OZONE THERAPY AND OXIDATIVE STRESS

Silvia A. Menéndez Cepero

Ozone therapy is closely related to the concept of cellular oxidation-reduction (redox) balance since among its properties is to produce an antioxidant effect, though, paradoxically, it is an oxidative therapy for diseases where an oxidative stress process is involved. On the other hand, the general mechanism of action of systemic ozone therapy is through production of a small and controlled (due to de antioxidant defenses of the organism) oxidative stress, which turns into a biological pulse for the cell. The systemic repetition of this pulse leads the organism to different therapeutic responses. Therefore, ozone therapy is closely related to the biological process known as oxidative stress. For this reason, a safe and therapeutic control system becomes a matter of great relevance for applications in systemic ozone therapy.

Generation of reactive oxygen species, called prooxidants, is a normal aerobic life’s attribute. The stable formation of prooxidants is balanced by a similar relation of their consumption by the enzymatic and non-enzymatic antioxidants. The term “oxidative stress” is currently used to indicate a series of complex biochemical processes which can affect the living matter under several distinct conditions and produce physiological as well as pathological effects. Oxidative stress can be defined as prevalence, within the living cell, of oxidizing species or activities over the cellular antioxidant defenses, and it is this pathological perspective which has received the greatest attention over the past few decades.

So far, a considerable amount of research conducted has focused on the role of oxidative stress as mediator of cell damaging effects of a wide range of prooxidant agents (chemical products, drugs, pollutants); several detailed reviews dealing with the mechanistic aspects of such cytotoxic processes have been published.

On the other hand, in recent years, a significant number of studies have underscored the role of oxidation-reduction reactions in regulating the molecular mechanisms involved in signal transduction. It is well defined that low physiological concentrations of prooxidant agents (transitory or regulated oxidative stress) can develop important functions within the cell. This novel perspective is particularly attractive for it will certainly be an advance in our understanding of the role played by the redox processes in human diseases.
A therapeutic control and toxic safety system is of great significance in the applications of systemic ozone therapy. The use of specific biochemical markers to control the applicable doses, in terms of safety and satisfactory therapeutic effects, has become increasingly important and is the basis for a non-empiric application of ozone in Medicine. However, as yet, there is not a generalized criterion on the specific biochemical markers that can be useful in the assessment in absence of occurrence of toxic effects, as well as on the beneficial effects from a systemic ozone therapy.

An important number of diseases is linked to oxidative stress, including a large number of physiological and pathological processes and phenomena, so diverse as inflammation, ageing, virus and bacterial infections, carcinogenesis, drug action, medicament toxicity, defense against protozoa, degenerative and neurodegenerative diseases. Therefore, oxidative stress, at a total or partial frequency, is the cause for several diseases, such as cancer, atherosclerosis, eye cataract, maculopathies, Alzheimer, Parkinson and others. But another large number of diseases such as diabetes, chronic renal failure, infection processes such as AIDS or virus hepatitis lead to an overproduction of ROS which aggravate the evolution of these diseases. They are all part of the basis for the application of the antioxidant therapy, that has gain high recognition at world scale in fighting against these pathologies.

In terms of safety, we must take into consideration that in normal cells there is a delicate balance (Fig. 4.1) between the oxidant activity and the antioxidant one. Notwithstanding, this balance can be disturbed by an excessive production of ROS or by deficiencies in the antioxidant defense systems of the organism itself or contributed by diet, leading toward an oxidative status. A prolonged status of oxidant activity is the cause that characterizes the establishment of the oxidative stress which will later lead to serious deleterious effects, as in the case of hyperoxia. Thus, it is convenient to know the pro-oxidant and antioxidant activities of the patient, as well as his/her metabolic capacity before and during the ozone therapy application. In this sense, knowledge of the redox status sets the real basis for selecting the ozone dosage at the beginning and during the treatment and, mainly, to analytically determine if there is a positive response to it.

**Figure 4.1.** Homeostatic balance of the oxidant and antioxidant activities in the organism.
Monitoring oxidative stress has become an interesting challenge to all clinical biochemists. In 1990, scientists from the USA and Canada gathered in Rockville (USA) to participate in a symposium with the purpose to exchange ideas about potential methods for the measurement of oxidative stress status in humans. Unfortunately, this forum did not reach to a consensus, perhaps because it was the first attempt to organize reflection on this target. However, it pointed out the need to develop reliable measures of oxidative stress in humans, and, in this sense, a set of methods were reviewed but no final conclusions were reached.

Nevertheless, control and monitoring (diagnosis) of oxidative stress is virtually impossible to directly assess in the organism, as a whole or intact, unless it is done in explants or tissue cultures. Due to this limitation, measurement of indirect variables are considered, involving the circulating concentrations of antioxidants (enzymatic and non enzymatic), products of the oxidation of lipids and proteins, sensitivity to lipoprotein oxidation or antibodies concentrations against oxidized biomolecules. A disadvantage of these measurements is that such markers can be influenced by the normal biological functions of the organism without having a relationship with any pathological process. Nevertheless, some of them have proven to have a direct relationship as markers of the antioxidant or prooxidant activity. A concept that must be clear is that a stand alone assessment of the markers of the antioxidant or prooxidant activity does not accurately reveal the redox balance status of the organism at a time given. It is necessary an overall analysis of both activities.

It is possible to find different procedures to measure the antioxidant and oxidant activity indices. There has been several antioxidant capacity indices proposed through the years with different acceptance and accuracy in the measurement of the antioxidant defense capacity. More than 50 indices are commonly used to assess oxidative stress which has led to develop over 100 methods. It has been reported that the use of procedures in which a sample capacity to resist oxidation is measured are unspecific methods and are not addressed to the relevant pathophysiological sources in oxidative stress; thus, consequently, their usefulness is limited to comparative studies. Moreover, complex and sensitive biochemical procedures have been proposed for determining minimum levels of oxidative stress in vivo. However, many of these methods are also relatively specific, elaborate, very expensive and require complex equipment, so that they must be used taking into consideration these characteristics. Industries producing diagnostic kits for clinical laboratories have launched themselves into millionaire research in trying to carry out simple cost-effective procedures, which can be
incorporated into routine clinical practice. Finally, highlighting what Abuja and Tatzber\textsuperscript{50} have recently stated “it is necessary to consider that assessing individual parameters does not fully cover the oxidative stress phenomenon, and much care should be taken when selecting a physiological and pathophysiological condition-dependent and adequate methodology”.

To select specific oxidative stress biomarkers for ozone therapy, it is necessary to revise the different chemical reactions where ozone can intervene when getting in contact with the biomolecules. Critical substrates for ozone are lipids, proteins and nucleic acids; however, lipids and proteins are the main targets in ozone reaction with blood plasma, rectal mucosa or biological fluids. The reaction with lipids almost exclusively occurs with presence of carbon-carbon double bonds in unsaturated fatty acids and in the side chain functionalities of several amino acid residues in proteins.\textsuperscript{57} These reactions produce different ozonation products\textsuperscript{58-61} with the task to transmit the ozone effects to distant sites in the organism.\textsuperscript{62-64} However, when the quantities of ozonation products surpass levels of the antioxidant system, a toxic effect is produced leading to tissue damage and disease occurrence. Therefore, the therapeutic effect depends on carefully measuring the ozone dosage to obtain the appropriate concentration of the ozonation products in order to obtain the desired effect without toxicity risk.\textsuperscript{65,66}

The mechanism through which these products produce the therapeutic effect is currently not well defined, but it is evident that a transitory oxidative pulse is implied here, acting as a cellular metabolic challenger. That is, these oxidized products can act as signal transduction molecules to start a series of metabolic cascades that end in the called “metabolic pulse” of ozone therapy.

The main products originated by lipid and protein ozonation are: Criegee ozonides, hidroxyhydroperoxides, hydrogen peroxide and aldehydes.\textsuperscript{58-61} To have an idea of the enzymatic antioxidant defenses we must have a look inside the metabolic path of the ozonation products. Criegee ozonides are catabolized by the enzyme glutathione S-transferase (GST) using glutathione (GSH) as a reducing agent, for the generation of aldehydes and oxidized glutathione (GSSG)\textsuperscript{67,68} (Fig. 4.2). Aldehydes are metabolized by the enzyme aldehyde dehydrogenase (ALDH) with oxidized nicotinamide adenine dinucleotide (NAD\textsuperscript{+} as cofactor\textsuperscript{69} or by means of enzyme GST as in the case of 4-hydroxynonenal using GSH as cofactor.\textsuperscript{70} Regeneration of GSH is achieved by the action of the glutathione reductase enzyme (GRd) (Fig. 4.2).
Hidroxy-hydroperoxides (ROOH) are metabolized through the enzyme glutathione peroxidase (GPx) with GSH as reducing cofactor (Fig. 4.3). In the applications of systemic ozone therapy by major autohemotherapy or rectal insufflation, a significant stimulation in the activity of this enzyme has been reported. However, organic hydroperoxides are also catalytically degraded by enzyme GST in presence of small quantities of GSH. The importance of GSH is even greater, if we take into account that glutathione concentration can regulate the catabolic actions of GPx and GST over ROOH. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) at physiological concentrations is catalyzed by enzyme GPx, but at high concentrations, catalase (CAT) is in charged of this action (Fig. 4.3).

The enzyme superoxide dismutase (SOD) catalyzes the dismutation of O\textsubscript{2}⁻ radical to hydrogen peroxide (Fig. 4.4). A simplified method to determine SOD for clinical use was developed in our laboratory. Though the superoxide radical is not directly produced in ozone reaction with the
biomolecules, it must be taken into consideration due to its direct relation to the formation of hydroxyl radical (HO\(^-\)), which is a more powerful oxidant. In vitro conditions, formation of hydroxyl radical has been proven from ozone reaction;\(^8^0\) however, that has not been the case in vivo.

\[
\text{SOD} \\
2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

Figure 4.4. Equation representing catabolism of the superoxide radical.

On the other hand, the nonenzymatic antioxidant defense line must also be considered. In this context, GSH has an antioxidant activity by itself, due to ozone high reactivity with the cysteine residue in GSH molecule,\(^5^1\) and antioxidant nutrients, such as α-tocopherol, ascorbic acid and β-carotene that protect the cell against lipid peroxidation.\(^5^2-5^5\) In addition, urates have been reported to have an important protecting activity against oxidation.\(^6^6\) All these antioxidant substances, acting as radical scavengers seem to be of vital importance in protecting the biological membranes against lipid peroxidation. A previous study showed that a significant increase in the content of blood GSH paralleled to the decrease in the lipid peroxidation activity after treatment with the systemic ozone therapy treatment by rectal insufflation in normo- and hypercholesteremic rabbits.\(^6^7\)

In figure 4.5 a sequence of reactions taking place while lipid peroxidation is shown. Lipid peroxidation is a process determined by the extent of peroxide-forming free radical mechanisms from ROS.\(^6^8\) Conjugated dienes (CD) arise as an early event of reactions of lipid peroxidation.
LH (polyunsaturated fatty acids)

Hydrogen abstraction

L (lipid free radical)

Energetic rearrangement

L' (conjugated diene free radical)

Peroxidation

L (conjugated diene peroxyl radical peroxilo)

Propagation

LOOH → Aldehydes

Figure 4.5. Sequence of reactions involved in lipid peroxidation.

Several products from lipid peroxidation, like aldehydes, react to thiobarbituric acid (chromogen compound), producing an easily quantified complex by spectrophotometry, which is currently used as one of the biomarkers of oxidative stress.\textsuperscript{89,90}

Proteins in plasma and interstitial fluids are also good substrates for ozone reaction during the applications of systemic ozone therapy. In this case, free amino acids and those of protein sequences are sensitive to ozone, particularly, cystein, tryptophan, methionine, tyrosine, phenylalanine and histidine. Rate constants for ozone reaction with these amino acids are 2-6 orders of magnitude greater than with leucine, valine amino acids and others.\textsuperscript{57,59,84,91} Proteins oxidized by ozone generate a group of metabolites which are Criegee ozonides, ROOH, hydrogen peroxide and aldehydes, being the same compounds generated by ozonation of lipids, as it was mentioned before.\textsuperscript{91} Therefore, oxidative activity in proteins can be assessed by measurement of protein hydroperoxides, oxidized amino acids, protein carbonyl groups, degradation of protein thiol groups and others.\textsuperscript{92-96}

Oxidative stress cannot be defined in universal terms for it is a complex biological process, which needs to be estimated from different angles. Consequently, it is currently accepted, as a universal criterion, that evaluation of the oxidative stress status in an individual requires a combination of methods, representing each a different type of oxidative stress, since many pathologies can be associated with different kinds of oxidative unbalance which compels the use of several assessment indices or biomarkers.\textsuperscript{97,98}
Bearing in mind the aforementioned considerations and the importance of the first line of antioxidant defense in the instauration and elimination of oxidative stress, we propose measurement of eight biomarkers or indices for their real evaluation in humans (Table 4.1). These indices can be routinely evaluated in a small blood sample using red blood cells and plasma. By analyzing this battery of biomarkers, we can be better aware of the way ozone therapy must be dosed and functions for each patient.

<table>
<thead>
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<th>Antioxidants</th>
<th>Prooxidants</th>
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<tr>
<td>Reduced glutathione (GSH)</td>
<td>Conjugated dienes (CD)</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>Thiobarbituric acid reactive substances (TBARS)</td>
</tr>
<tr>
<td>Glutathione S-transferase (GST)</td>
<td>Total hydroperoxides (THP)</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td></td>
</tr>
<tr>
<td>Catalase (CAT)</td>
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The occurrence of compensatory homeostasis among the different biomarkers in a given tissue can lead to wrong conclusions with regard to the physiological meaning of changes in the measurement of a specific biomarker. This has aroused increasing interest in clinical as well as in ozone therapy specialists and scientists, in general, in diagnosing oxidative stress in humans through reliable, feasible and acceptable cost-effective systems. After more than 10 years of research at the Ozone Research Center (Cuba), a diagnosis system has been developed for the interpretation of the already mentioned eight indices, with the use of advanced techniques of artificial neural network for the solution of complex processes. This procedure is based on integrating the different complementary data of the system through the application of a computer program that finally provides with four new indices (Table 4.2).
Table 4.2. Indices resulting from the application of a computer program based on the different data of the system.

<table>
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<th>Total antioxidant activity</th>
<th>Total prooxidant activity</th>
<th>Redox index</th>
<th>Grade</th>
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Total antioxidant activity index characterizes the total level of the organism’s antioxidant capacity, taking into consideration the antioxidant defenses contributed by the enzymes as well as the non enzymatic ones. Total prooxidant activity index indicates the level of the whole oxidative activity to which the cells are submitted. The redox index represents the numerical value of the resulting vector between the oxidant and prooxidant capacities. Therefore, it is the value that quantifies the real direction between both activities. The grade shows the level of oxidative stress which the organism is experiencing and consists of a scale of 5 grades representing oxidative clinical status (Table 4.3). This system has the advantage of, not only verifying if the person is undergoing an oxidative stress but also of evidencing the stress level, which is very important, since it allows the specialist in question to control the ozone dosage and effectiveness as well as safety of the therapy.

Table 4.3. Grades of oxidative stress and their clinical meanings.

<table>
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<tr>
<th>Grade</th>
<th>Significance</th>
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<tbody>
<tr>
<td>0</td>
<td>Absence of oxidative stress</td>
</tr>
<tr>
<td>1</td>
<td>Light oxidative stress</td>
</tr>
<tr>
<td>2</td>
<td>Moderate oxidative stress</td>
</tr>
<tr>
<td>3</td>
<td>Severe oxidative stress</td>
</tr>
<tr>
<td>4</td>
<td>Very severe oxidative stress</td>
</tr>
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Most oxidative components produced in the organism are transported to blood plasma, urine or breath; therefore, the analysis of the oxidative level in plasma is a measure of the oxidant aggression that the cells have to combat. Quite the opposite, by analyzing the antioxidant level in blood cells is a measure of their capacity to defend themselves against the external oxidative action. These concepts are considered in the program together with the interrelation among the biomarkers and the intercross among them just like it occurs in the neural networks. This software has been registered in the Center of Copyright of the Republic of Cuba.

Assessment of oxidative stress status during and at the end of the ozone therapy treatment facilitates customizing the therapy and ensuring a maximum result. Thus, the biochemical control system proposed is very useful in certifying an optimum use of the ozone therapy applications; though any other system indicating the oxidative stress status can be helpful as well. The real proposal is that whenever systemic ozone therapy is used, a control system for oxidative stress must be applied.

Application of systemic ozone therapy to diagnosis of oxidative stress is an important item in terms of defining the patient’s biological status before beginning treatment and, with clinical data at hand, for decision making in regard with the initial doses as well as the control and monitoring of treatment. Specialists can rely on diagnosis of oxidative stress to select the most appropriate application, address treatment to obtain an oxidative preconditioning, directly select the adequate ozone doses or concentrations to attain the aforementioned benefits by ozone therapy and, finally, to extend or suspend the treatment scheme. If there is not a possibility to obtain the oxidative stress diagnosis, it does not mean that ozone therapy can not be applied; it is that we can not count on the relevant information to customize treatment. In such case, it would be better to use low doses and to advance slowly, according to the clinic, in the dosage. Clinical assessment made by the specialist jointly with oxidative stress diagnosis provided by the clinical laboratory, are the main aspects to elaborate the treatment protocol for each patient.

References