Chapter 4
How Does Ozone Act? How and Why Can We Avoid Ozone Toxicity?

This is one of the most important chapters because I believe that, if the ozonetherapist understands how ozone reacts with body fluids and cells, he can achieve useful therapeutic results. The administration of ozone causes a number of biochemical, pharmacological and psycho-neuro-immunological reactions to take place in a patient who is an essential part of the process.

Although oxygen represents the bulk (95–98%) of the gas mixture, by considering the enormous dilution of the small reinfused oxygenated-ozonated blood with venous blood, it has a negligible role. While, only thanks to oxygen we can live, this gas has a negative effect on the long run because cell respiration allows the formation of reactive oxygen species (ROS), among which, hydroxyl radical ($OH^{\bullet}$) is one of the most destructive radical compounds for precious enzymes and DNA. Halliwell (1994) has calculated that, even at rest, a human being produces about 5 g of anion superoxide ($O_2^{-\bullet}$), which is the father of several radical molecules. Anion superoxide is physiologically produced in the mitochondria, from Complex I and II (Kowaltowski et al., 2009) but other ROS, such as hydrogen peroxide, hypochloric acid and nitric oxide are continuously generated by various oxidases and myeloperoxidase and, in trace amounts, have a crucial defensive role against pathogens. On the other hand almost every one knows that ageing, the metabolic disorders (atherosclerosis, diabetes, cell degeneration) can be worsened by an excessive production of ROS and, only in part, we can prevent their damageable effects. Ironically, even the partial lack of oxygen (hypoxia), observable in ischemic vascular diseases, represents the cause of death due to limb ischaemia, heart infarction and stroke. Moreover, hypoxia enhances neoplastic metastatisation and ultimately leads to death.

Ozone, the triatomic oxygen, synthesized in the stratosphere to protect us from excessive UV radiation, can be precisely produced with a medical generator but it is up to us to use it proficiently as a real drug. As ozone is one of the most potent oxidants; the third in the chemical scale, we must learn how to tame it and **the scope of this chapter is to define its therapeutic coefficient, or**, in simple words, **to distinguish the therapeutic from the toxic dose**.

When I ask physicians how ozone acts, I receive odd answers: a favoured one is the esoteric idea that ozone, during its decomposition to oxygen, will transfer some energy to the body thus invigorating it, and another is that ozone will be

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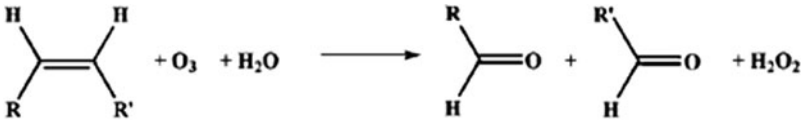
absorbed and, after entering into the cells, will turn them on. In comparison to other complementary approaches based on philosophical postulations or unverifiable hypothesis, a positive characteristic of **ozonetherapy** is that it **can undergo the most objective scientific investigation carried out with normal biochemical, pharmacological and clinical methods**. It has been unfortunate that for several decades, empiricism and the lack of basic studies have delayed an understanding of the mechanisms of action. Moreover, dangerous, even deadly infusion of ozone by quacks, a good dose of prejudice and **the inconsistent dogma that "ozone is always toxic"** are responsible for the strong and dull opposition of conventional medicine to the use of ozonetherapy. However I will persevere in my endeavour and I feel confident that this wrong belief will change in the future.

At the moment my duty is to schematically try to demonstrate that ozone obeys perfectly well to common physical, chemical, physiological and pharmacological notions and that its activities modulating several cellular functions are already known.

First of all, ozone, as any other gas, readily dissolves in the water either of the plasma (the liquid part of blood), or into the extracellular fluids, or into the thin layer of water covering the skin and particularly the mucosae of the respiratory tract, gut, vagina, etc. At normal temperature and atmospheric pressure, owing to its high solubility and depending upon its relative pressure, ozone dissolves into the water but, unlike oxygen, DOES NOT EQUILIBRATE with the ozone remaining in the gas phase. This happens because **ozone, being a potent oxidant, REACTS IMMEDIATELY with a number of ions and biomolecules present in biological fluids, namely antioxidants, proteins, carbohydrates and**, ***preferentially, polyunsaturated fatty acids (PUFAs) bound to albumin***. In fact phospholipids and cholesterol present either in cell membranes or/and lipoproteins are shielded by antioxidants and albumin molecules (Bocci and Di Paolo, 2009; Travagli et al., 2010b).

The reaction of ozone with so many molecules implies several fundamental processes occurring at the same time: some of the ozone dose is unavoidably consumed during oxidation of ascorbic and uric acids, sulphydryl (SH)-groups of GSH, proteins and glycoproteins present in the water of plasma. The other fundamental and well characterized reaction is known as "LIPID PEROXIDATION" (Pryor et al., 1995). In the hydrophilic plasma environment, one mole of an unsaturated olefin (particularly arachidonic acid transported by albumin or present in plasma triglycerides and chylomicrons) and one mole of ozone give rise to two moles of aldehydes and one mole of hydrogen peroxide (H_2O_2).



These reactions, completed within seconds, use up the total dose of ozone that generates hydrogen peroxide, and a variety of aldehydes known as LIPID OXIDATION PRODUCTS (LOPs).

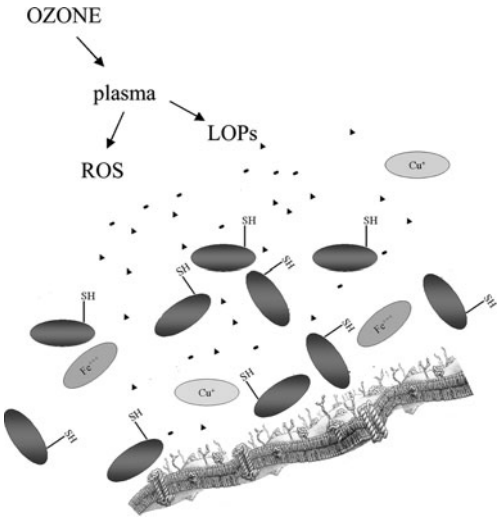


Fig. 4.1 The scheme helps to imagine the multiplicity of substrate reacting with ozone dissolved in plasmatic water. *Small circles*, *triangles*, and *squares* symbolyze hydrosoluble antioxidants present in 100 ml of human blood (uric acid 4.5 mg/dl, ascorbic acid 1.5 mg/dl, glucose 80 mg/dl, etcy). Large albumin molecules (4,000 mg/dl) exposing -SH groups form a cloud over the cell membrane and protect it. Molecules such as transferring and ceruloplasmin bind Fe^{3+} and Cu^{+} and prevent formation of OH^{-}. The exogenous addition of 4–8 mg of ozone to 100 ml of blood is transitory and controlled by antioxidants. In contrast, the endogenous production of ROS is continuous and barely quenched by intracellular antioxidants

The following scheme (Fig. 4.1) has been drawn to depict how ozone dissolved in water reacts simultaneously with hydrosoluble antioxidants and lipids bound to albumin. It also shows how ozone, at therapeutic concentrations, cannot reach the phospholipids bilayer constituting the erythrocyte membrane well shielded by albumin molecules. It appears obvious that artificial experiments performed with saline-washed erythrocytes have shown a damage to the cell membrane and it is unfortunate that these results have wrongly led to believe that ozone is cytotoxic.

4.1 From Now on, Ozone Is Exhausted and Only ROS (Mostly Hydrogen Peroxide) and LOPs Are Responsible for the Successive and Multiple Biochemical Reactions Happening in Different Cells All Over the Body

Therefore it should be clear that **some of ozone dose is neutralized by the antioxidants present in plasma and only the reaction with PUFA is responsible for the biological and therapeutic effects**. This should clarify why a very low ozone dose can be ineffective or equivalent to a placebo. Moreover, after ozonation of human blood, the antioxidant capacity measured in plasma decreases no more than

30% after about 5 min but returns to the normal value during the following 15 min, thanks to the rapid reduction of the oxidized antioxidants operated by erythrocyte (Bocci et al., 1998b; Bocci and Aldinucci, 2006). This result emphasizes that even the higher ozone dose (80 mcg/ml gas per ml of blood) never overwhelms the antioxidant capacity of plasma and insures against any damage to blood cells.

ROS include several radicals as anion superoxide ($O_2^{\bullet -}$), nitrogen monoxide ($NO^{\bullet}$), peroxynitrite ($O{=}NOO^{-}$), the already mentioned hydroxyl radical and other oxidant compounds such as hydrogen peroxide and hypoclorous acid (HClO). ***All of these compounds are potentially cytotoxic*** (Fridovich, 1995; Pullar et al., 2000; Hooper et al., 2000), ***luckily have a very short half-life (normally a fraction of a second) and both the plasma and cells have antioxidants able to neutralize them, if their concentrations do not overwhelm the antioxidant capacity. This concept emphasizes why the ozone dose must be precise and well calibrated against the antioxidant capacity of blood thus capable of triggering useful reactions without procuring any damage***.

LOPs generated after peroxidation of a great variety of PUFAs are heterogenous and briefly are represented by peroxyl radicals ($ROO^{\bullet}$), a variety of hydroperoxides (R–OOH) and a complex mixture of low molecular weight aldehydic end products, namely malonyldialdeyde (MDA), and alkenals, among which 4-hydroxy-2,3 transnonenal (4-HNE), which is potentially cytotoxic. The chemistry and biochemistry of these compounds has been masterfully described by Esterbauer's group (1991). If one thinks about the wealth and chemical heterogeneity of lipids, glycolipids and phospholipids present in plasma, it becomes difficult to imagine how many potent, potentially noxious, compounds can be generated by the lipids reacting with ozone. During one of my several disputes with American referees, a distinguished scientist wrote: "It is grotesque to think that any Western World Drug Regulating Agency would condone infusing the hodgepodge of ozonized products to treat diseases, although it is probable that the products would initiate and/or modulate a wide spectrum of inflammatory-immune processes to varying degrees".

In my opinion, **this referee missed what I believe is the formidable strength of ozonetherapy: provided that we can control (by using precise ozone concentrations exactly related to the blood volume and antioxidant capacity) the amount of LOPs, we can achieve a multitude of biological effects unthinkable with a single drug. Indeed a great expert in antioxidants, Prof Lester E. Packer, University of California at Berkeley wrote me that the hypothesis that a small dose of ozone can elicit a number of antioxidant responses useful to the organism is quite reasonable and in line with current thinking**.

The next simple scheme ought to fix in the reader's mind this crucial point and the sequence of events eventually leading to the therapeutic results: ROS are produced only during the short time that ozone is present in the glass bottle, ***ex vivo***, **and they yield EARLY biological effects on blood, whereas LOPs, which are simultaneously produced, have a far longer half-life and, during the reinfusion of ozonated blood in the donor, they reach the vascular system and practically all the organs where they trigger LATE effects** (Figs. 4.2 and 4.3).

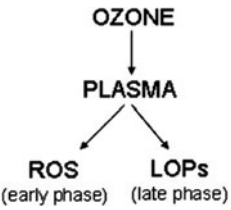


Fig. 4.2 The scheme intends to show that ozone dissolved in the plasmatic water reacts immediately with a number of biomolecules and disappears. The compounds generated during the reactions (ROS and LOPs) represent the "ozone messengers" and are responsible for the biological and therapeutic effects

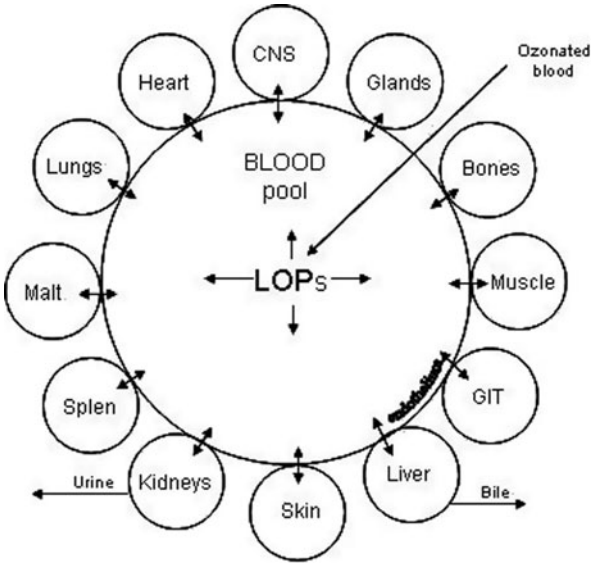


Fig. 4.3 The multivaried biological response of the organism to ozonized blood can be envisaged by considering that ozonized blood cells and the generated LOPs interact with a number of organs. Some of these represent real targets (liver in chronic hepatitis, vascular system for vasculopathies), while other organs are probably involved in restoring normal homeostasis. CNS: central nervous system, GIT: gastrointestinal tract, MALT: mucosal associated lymphoid tissue

We have come to a critical point: how can we reconcile the production of toxic compounds with the idea that these compounds exert important biological and therapeutic effects?

Let us first examine the behaviour and pharmacodynamic of hydrogen peroxide, which in practical terms is the most important ROS. As soon as ozone dissolves in the plasmatic water and reacts with PUFAs, **the concentration of hydrogen peroxide starts to increase but, just as rapidly, decreases because this unionized molecule diffuses quickly into erythrocytes, leukocytes and platelets, where it triggers several biochemical pathways**.

Does the increased intracellular concentration of hydrogen peroxide become toxic for the cell? **Absolutely no! Because, at the same time, it undergoes reduction to water** in both plasma and intracellular water, thanks to the presence of powerful antioxidant enzymes such as catalase, glutathione-peroxidase (GSH-Px) and free reduced glutathione (GSH). Perhaps for a few seconds, the chemical gradient between plasma and the intracellular concentration of hydrogen peroxide has been estimated to range from 1 to 4–5 μM equivalent to about 10% of its plasma concentration, which avoids any toxicity (Antunes and Cadenas, 2000; Stone and Collins, 2002; Stone and Yang, 2006; Forman, 2008). The transitory presence of hydrogen peroxide in the cytoplasm means that it acts as one of the ozone chemical messengers and that **its level is critical: it must be above a certain threshold to be effective but not too high to become noxious. In our studies**, performed with human blood exposed to ozone concentrations ranging from 20 to 80 mcg/ml per ml of blood, **the process of hydrogen peroxide generation, diffusion and reduction was found always extremely transitory** (Bocci et al., 1993a, b, 1998a, b) even though Halliwell et al. (2000a, b) consider this molecule physiologically ubiquitos in the body.

Moreover hydrogen peroxide is recognised as an intracellular signaling molecule able to activate a tyrosine kinase, which phosphorylates a transcription factor (Nuclear Factor KB, NFKB), which allows the synthesis of a number of different proteins (Baeuerle and Henkel, 1994; Barnes and Karin, 1997). Basically hydrogen peroxide functions by oxidizing cysteines (Rhee et al., 2000), and we and Others have found that it acts on blood mononuclear cells (Bocci and Paulesu, 1990; Bocci et al., 1993b, 1998a; Reth, 2002), on platelets (Bocci et al., 1999a), on endothelial cells (Valacchi and Bocci, 2000) and on erythrocytes (Bocci, 2002).

ROS entering into the erythrocytes are almost immediately reduced (hydrogen peroxide to water and lipoperoxides to hydroperoxides) at the expense of GSH. The enormous mass of erythrocytes can easily mop up hydrogen peroxide and, within 10–15 min, marvellously recycle back oxidized antioxidants in reduced form (Mendiratta et al., 1998a, b). While **glutathione reductase** (GSH-Rd) **utilises the reduced nicotinamide adenine dinucleotide phosphate** (NADPH, this coenzyme serves as an electron donor for various biochemical reactions) **to recycle oxidized glutathione (GSSG) to the original level of GSH**, *the oxidized NADP is reduced after the activation of the pentose phosphate pathway, of which glucose-6-phosphate dehydrogenase (G-6PD) is the key enzyme*. Thus, glycolysis is accelerated with a consequent increase of ATP levels. Moreover the reinfused erythrocytes, for a brief period, enhance the delivery of oxygen into ischemic tissues because of a shift to the right of the oxygen-haemoglobin dissociation curve due either to a slight decrease of intracellular pH (Bohr effect) or/and an increase of 2,3-diphosphoglycerate (2,3-DPG) levels (Fig. 4.4).

There is an ample literature regarding the cytotoxicity of LOPs (Poli et al., 2008). These compounds, when tested either in tissue culture, or examined in the context of the delicate respiratory system, are toxic even at a concentration of 1 μM. Surprisingly, submicromolar concentrations (0.01–0.5 μM) tested in several cell types can stimulate proliferation and useful biochemical

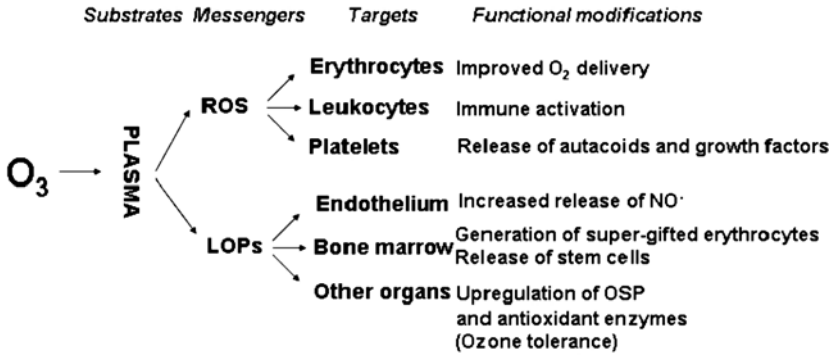


Fig. 4.4 A summary of the biological effects elicited during exposure of human blood to oxygen-ozone, *ex vivo* and during its reinfusion in the donor

activities. These findings lead to believe that toxicity of ozonated lipid products depends upon their final concentrations and tissue-localization, so that they can act either as injurious or useful signals (Dianzani, 1998; Parola et al., 1999; Bosch-Morell et al., 1999; Larini et al., 2004; Aldini et al., 2006, 2008). Blood, in comparison to the lungs, is a much more ozone-resistant "tissue" and we have never observed any damage. **However**, when we reinfuse ozonated blood, what is the fate of LOPs? **We have often measured the kinetic of their disappearance from blood and their half-life in six patients with age-related macular degeneration (ARMD) was equivalent to 4.2 ± 1.7 min. On the other hand, if the same ozonated blood samples were incubated in vitro, levels of LOPs hardly declined during the next 2 h, a result clarifying their toxicity in static cell cultures. As far as cholesteryl ester hydroperoxide is concerned**, Yamamoto (2000) **has emphasized the role of the enzymatic degradation and hepatic uptake**. Thus LOPs toxicity in vivo is irrelevant for the following processes:

(1) **FORMATION OF ALBUMIN-4-HNE ADDUCTS**. Assuming to ozonate 200 ml of blood with an ozone dose of 8 mg, the presence of about 5 g of albumin (Cys 34) is sufficient to form adducts with 4-HNE. Moreover in a total body pool of about 320 g ofd albumin, the ozonated aliquot is less than 1% (Aldini et al., 2006).
(2) **DILUTION** (up to 150–200 folds) of these compounds in blood and body fluids rapidly lowers their initial concentration to pharmacological, but not toxic levels. Obviously the ozone dose must be within the therapeutic range.
(3) **NEUTRALISATION** of LOPs due to the antioxidant capacity in body fluids and cells.
(4) **DETOXIFICATION** of LOPs due to the interaction with billions of cells endowed with detoxifying enzymes such as aldehyde- and alcohol-dehydrogenases, aldose reductase and GSH-transferases (GSH-T) (Siems and Grune, 2003; Awasthi et al., 2005)

(5) **EXCRETION** of LOPs into the urine and bile after hepatic detoxification and renal excretion (Alary et al., 2003).
(6) **BIOACTIVITY** without toxicity. As already mentioned, **submicromolar concentrations of LOPs can act as physiological messengers able to reactivate a biological system gone awry**.

From a pharmacokinetic point of view, trace amounts of LOPs, can reach all organs and particularly the bone marrow and the Central Nervous System (Fig. 4.3). **LOPs are extremely important in informing cells of a minimal and calculated oxidative stress eliciting the adaptive response**. In regard to erythrocytes, LOPs can influence the erythroblastic lineage, allowing the generation of cells with improved biochemical characteristics. These "supergifted erythrocytes" as I called them, due to an induction of glucose-6-phosphate dehydrogenase, a higher content of 2,3-DPG and antioxidant enzymes, during the following weeks, are able to deliver more oxygen into ischemic tissues. The consequence of repeated treatments, obviously depending upon the volume of ozonated blood, the ozone concentration and the schedule is that, after a few initial treatments, a cohort (about 0.8% of the pool) of "supergifted erythrocytes" will enter daily into the circulation and, relentlessly, will substitute old erythrocytes generated before the therapy. This means that, during prolonged ozonetherapy, the erythrocyte population will include not only cells with different ages but, most importantly, erythrocytes with different biochemical and functional capabilities. In the course of ozone therapy, we have already measured a marked increase of G-6PD and other antioxidant enzymes in young erythrocytes (Bocci, 2004). The process of cell activation is very dynamic and don't last for ever because blood cells have a definite life-time and a limited biochemical memory; therefore, the therapeutic advantage MUST BE MAINTAINED WITH LESS FREQUENT TREATMENTS.

Ozone toxicity to blood, biological fluids and internal organs can be totally avoided when the ozone dose reduces only in part and transitorily the multiform and potent antioxidant capacity. The antioxidant system has evolved during the last two billions years as an essential defence against oxygen: it is made up of scavengers components, namely albumin, vitamins C and E, uric acid, bilirubin, cysteine, ubiquinol, alpha-lipoic acid and of intracellular antioxidants, such as GSH, thioredoxin and enzymes (superoxide dismutase, SOD; GSH-Px, GSH-Rd, GSH-T, catalase, etc.) and proteins such as transferrin and caeruloplasmin, able to chelate free iron and copper that, otherwise, can favour the formation of hydroxyl radicals. **The wealth and the variety of extracellular and intracellular antioxidants**, thoroughly described by Chow and Kaneko (1979), Halliwell (1994, 1999a, b, 2001), Frei (1999), Holmgren (1989), Di Mascio et al. (1989), Jang et al. (1997), Packer et al. (1997), Bustamante et al. (1998) and Chae et al. (1999), **are able to explain how bland amounts of ozone can be tamed with the results of stimulating several biological systems without deleterious effects**. ***Until this key point is understood, the dogma of ozone toxicity will continue to linger***.

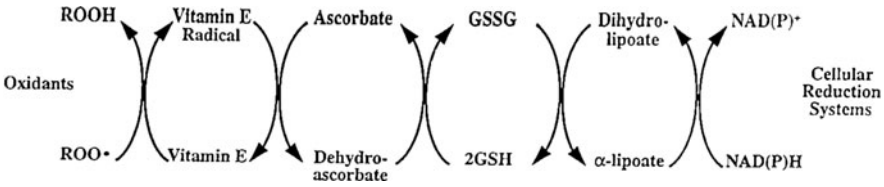
The reader can appreciate the complexity of this system in Table 4.1

Table 4.1 The antioxidants system

Non enzymatic			
Hydrosoluble	Liposoluble	Chelating proteins	Enzymatic
Uric acid	Vitamin E	Transferrin	Superoxide dismutases (SOD)
Ascorbic acid	Vitamin A	Ferritin	Catalase
Glucose, cysteine	Carotenoids	Caeruloplasmin	Glutathione peroxidases
Cysteamine, taurine	Coenzyme Q	Lactoferrin	Gluthatione redox system
Tryptophane	α-lipoic acid	Haemopessin	Reducing equivalents via NADPH and NADH
Hystidine	Bilirubin	Albumin	
Methionine	Thioredoxin		
GSH	Bioflavonoids		
Plasma proteins	Melatonin		
	Lycopene		

The interaction among antioxidants, enzymes and the metabolic system is very important as it allows their rapid regeneration and the maintenance of a normal antioxidant status.

The following scheme, drawn by Prof. L. Packer, beautifully illustrates the cooperation among various antioxidant system in order to neutralize a lipoperoxide radical $ROO^{\bullet}$ (shown on the left hand side) to a less reactive hydroperoxide, ROOH. The reducing activity is continuously generated by cellular metabolism via the continuous reduction of $NAD(P)^{+}$ to NAD(P)H.



It suffices here to say that, during the transient exposure of blood to appropriate concentrations of ozone, the antioxidant reservoir decreases no more than 35% in relation to ozone doses between 10 and 80 mcg/ml of gas per ml of blood. **It is important to add that this partial depletion is corrected in less than 20 min thanks to the recycling of dehydroascorbic acid, GSSG, alpha-tocopheryl radical to the reduced compounds**.

4.2 Conclusions

What happens when human blood is exposed to a therapeutic dose of oxygen-ozone?

Both gases dissolve in the water of plasma depending upon their solubility, partial pressure and temperature. While oxygen readily equilibrates between the gas phase and erythrocytes, the ten-fold more soluble ozone cannot equilibrate because

IT REACTS with biomolecules (PUFA, antioxidants) present in the plasma. The reaction yields hydrogen peroxide (among other possible ROS) and lipid oxidation products (LOPs). The sudden rise in plasma of the concentration of hydrogen peroxide generates a gradient, which causes its rapid transfer into blood cells where, in a few seconds, it activates several biochemical processes and simultaneously hydrogen peroxide undergoes reduction to water by the efficient intracellular antioxidant system (GSH, catalase, GSH-Px). This critical step corresponds to a controlled, acute and transient oxidative stress necessary for biological activation, without concomitant toxicity, provided that the ozone dose is compatible with the blood antioxidant capacity.

While ROS are responsible for *immediate* biological effects (Fig. 4.1), LOPs are important as *late* effectors, when the blood, ozonated *ex vivo*, returns into the circulation upon reinfusion (Figs. 4.2 and 4.3).

LOPs can reach any organ, particularly the bone marrow where, after binding to receptors in submicromolar concentrations, elicit the *adaptation to the repeated acute oxidative stress*, which is the hallmark of ozonated autohemotherapy. Upon prolonged therapy, LOPs activity will culminate in the upregulation of antioxidant enzymes, appearance of oxidative stress proteins (haeme-oxygenase I as a typical marker) and probable release of stem cells, which represent crucial factors explaining some of the extraordinary effects of ozonetherapy (Chapter 8).

It must be emphasized that BLOOD EXPOSED TO OZONE UNDERGOES A TRANSITORY OXIDATIVE STRESS absolutely necessary to activate biological functions without detrimental effects. The stress must be adequate (not subliminal) to activate physiological mechanisms, BUT NOT EXCESSIVE to overwhelm the intracellular antioxidant system and cause damage. Thus, an excessive ozone dose (>160 mcg/ml gas per ml of blood) or incompetence in manipulating this gas can be deleterious. On the other hand, very low ozone doses (below the threshold), are fully neutralised by the wealth of plasma antioxidants and can produce only a placebo effect.

The concept that ozonetherapy is endowed with an acute oxidative stress bothers the opponents of this approach because they consider it as a damage inflicted to the patients, possibly already under a chronic oxidative stress. THEY DO NOT BELIEVE THAT OZONETHERAPY INDUCES A MULTIVARIED THERAPEUTIC RESPONSE ALREADY WELL DOCUMENTED IN SOME DISEASES. Moreover THEY DO NOT DISTINGUISH *THE CHRONIC OXIDATIVE STRESS (COS)* DUE TO AN ENDOGENOUS AND UNCONTROLLED HYPEROXIDATION LINKED TO SEVERAL PATHOLOGIES *WITH THE SMALL AND TRANSIENT OXIDATIVE STRESS that we can precisely perform EX VIVO with the ozone dose*.

The THERAPEUTIC RESPONSE achieved after these repeated oxidative stresses can be envisaged as a PRECONDITIONING EFFECT eventually able to reequilibrate the redox system altered by pathogenetic stimuli.