to take advantage of ozonotherapy when the best orthodox treatment fails; as an example why patients with either hind-limb ischemia (III-IV grade) facing amputation or chronic hepatitis patients, who do not tolerate IFN, should not try autohemotherapy?

### 5.1. Infectious states

Although in these days it appears surprising, owing to diffused antibiotic-resistant bacteria, ozone is profitably used under the form of either ozonated physiological solution, or bidistilled water, or oil for the treatment of war wounds, anaerobic infections, trophic ulcers and burns (157). Abscesses, anal fissures, decubitus (bed sores), fistulae, fungus diseases, furunculosis, gingivitis, inveterate osteomyelitis, peritonitis, sinusitis, stomatitis, vulvovaginitis and wound healing disturbances are claimed to improve with the treatment because ozonated solutions display a cleansing effect and act as a powerful disinfectant to which even antibiotic-resistant or anaerobic bacteria cannot resist. It appears that ozonated solutions control bleeding, improve the metabolism and reduce the infection (8,21,22,27,30,158-164). However, in toxic and septic shock where there is already a redundant production of inflammatory cytokines, O<sub>3</sub>-AHT should be performed with caution possibly at low O<sub>3</sub> concentration (40 µg/ml) improving tissue oxygenation but avoiding further stimulation of cytokine production.

It is reasonable to postulate (section 2.3. and 3.6.) that ozone can induce the expression and release of several growth factors such as PDGF, TGFβ, bFGF, MCP-1/JE and KGF (85,87,88,165). These are the main cytokines that play a major role in stimulating particular facets of the healing process and the endogenous release is likely to accelerate wound healing (86).

### 5.2. Pathologies linked to immune depression

There are several pathological states where the infectious agent, usually a virus, remains more or less active because the immune system is unable to eliminate it. Chronic hepatitis B and C constitute a serious problem because of their widespread diffusion and the tendency to evolve within 1-2 decades towards cirrhosis or/and hepatocarcinoma. There is no doubt that the drug of choice has become IFNα (166). However, in order to achieve a therapeutic success, one must follow a precise schedule (s.c. administration of 6 MegaUnits every other day or daily) for 6 up to 18 months but unfortunately some patients do not comply with it because of side-effects, particularly troublesome in elderly patients. Owing to various reasons (virus genotype, anti IFN-antibody formation, virus coinfection, etc), some patients respond poorly or not at all and complete responses range between 25 and 33%. Because the treatment is also expensive, there are millions of people worldwide who do not receive

ve any therapy and, we should not forget about the tragic outcome with fialuridine, a nucleoside analogue tested for the treatment of hepatitis B (167).

Is there a rational basis for using O<sub>3</sub>-AHT? There is no doubt that ozone has virucidal activity because through peroxidation of phospholipids and lipoproteins present in the envelope, the viral integrity is compromised (168,169). Oxidation of viral ligands may further impair their binding to specific cell receptors (170,171). In practice, however, the direct virucidal effect remains of dubious relevance: during ozonation of blood *ex vivo*, the concentration of generated ROS may inactivate free viral particles in plasma but after reinfusion, owing to the ensuing dilution and inactivation by antioxidants, for how long ROS remain active? Moreover most of the viral load is located intracellularly (liver, lymphoid system, neurones) and is paradoxically protected by the antioxidant system *in vivo*. Thus other mechanisms may be operative: probably the most relevant is the stimulation of humoral and cell-mediated immunity via induction of relevant cytokine such as IL-2, 4, 6, 12, TNF $\alpha$  and IFN $\gamma$  (subsection 2.2.). Furthermore, infected cells may become more prone to be recognized and killed by various cytotoxic effector cells. In conjunction with hepatocyte death, improved liver metabolism and release of hepatocyte growth factor (HGF) and TGF $\alpha$  may stimulate liver regeneration (172) and accelerate recovery of hepatic functions. Another possibility is that ozone generates immunogenic viral particles that may continuously stimulate the synthesis of antibodies against viral mutants. In subsection 3.6. the overexpression of antioxidant enzymes during prolonged autohemotherapy as a defensive response has been entertained. While this occurs in normal cells, virally-infected and cancer cells, which usually are in a hyperoxidative state (173) may be at a loss and unable to increase SOD, CAT and GSH Px - levels; if this occurs *in vivo* these cells will probably switch on the apoptotic mechanism. Thus, O<sub>3</sub>-AHT so far carried out in uncontrolled and not standardized studies, (7,8,28) may lessen clinical severity or duration of acute and chronic hepatitis, herpes virus, herpes zoster infection (174). HIV infection poses considerable problems because an increased oxidative status favours HIV replication (175-178) and O<sub>3</sub>-AHT could be deleterious (81), unless is able to eliminate infected cells and stimulate an antioxidative state. The aim is to reduce viral load or to induce viral quiescence and it would be useful to evaluate whether the simultaneous employment of IFNs, antiretroviral drugs and O<sub>3</sub>-AHT entails a synergistic effect and achieve a stabilization of the disease.

It does not come as a surprise that ozone inhibits growth of cancer cells *in vitro* (179) but it remains doubtful that it could obtain a direct effect *in vivo*. A reasonable possibility is that human cancer cells have an impaired antioxidant system and they are incapable of exercising an effective peroxide inactivation. Moreover, if the upregulation of antioxidative enzymes occurs in normal but no in neoplastic cells, it may interrupt the vicious circle described by

Toyokuni et al (140) as a "persistent oxidative stress" able to amplify the oncogenic process. The immunomodulatory activity of O<sub>3</sub>-AHT may represent an additional advantage either for the treatment of minimal residual disease or even to slow progressing metastatic cancer in elderly patients, where a palliative monochemotherapy appears useless and worsens the quality of life. While no controlled data have been published (7,8,180,181) to prove that O<sub>3</sub>-AHT is outright beneficial for solid tumors, it remains the tantalizing possibility that it could be useful for many patients who feel abandoned after having unsuccessfully tried orthodox therapies.

For autoimmune diseases such as either rheumatoid arthritis or multiple sclerosis, there are new encouraging therapeutic development with either anti-TNF $\alpha$  antibodies (182) or IFN $\beta$  (183), respectively, but these treatments, although they have a scientific foundation are still at an experimental stage. It has been claimed (8) that either low or high dosages of ozone display either an immuno-stimulatory or inhibitory effect, respectively, implying the possibility of correcting a cytokine imbalance between the production of pro-inflammatory (IL-2, TNF $\alpha$ , IFN $\gamma$ ) and immunosuppressive cytokines namely IL-4, TGF $\beta$  and IL-10 (184-186). A prevalent secretion of the latter could inhibit autologous cytotoxic T cell clones but so far we have been unable to support this claim with experimental proof. Therefore great caution must be exercised at this stage for avoiding an exacerbation of the autoimmune process. It remains also uncertain whether the increased production of ROS at the lesional sites might be quenched by an overexpression of antioxidant enzymes. As too often happens, the validity of O<sub>3</sub>-AHT remains in the realm of fiction also because ozonetherapists often use two or if not three complementary approaches simultaneously.

### 5.3. Ischemic conditions

Owing to various causative agents, several circulatory disturbances (hind-limb ischemia, heart-brain-retinal-ischemia) develop with the common denominator of either acute or chronic ischemia. As it is summarized in Table IV a complex series of partly hypothetical and partly demonstrated biological effects can explain the clinical improvement observed after O<sub>3</sub>-AHT using an ozone concentration of 10-30  $\mu$ g/ml. Unfortunately, it is neither known which is the most suitable anticoagulant, nor the optimal blood volume, nor the ozone concentration, nor the schedule. The reported clinical results in acute cerebro-vascular disorders (29), chronic ischemic cardiopathy (187) and particularly in hind-limb ischemia (III-IV stages) have been impressive, particularly combining O<sub>3</sub>-AHT and topical treatment with either O<sub>2</sub>-O<sub>3</sub> ozonated water, but are anecdotal (27,30) and systematic, controlled studies remain to be done. A preliminary investigation by Verrazzo et al. (188) has shown that O<sub>3</sub>-AHT induces a favourable improvement of rheologic parameters.

Other serious hypoxic conditions such as those due to acute respiratory distress syndrome, pulmonary fibrosis, and



emphysema may benefit by this approach but have never been evaluated.

#### 5.4. Degenerative diseases associated with aging

Cancer and immune system decline have been discussed in subsection 5.2. and cardiovascular disease in subsection 5.3. although in principle they could be included here. The progressive prolongation of human half-life is accompanied by an increase of degenerative diseases and those of the CNS are decisively crippling. There is substantial evidence (39,115,119,122,124,136,189,190) suggesting that, in association with several cofactors, a lifetime oxidative damage may be responsible for neurodegenerative disorders such as Parkinson's disease, senile dementia, optic nerve dysfunction, primary open angle glaucoma, neurosensorial bilateral hypoacusia and maculopathies. At first glance, it seems crazy to propose a treatment based upon a brief and calculated oxidative stress in neurodegenerative diseases but in fact this approach may break and reverse an irreversible situation. Clinical investigations have been carried out at the Ozone Research Center, Havana (Cuba) and have been reviewed by Gomez Moraleda (191). As far as the improvement of senile dementia is concerned, a double blind study was performed on 60 patients, of which 30 were treated with O<sub>3</sub>-AHT and 30 control patients with O<sub>2</sub>-AHT. Evaluation of three parameters: mental condition, self-medication capacity and daily life activities showed an improvement of 83, 83 and 90% in the treated group. Use of O<sub>3</sub>-AHT in optic nerve dysfunction, primary open angle glaucoma, cochleo-vestibular syndrome and ischemic cerebro-vascular disease yields improvement ranging from 50 up to 100 %. What explanations have been advocated for explaining results that seem too good to be true? As discussed in section 2.2., O<sub>3</sub>-AHT activates simultaneously several mechanisms: the improved blood flow and oxygen supply to hypoxic tissue may stimulate aerobic glycolysis in hypofunctional cells that, by resuming a normal metabolism might restore a normal ATP content, GSH/GSSG ratio and upregulate the enzymatic redox system. Induction and release of neuronal growth hormones remain speculative but it is not a too farfetched idea. The possibility that Alzheimer's disease, associated with a deposition of insoluble  $\beta$ -amyloid aggregates, reflects an NO<sup>•</sup>/superoxide imbalance (subsection 2.2.) has been entertained by Thomas et al (192). The therapeutic implication is that a prevalence of NO<sup>•</sup> over superoxide appears advantageous and may inhibit aggregation. This may be achieved by the administration of either exogenous SOD mimetics or/and antioxidants but, interestingly, O<sub>3</sub>-AHT, by inducing SOD and production of NO<sup>•</sup> at the same time, could correct the imbalance.

Two cautionary annotations appear in order: the first is that functional recovery may be achieved only in initial or not too advanced patients and secondly, that an optimal O<sub>3</sub>-AHT schedule has not yet been worked out. It seems reason-

able to start with a low dose of blood and O<sub>3</sub> (concentration 30-40  $\mu$ g/ml) that could be gradually scaled up to 300 g blood and 40-60  $\mu$ g/ml ozone for preventing a possible deterioration due to ischemia-reperfusion injury. If an improvement really occurs, it may be necessary to continue the treatment (once weekly or biweekly?) for life.

Retinal maculopathies deserve a short discussion on their own. Open studies carried out using O<sub>3</sub>-AHT in both age-related degenerative retinal maculopathy and retinitis pigmentosa yield an improvement of visual activity in about 2/3 of patients (191,193). A controlled clinical trial on a large number of patients is ongoing at the University Hospital of Siena in collaboration with the Department of Ophthalmology and is yielding a similar improvement. However a recent evaluation of 10 patients with retinitis pigmentosa subjected in Cuba to a regimen composed of electrical stimulation, O<sub>3</sub>-AHT and ocular surgery is not confirmatory and actually suggest that this complex intervention, in comparison to simple vitamin A supplementation may worsen the course of the disease (6,194). Thus, this problem remains open in so far the study has examined a genetic disease and a too complex protocol, where it is not possible to clarify the exclusive role of O<sub>3</sub>-AHT.

#### 5.5. Acute and chronic articular diseases including discal hernias

During the last few years a number of orthopedician have begun treating acute and chronic polyarthritis (195), discal hernia with intra-articular or periarticular insufflation of small volumes of ozone with very encouraging results. In a review article, Siemsen (196) has reported that application of medical ozone in acute and chronic painful diseases of the joints is a complementary method of treatment for obtaining rapid pain relief, decongestion, disappearance of edema, a reduction of local temperature and an increase in mobility. The treatment bears neither risk, nor side-effects. Several mechanisms of action have been put forward as inhibition of prostaglandin synthesis, prompt neutralization of ROS owing to overexpression of SOD, enhanced release of cytokine antagonists or/and of immunosuppressive cytokines able to inhibit cytotoxic cells (81).

## 6. CONCLUSIONS AND FUTURE PROSPECTS

In a superbly written article entitled "Complementary medicine: from quackery to science?" Prof. Edzard Ernst (197) has pointed out that time has come "to submit to scientific scrutiny treatments that appear potentially useful. The obvious way to do this is to conduct randomized controlled trials taking into account the peculiarities of the remedies that are being tested". That is exactly what is badly needed for justifying the use of ozonotherapy but my effort and proposals (24,81,153,198) so far have been to little avail. The main aim of this review has been to show that we now begin

to understand at least some of the basic mechanisms that govern the biological effects of ozone. Rather than to affirm that ozone is toxic any way you deal with it, I would say that ozone must be considered as a sort of double-edged sword with some of the generated ROS acting as useful physiological mediators able to reactivate the metabolism and the immune system. A provocative concept is that ozone, if used correctly and for a prolonged time, could act as a multiform bioregulator able to readjust an unbalanced metabolism and the immune status.

I believe that by leaving indefinitely ozonotherapy in limbo we will do a disservice to Medicine and patients and I hope that this review will serve to dissipate some prejudices, critically discuss new ideas and possibly stimulate new investigations. I have already said that I fully agree with skeptics about the poor quality and quantity of the scientific work published on ozonotherapy. This includes clinical work and it was really difficult to summarize section 5. because most of the data, although apparently encouraging, are not scientifically documented. Most of them are hardly comparable because no constant attention has been paid to observe a stoichiometric relationship between precise blood weight, volume, ozone concentration and time of exposure. Controls with either O<sub>2</sub>-or air-AHT have been rarely performed. Talking with ozonotherapists, to my disappointment, I have often realized that the result of the treatment may either be a placebo effect or, being mixed with other complementary approaches, does not allow any conclusion. Ozonotherapists frequently have a resentment towards the medical establishment because they feel disregarded, occasionally prosecuted owing, they say, to the overwhelming power of pharmaceutical industry, the medical centers and association and by the fact that ozone is non-patentable and the therapy is inexpensive in comparison to orthodox medical procedures. Most of these comments are inappropriate and it is useless to accuse others for excusing its own inadequacy.

Having said this what can we do? Besides continuing the evaluation of the biological mechanisms great attention must be paid to use ozone with care, precision and efficiency keeping well in mind the axiom: *primum non nocere*. This can be achieved by using either modern medical generators that check in real time the ozone concentration and volume and by adequately preparing physicians to use this drug. Indeed, ozone must be considered as a peculiar drug with all its inherent toxicity and being well aware that only by using standardized procedures we can obtain reproducible results and progress. This obviously implies the urgent need to evaluate in a controlled fashion ozone dosing and schedules of administration in relation to clinical benefits and possible toxicity. Moreover it is not enough to observe that some diseases improve with ozonotherapy: because there are no data on the follow-up, it is compelling to evaluate if the beneficial effect can be maintained or resumed. Only by obtaining valid results, we can change the aptitude of governmental agencies and perhaps

obtain some badly needed financial support for carrying out further research. I never miss the opportunity of emphasizing that we should not consider ozonotherapy as the *omnia sanans* and we should use this as a complementary approach when either orthodox therapy fails to be beneficial or to reduce the symptoms of terminal illnesses.

Finally, I could not finish better this article without quoting Ernst's final sentence: "*If we manage to intensify these efforts, we should one day be in a better position to tell the fraudulent or harmful from the effective and safe. It seems to me that this is not merely a practical necessity but an urgent ethical duty that physicians and complementary practitioners alike owe to their patients*".

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#### ABBREVIATIONS

ATP, adenosin tri-phosphate; CAT, catalase; CPD, citrate-phosphate-dextrose; 2-3 DPG, 2-3 diphosphoglycerate; bFGF, basic fibroblast growth factor; G3-PD, glyceraldehyde 3-P-dehydrogenase; G-6-PD, glucose-6-phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony stimulating factor; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSH-R, glutathione reductase; GSH-S-tr, glutathione S-transferase; GSSG, oxidized glutathione; Hb, hemoglobin; HGF, hepatocyte growth factor; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HIV, human immunodeficiency virus; KGF, keratinocyte growth factor; IFN, interferon; IL, interleukin; LDH, lactate dehydrogenase; LDL, low density lipoproteins; LLFL, lung lining fluid layer; MCP-1/JE, monocyte chemoattractant protein 1; NAC, N-acetyl cysteine; NF-KB, nuclear factor kappa B; NO<sup>•</sup>, nitric oxide; O<sub>2</sub><sup>•-</sup>, anion superoxide; O<sub>2</sub>-O<sub>3</sub>, oxygen-ozone; O<sub>3</sub>-AHT, major ozonated-autohemotherapy; OH<sup>•</sup>, hydroxyl radical; PBMC, peripheral blood mononuclear cells; PDGF, platelet-derived growth factor; PK, pyruvate kinase; 6-PGD, 6-phosphogluconate dehydrogenase; ROS, reactive oxygen species; SCE, sister chromatid exchange; SODs, superoxide dismutases; TBA, thiobarbituric acid; TGF-α, transforming growth factor α; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

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