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OPINION

# To What Extent Does Ozone Therapy Need a Real Biochemical Control System? Assessment and Importance of Oxidative Stress

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Ozone therapy is not officially allowed in many countries, but private medical services are using this therapy worldwide. However, appropriate control systems to assess the benefits and risks of systemic ozone therapy are not always used and in such cases the treatment is based on anecdotal reports. Oxidative stress phenomenon is becoming a highlighted biological process for ozone therapy because it is deeply involved in its mechanism of action. On the contrary, ozone therapy is an efficient regulator of the oxidative stress processes. In terms of therapeutic effects, it is convenient to know the metabolic status of the organism to face new oxidative challenges before and during ozone therapy applications. Oxidative stress is also important because it is involved as a cause or effect of many diseases. Since the 1990s, there has been the necessity of developing reliable systems for measuring oxidative stress in humans. In this sense, we have proposed a system for oxidative stress diagnosis that can serve as a control system for systemic ozone therapy applications. The system is based on the blood measurement of eight biomarkers (GSH, GPx, GST, SOD, CAT, DC, SRATB, and HPT) and the interpretation of these values by a computer-developed algorithm yielding four new indices (total antioxidant activity, total prooxidant activity, redox index and grade of oxidative stress). The system shows the patient's redox status and estimation of the oxidative stress level, with this information being relevant regarding implications on dosage and therapeutic effectiveness of ozone therapy. 

*Key Words:* Ozone therapy, Oxidant state, Antioxidant state, Antioxidant enzymes, Oxidative stress.

## Introduction

One of the reasons why ozone in medicine has not been approved as a common practice is its use without an appropriate control. Therefore, a safety and therapeutic control system becomes a matter of great relevance for applications in systemic ozone therapy. The use of specific biochemical parameters to control the applicable dose in terms of safety and therapeutic effects has been gaining importance (1,2) and would support the bases for nonempiric application. However, there is not yet a generalized criterion of those specific biochemical indices that may be useful in assessing the safety and therapeutic effect of systemic ozone therapy.

### *Ozone Therapy and Oxidative Stress*

The main ozone therapy mechanism of action is based on an extremely transitory and regulated oxidative stress imposed *ex vivo* (3). At the same time, ozone therapy acts as an efficient oxidative stress regulator stimulating the antioxidant system of the cell. Therefore, the oxidative stress phenomenon is deeply involved in ozone therapy and a test or system for assessing this process will be of great relevance. As reactive oxygen species (ROS) attack a variety of organic substrates, oxidative stress can be evaluated by measuring reaction products of oxidative damage. Hence, it is convenient before and during application of ozone therapy to know the patient's redox status in order to control the safety doses

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of ozone to be applied in each application. Also, in terms of therapeutic effects, it is convenient to know the metabolic status of the organism to face new oxidative challenges before and during ozone therapy applications.

The comprehensive term "oxidative stress" is currently used to indicate a complex series of biochemical processes that can affect living matter under several distinct conditions and produce both physiological and pathological effects. Oxidative stress can be defined as the prevalence within the living cell of oxidizing species or activities over the cellular antioxidant defenses, and it is this pathological perspective that has received great attention over the past decades (4,5). Until recently, a considerable amount of research has, in fact, focused on the role of oxidative stress in mediating the cell-damaging effects of a wide range of prooxidant agents (chemicals, drugs, pollutants). Several detailed reviews dealing with the mechanistic aspects of these cytotoxic processes have been published (6–9).

On the other hand, a number of studies in recent years have highlighted the role of oxidation/reduction (redox) reactions in the regulation of molecular mechanisms involved in cellular signal transduction (10–14). It is becoming clear that low physiological levels of oxidant agents (transitory or regulated oxidative stress) can have physiological roles within cells (15). This novel perspective is particularly intriguing, as it will surely advance our understanding of the role played by redox processes in human disease. Oxidative stress, if often partly or totally, is a cause of various diseases such as cancer, atherosclerosis, cataract, maculopathy, Alzheimer, Parkinson and others, but a great number diseases such as diabetes, chronic renal insufficiency, and infectious processes such as AIDS or viral hepatitis create a secondary ROS overproduction that worsens the evolution of the diseases (16,17).

Monitoring oxidative stress status becomes an interesting challenge for clinical biochemists. In the year 1990, 16 scientists from the U.S. and Canada gathered in Rockville, MD to participate in a symposium for exchanging ideas on potential methods of measuring the oxidative stress status of humans. This workshop did not reach a consensus, perhaps because it was the first effort to organize thinking about this field (18). However, it points out the necessity of developing reliable measures of oxidative stress in humans; in this sense, a group of methods were revised without arriving at a real conclusion (19). Since then, many sophisticated and sensitive biochemical procedures have been developed for the determination of even minimal levels of oxidative stress *in vivo* (19–25). More than 50 indices are in common use to assess oxidative stress, thus implementing more than 100 methods.

In my opinion the problem should be faced from two points of view in order to create a system for oxidative stress assessment that can serve as a biochemical control for medical ozone applications: (1) identification of appropriate biochemical indices and (2) interpretation of selected biochemical indices.

*Identification of Appropriate Biochemical Indices*

Critical substrates for ozone are lipids, proteins, carbohydrates and DNA; however, lipids and proteins are the main targets in the ozone reactions with blood plasma, rectal mucosa or biological fluids. The reaction with lipids occurs almost exclusively with the carbon-carbon double bonds present in unsaturated fatty acids, and the reaction with proteins is with the side-chain functionalities of several amino acid residues (26). These reactions produce different ozonation products (27–30) that have the task to relay the ozone effects to distant sites in the organism (31–33). However, when the amount of ozonation products overwhelms the antioxidant system, a toxic effect leading to tissue damage and diseases is established. Therefore, the expected therapeutic effects from ozone therapy depend on a careful measurement of the ozone dose to obtain an appropriate concentration of the ozonation products to have the desired effect with no toxicity (34,35). The mechanism of the products producing the therapeutic effect is not yet clear, but it is evident that a transient oxidative pulse acting as a cellular metabolic challenger is implicated. In other words, these oxidized products can act as signal transduction molecules to initiate a series of metabolic cascades that end with the called biological pulse due to ozone therapy.

The different ozonation products from lipids and proteins are Criegee ozonides, hydrogen peroxides, hydroxyhydroperoxides and aldehydes (27–30). To have an idea of the enzymatic antioxidant defense, one has to look inside the metabolic pathway of ozonation products. Criegee ozonides are catabolized by the enzymes glutathione S-transferases (GST) (36,37) using glutathione (GSH) as reduced cofactor, yielding aldehydes and oxidized glutathione (GSSG) (Figure 1). The glutathione S-transferases are a family of isoenzymes catalyzing the conjugation of glutathione with a large variety of xenobiotics as well as endogenous substrates (38–40). Aldehyde formation and formation of GSSG have been established by coupling of an aldehyde dehydrogenase assay or a glutathione reductase assay to the GST-catalyzed reaction of ozonides with GSH (41). Aldehydes are metabolized by the enzyme aldehyde

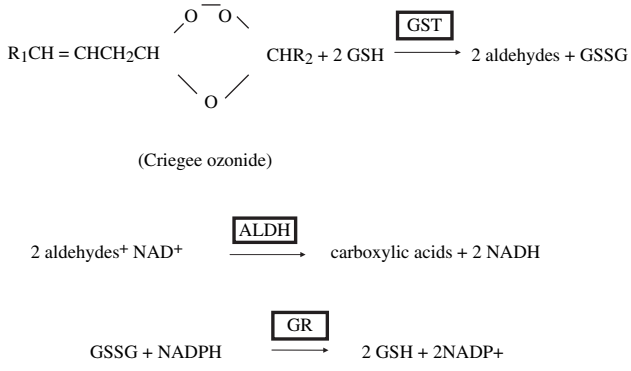


**Figure 1.** Metabolic pathway for Criegee ozonides and aldehydes.

dehydrogenase (ALDH) with oxidized nicotinamide adenine dinucleotide ($NAD^+$) as cofactor (41) or the enzyme GST as occurs in the case of 4-hydroxynonenal using GSH as cofactor (42). GSH regeneration is achieved by glutathione reductase enzyme (GR) (Figure 1).

Hydroxyhydroperoxides (ROOH) are mainly metabolized through the enzyme glutathione peroxidase (GPx) with GSH as a reducing cofactor (Figure 2) and the regeneration of GSH is also obtained by the anaplerotic enzyme glutathione reductase. A stimulation of GPx activity during the application of systemic ozone therapy has been previously reported (43–46). However, organic hydroxyperoxides are also catalytically decomposed by the GST in the presence of a small amount of glutathione (47–49). Furthemore, the concentration of glutathione may regulate the catabolyzing action of GPx or GST on hydroxyhydroperoxides. Hydrogen peroxide ($H_2O_2$) at a very low concentration is catalyzed by GPx but at high concentrations the catalase enzyme (Cat) is involved (50,51) (Figure 2).

The enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide radical ($O_2^{-\cdot}$) to hydrogen peroxide (52) (Figure 3). A simplified blood method to determine SOD has been developed in our laboratory (53). Although superoxide radical is not directly produced by ozone reaction with biomolecules, it must be taken into account because of its direct relation to form the hydroxyl radical ($HO^{\cdot}$) that is a more powerful oxidant. Ozone reaction forming hydroxyl radical has been proved in *in vitro* conditions (54), but until now it has not been detected in *in vivo* conditions.

On the other hand, the nonenzymatic antioxidant defense line must also be taken into account. In this context GSH has an antioxidant activity by itself because of the high reactivity of ozone with the cysteine residue in GSH molecule (55), and the antioxidant nutrients like α-tocopherol, ascorbic acid and β-carotene protect the cells against lipid peroxidation (56–59). Urate has also been reported to have an important antioxidant protective activity (60). All of these antioxidant substances, acting as radical scavengers, appear to be of vital importance in the protection of biological membranes against lipid peroxidation. A previous study showed that a significant increase in the content of blood GSH paralleled with a decrease in lipid peroxidation activity after ozone therapy treatment of rabbits (61).

The analysis allows us to assume that the changes in the distinct catabolic compounds of the ozonation products may serve as possible indices for oxidative stress estimation.

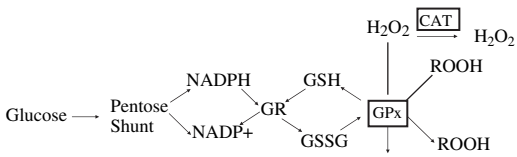


**Figure 2.** Metabolic pathway for hydroxyhydroperoxide and hydrogen peroxide.

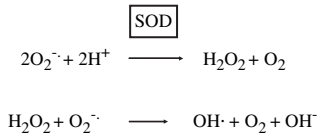


**Figure 3.** Equation for superoxide radical and hydrogen peroxide catabolism.

Nonetheless, the oxidation products from the different biomolecules should also be analyzed as possible biochemical indices. Thus, oxidant activity can be followed by means of the metabolites from lipid peroxidation reactions. The sequence of reactions during lipid peroxidation is shown in Figure 4. Lipid peroxidation is a process determined by the extent of peroxide-forming free radical mechanisms (62). Conjugated dienes (CD) arise as an early event of reactions of lipid peroxidation.

Several end products of lipid peroxidation such as aldehydes react readily with thiobarbituric acid (TBA) and are defined as thiobarbituric acid reactive substances (TBARS) serving as a measurement of tissue oxidative stress (63,64). Fluorescent chromolipids (FC) are inactive compounds formed by the reaction of aldehydes with free amino group in proteins and can easily be measured with a spectrofluorometer. In general, diene conjugation is a measure of early events of lipid peroxidation reactions. TBARS measures end products of lipid peroxidation and FC are products formed by reaction of end products of lipid peroxidation with the amino groups (65). The CD/FC ratio has been used as an effective measurement of peroxidation phase (2), but

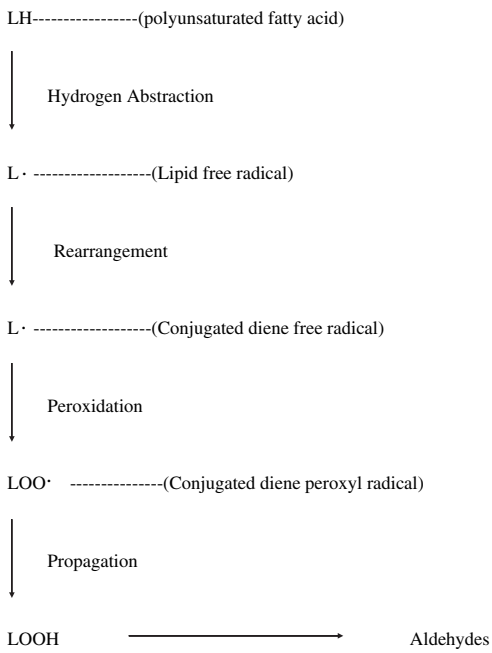


**Figure 4.** Figure showing the lipid peroxidation events.

its real effectiveness needs to be proven in different biological situations.

Proteins in plasma and interstitial fluids are also good substrates for ozone reaction during ozone therapy applications. In the case of free amino acids and the amino acids in proteins, cysteine, tryptophan, methionine, tyrosine, phenylalanine and histidine residues are particularly sensitive to oxidation by ozone; rate constants for reaction of ozone with these amino acids are 2–6 orders of magnitude larger than with leucine, valine and similar amino acids (26,55,66). Proteins undergoing oxidation generate a variety of products from ozone reaction with such amino acids; they are mainly Criegee ozonides and hydrogen peroxides with intermediate metabolites that are hydroxyhydroperoxides and aldehydes (66), similar to what was stated before for lipids, but ending the reactions in new products depending on the type of amino acid that undergo oxidation (67). Thus, oxidant activity in proteins can be evaluated by measurement of protein hydroperoxides, oxidized amino acids, protein carbonyl group content and protein-thiol group degradation (68–72).

There are several studies demonstrating that oxidative stress cannot be defined in universal terms because this is a biologically complex process that needs to be estimated from different viewpoints (73–75). Therefore, now it is universally accepted that assessment of oxidative stress status of an individual may require a combination of methods, each one representing a different type of oxidative stress because different pathologies may be associated with different types of oxidative stress and need to be evaluated by different indices (74).

Having in mind all of the before considerations and taking into account that the first line of defense is the most important against oxidative stress in humans, we have proposed eight possible relevant blood indices or biomarkers for assessing the real oxidative stress in a human (Table 1).

All of these biomarkers shown in Table 1 can be routinely measured using both blood red cells and plasma samples. The occurrence of compensatory homeostasis among different antioxidants in a given tissue can lead to erroneous conclusions regarding the physiological meaning of changes in the measurement of a particular antioxidant. Analyzing this battery of indices, one will have a better knowledge on how ozone therapy should be applied and how it works in each patient.

**Table 1.** Biochemical indices for a proposed system for measuring the oxidative stress status in humans from a blood sample

| Antioxidants | Prooxidants |
|---|---|
| Reduced glutathione (GSH) | |
| Glutathione peroxidase (GPx) | Conjugated dienes (CD) |
| Glutathione S-transferase (GST) | Total hydroperoxides (THP) |
| Superoxide dismutase (SOD) | Thiobarbituric acid reactive substances (TBARS) |
| Catalase (CAT) | |

### *Interpretation of Selected Biochemical Indices*

Because of the complexity of the oxidative stress phenomenon, the analysis of the antioxidant and prooxidant indices cannot be simply done. In a simple manner, the increment in antioxidant parameters can be assumed as a positive or negative response to a given oxidant stimulus; on the contrary, the increment in prooxidant parameters can be assumed as a negative response. However, biological phenomena in a few cases are simple and in the majority of occasions are very complex with interrelationships between many particular biological events that give a resulting effect as final response. This is the key point in the way of interpretation of the different indices or parameters to assess the oxidative status. Many authors have patented different methods for the analysis of the different indices measured for assessing the oxidative stress. Some of them based their analysis by comparison to reference values from apparently normal donors, others by making diagnostic graphic plots of a huge number of assayed parameters, and others by using cells isolated in a noninvasive assay (76–79). All of these diagnostic systems have their advantages and disadvantages, depending on the feasibility of the user. These systems apparently give an idea if an oxidative stress exists, but none is really able to predict the grade or level of redox status. For ozone therapy applications it is very important to know the patient's level of oxidative stress.

An example on how interpretation from antioxidant and prooxidant parameters is done at present is shown in Figure 5. These values were obtained from a cardiac patient who suffered myocardial infarction 6 months previously. He was treated with ozone major autohemotherapy with a protocol comprising volume of blood 200 mL, ozone concentration 50 μg/mL/mL of blood, at a frequency of 2 or 3 days a week.

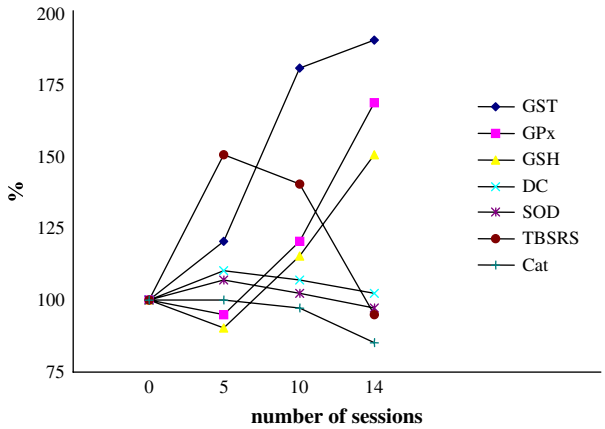


**Figure 5.** Response of antioxidant and prooxidant parameters during ozone major autohemotherapy application in a cardiopathic patient.

The response was plotted in terms of percent vs. number of ozone therapy sessions. Before starting the therapy, blood sample was drawn and prooxidant and antioxidant values were analyzed, corresponding to 100% of each parameter.

After five sessions of treatment, blood analysis was repeated and compared with the first one in order to determine the individual specific changes after ozone treatment. There was a slight increase in the prooxidant state, given by a high level of TBARS and a small increase in DC, along with a small increase in the antioxidant enzyme GST but no significant changes in GPx, Cat and SOD with a diminished level in GSH. Evidently, there were uncertain changes. After 10 treatment sessions, a significant increase in the antioxidant parameters GST, GPx and GSH with a decay in SOD and Cat was observed; the prooxidant indices DC and TBARS were slightly decreased in comparison with the prior analysis. Results were similar at the end of 14 sessions but with a high increase in GPx and GSH, and a high decrease in CAT and TBARS indices. Although it looks like an induction in antioxidant activities occurred at the end of the treatment, there was no clear evidence or interpretation of this phenomenon because many biological interrelations were not taken into account. This is the main reason why I am stating that ozone therapy needs a control system including all possible biological aspects related to oxidative stress. Therefore, our laboratory developed a computer algorithm taking into account all the biological relationships between antioxidant and prooxidant activities, which gives a total measurement of both activities with a redox index and grade of oxidative stress.

### *Proposal of a Novel System for Oxidative Stress Diagnosis*

There is growing interest by scientists, ozone therapy and clinical medicine specialists for a reliable and cost-effective system in which the interpretation of the included parameters is effective in realistically estimating changes and level of oxidative stress in humans.

A system based on the technique of neuronal artificial net has been developed in our laboratory for the interpretation of the proposed indices. This new technique is based on the integration of different but complementary biochemical parameters that cover the main aspect of the biological oxidative stress. A computer program has been constructed which yields four new relevant indices for final oxidative stress diagnosis. These indices are shown in Table 2.

**Table 2.** Resulting indices from oxidative stress diagnostic computer program

| |
|---|
| Total antioxidant activity |
| Total prooxidant activity |
| Redox index |
| Grade |

**Table 3.** Grades of oxidative stress and their clinical meaning

| Grade | Meaning |
|---|---|
| 0 | No oxidative stress |
| 1 | Light oxidative stress |
| 2 | Moderate oxidative stress |
| 3 | Severe oxidative stress |
| 4 | Very severe oxidative stress |

Total antioxidant activity index characterizes the level of the antioxidant capacity of the whole organism, taking into account the enzyme and nonenzyme antioxidant defenses. Total prooxidant activity index indicates the level of oxidative activity to which the cells are submitted. Redox index represents the numerical value of the resulting vector from both activities. Grade index displays the level of oxidative stress that the organism is undergoing and is composed of five grades indicating five clinical oxidative statuses (Table 3).

Although HPT biomarker was not measured in the case of Figure 5, we made an arrangement in the computer program to evaluate the results from the biomarkers analyzed in the blood of that patient before, during and after ozone therapy applications. As can be seen in Table 4, the level of oxidative stress status in each point was possible to be assessed without any doubts or misinterpretations.

A slight increase in the oxidative stress level was achieved after five sessions of treatment; however, a significant increase in the total antioxidant activity produced a remarkable increase in the redox index after ten sessions, with a minor level of oxidative stress grade. It is evident that an improvement in the oxidative stress status at the end of 14 sessions occurred because the patient had one grade less than before treatment. Clinical status was well correlated with the redox status given by the program.

Many of the oxidized damage components produced throughout the body are transported to the blood plasma, urine or breath; thus, analyzing the oxidative level of plasma is a measure of possible oxidant attack at which cells have to face. By contrast, analysis of the antioxidant level inside the red cell is a measure of the defense capacity against the oxidative action outside the cell. These concepts

**Table 4.** Oxidative stress diagnosis using the computer program for values obtained with the patient in Figure 5

| Ozone therapy session number | Total antioxidant activity (units) | Total prooxidant activity (units) | Redox index | Grade of oxidative stress |
|---|---|---|---|---|
| 0 | 128.38 | 167.96 | 0.764 | 2 |
| 5 | 123.45 | 178.69 | 0.691 | 3 |
| 10 | 138.92 | 175.21 | 0.790 | 2 |
| 14 | 144.28 | 169.70 | 0.850 | 1 |

are undertaken by the program along with the interrelationship between each group of indices and the crossover between them.

This oxidative stress diagnostic system has the advantage that does not only give a measure if a person is suffering from oxidative stress, it additionally shows the level of oxidative status that is a very relevant point because it allows the physicians to control the ozone doses and safety of the therapy.

In conclusion, oxidative stress phenomenon is a very important biological process that all ozone therapists should have in mind when using systemic ozone therapy: first, because this therapy works by mean of a transient and controlled exogenous oxidative stress produced from a myriad of ROS and second, because the transient oxidative stress needs to be controlled by the ozone dosage in order not to cause toxic or only placebo effects. And third, and most important, the redox status of the patient should be assessed before, during and after systemic ozone therapy applications.

A reliable system that allows knowing the patient's redox status and estimation of the oxidative stress level has been developed in our laboratory, with implications on the dosage and therapeutic effectiveness of ozone therapy.

Ozone therapists should have in mind that safe dosage in ozone therapy is not always the therapeutic dosage; hence, biochemical findings in relation to the patient's ailment, therapy objective and pro-oxidant/antioxidant balance should be analyzed as an entire system for safety and therapeutic actions. Interpretation of these parameters is the most important part of any system evaluating the oxidative level in a patient. Decay in the oxidant state and/or the increase in the antioxidant state is not only a homeostatic response of the cells, but also represents the safety and effectiveness of applied ozone dose. For that reason, a biochemical control system will be very useful in certification of the optimal use of ozone therapy applications, although any measurement, given an idea of the oxidative stress status, can be used. The real proposal is that when using systemic ozone therapy, one control system of the oxidative stress must always be applied.

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