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# Ozone Dosage is the Key Factor of Its Effect in Biological Systems

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## Abstract

The applications of ozone are not only restricted to environmental remediation or industrial areas. This gas has been applied in medicine to treat several diseases, where positive effects have been confirmed by many clinical studies. According to the European Medical Society of Ozone and the National Center of Scientific Investigation in Cuba, it has not been possible to validate ozone's effectiveness by traditional analytical methods. Thus, this investigation proposed evaluating the effect that ozone has on biological substrates (murine models with induced carcinogenic tumors, inflammation, and wounds), studying the variations that ozone (dissolved in physiological solution or ozonated vegetable oils) provokes over the total unsaturation of lipids (TUL), and by using the so-called method double bond index (DB-index), make a correlation with the dynamic reactions obtained by several analytical methods according to each experimental stage considered in this study.

**Keywords:** ozone therapy, cancer, ozonated oils, inflammation, wound healing, total unsaturation, double bond index

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## 1. Introduction

Ozone is a gaseous molecule formed by three oxygen atoms; it has a blue color (when dissolved in water) with a strong acrid aroma and a molecular weight of 48 mg/mole. The ozone molecule has a cyclic structure with a distance between atoms of  $1.25\text{\AA}$ . It has a solubility of 49/100 ml of water (at  $0^{\circ}\text{C}$ ), that is 10 times greater than the oxygen solubility (4.89/100 ml of water) [1].

Ozone concentration on air (mg/L)	Symptomatology
0.1	Respiratory tract and eyes irritation
1.0–2.0	Rhinitis, cough, headaches, nausea and vomiting
2.0–5.0 (10–20 min)	Dyspnea and bronchial spasms
5.0 (60 min)	Acute pulmonary edema and occasionally respiratory paralysis
10.0	Death in 4 h
50.0	Death in minutes

\*The ozone toxicity on the respiratory system should not be extrapolated to the circulatory system due to the differences in biochemistry and the metabolic regimen [3].

**Table 1.** Toxic effects of ozone in gaseous phase\*.

The excessive emissions of NO, NO<sub>2</sub>, CO, CH<sub>4</sub>, H<sub>2</sub>SO<sub>4</sub>, among others, have favored the increase in ozone concentration in the tropospheric space, above 0.1 ppmv. The reactions between the chemical compounds abovementioned give rise to the so-called photocatalytic smog, which has become the main toxic substance for lungs, eyes, nose, and skin. There are several symptoms that could appear according to ozone concentration that exists in the air (**Table 1**) [1, 2].

## 2. Ozone in medicine

According to the European Ozone Medical Society and the National Center for Scientific Research in Cuba, among others, the following diseases can be treated with ozone: abscesses, acne, AIDS, allergies, anal fissures, arthritis, asthma, cancerous tumors, cerebral sclerosis, problems in the circulatory system, cirrhosis of the liver, corneal ulcers, cystitis, diarrhea, fistulas, boils, gangrene, gastric ulcers, intestinal disorders, glaucoma, hepatitis, herpes, hypercholesterolemia, colitis, mycosis, and osteomyelitis [2].

Unlike research on the application of ozone at the industrial level, studies in the medical field are scarce. Moreover, the studies describing the interactions of ozone with substances of biological origin and their kinetic implications have not described the entire reaction mechanisms. One of the most important premises in the application of ozone for medical aspects establishes the induction of an extraordinary and temporary response of the body systems associated with peroxidation of lipids and the antioxidant scheme of the organism [1–3].

However, it is suspected that the oxidative effect of ozone causes different effects on the immune system, sympathetic and parasympathetic systems among others. It is well known that the presence of compounds derived from oxidation reactions in the human body produces a cascade of biochemical reactions that has been clearly explained but not associated with the presence of ozonation-derived byproducts. This condition occurs in many events that compromise the health of the human being, such as deep wounds, appearance of neoplasms, and so on. However, the mechanism of reactions through which the cascade of

biochemical reactions occurs is not entirely known with certainty [3–5]. Even though it is accepted that ozone (under the adequate dosing strategy) produces a significant number of benefits in the human body because it is dissolved in oxygen, it increases oxygenation in the blood, improves circulation, stimulates oxygenation in tissues, and so on, it has not been established what are the mechanisms that generate such important advantages from the clinical point of view [1, 2].

### 3. How ozone acts and how its toxicity can be avoided

Oxygen is essential for life; nevertheless, this gas has long-term negative effects. Reactive oxygen species (ROS) are formed during cellular respiration. The hydroxyl radical  $\text{OH}^\cdot$  is the most destructive ROS for enzymes and deoxyribonucleic acid (DNA). The aging process and metabolic disorders (arteriosclerosis, diabetes, cellular degeneration, etc.) can be worsened by the presence of ROS. The application of an excessive ozone dose used in medical therapy may aggravate the ROS effect on the body. This process can be prevented if there are proper control methods in ozone dosage, regardless of the medical ozone technique [1].

Notice that, in the ozone-oxygen mixture, the former is not equilibrated with ozone, because ozone reacts immediately with a certain number of molecules in biological fluids, mainly antioxidants, proteins, carbohydrates, and specifically the polyunsaturated fatty acids (PUFAs) [3].

The reaction kinetics and the sequence of such reactions are uncertain, and it has been briefly described [1, 6–8]. The subsequent formation of byproducts that may be responsible for the clinical effects of ozone and the accumulation of final products must be controlled in order to avoid some of the undesirable side effects of the therapies based on ozone.

It is widely accepted that the main reactions of ozone with biological molecules are executed according to the following stages [1]: (1) ozone reacts with ascorbic acid, uric acid, sulfhydryl groups ( $\text{SH}^-$ ) from proteins, and glycoproteins generating ROS, which trigger several biochemical stages in the blood *ex vivo*. The ROS are neutralized 0.5–1.0 min later by the antioxidants of the system and (2) ozone reacts with the double bonds ( $>\text{C}=\text{C}<$ ) of arachidonic acid and triglycerides in the plasma, which produces a molecule of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and two aldehyde molecules known as lipid peroxidation products (pPOL).

According to these stages, it is possible to claim that not ozone but ROS and pPOL are the compounds responsible for the multiple biochemical reactions that occur in the cells of the body, in particular, in particular, the second reaction, which has been characterized as the key factor of the therapeutic effects of ozone. In this way, the study of the ozonation byproducts formation improves the understanding of the clinical effects, which is helpful to choose the better ozone's application scheme in a medical treatment.

As soon as ozone dissolves in biological fluids, it reacts with PUFAs, and then the concentration of hydrogen peroxide increases. However, with a similar rate, it begins to diminish as

the molecule diffuses quickly toward erythrocytes, leukocytes and platelets, while several antioxidative processes are performed. Due to the presence of enzymes such as GSH-Px and GSH, the intracellular concentrations of hydrogen peroxide are reduced within the plasma and the intracellular fluid [1, 3].

The activity of the pPOL, under prolonged therapies, can give rise to the regulation of antioxidant enzymes, the appearance of oxidative proteins, and the release of stem cells, which is considered a crucial factor to explain some effects of ozone applications as medical therapy [1].

## 4. Ozone application pathways

The therapeutic indications of ozone are based on the theory that at low concentrations of this gas (in the gaseous phase), some significant phenomena occur within the cell. It has been proved that at concentrations of 5–10 mg/L or lower, there are therapeutic effects with a wide margin of safety in the patient. At present, concentrations ranging from 5 to 60 mg L<sup>-1</sup> are accepted for the medical application of ozone [9].

### 4.1. Direct methods

*Rectal insufflation:* The gaseous mixture is introduced to human body by the rectum, and it is absorbed in the bowels.

*Intramuscular injection:* In this technique, 10 mL of gaseous mixture are injected in the buttocks of the patient.

*Major and minor autohemotherapy:* This technique has been used since 1960. The minor autohemotherapy requires 10 mL of blood, which is put in contact with the gaseous mixture to finally return it to the human body. The major autohemotherapy requires 50–100 mL of blood, which is put in contact with the gas for a few minutes to then return it to the patient.

*Ozone bag:* A plastic bag is placed around the treated area. The gaseous mixture is pumped into the bag, then the gas is absorbed by the human body through the skin. This is one of the methods where the reaction occurs in two stages, one is absorption by the skin and the second is the direct reaction of the skin compounds with ozone. That implies a combined model that involves mass transfer of ozone and its reaction with the bio-substrate [1, 2, 9].

### 4.2. Indirect methods

*Ozonated water:* Ozonated water is used to wash wounds, skin burns and skin infections.

*Intra articular injection:* The ozonated water is injected directly between the joints and is used for the treatment of arthritis and rheumatism.

*Ozonated oil:* The ozonated oil is applied as an ointment for long times with low ozone doses [1, 2, 9].

## 5. Control methods for ozone's therapeutic applications

Oxidative stress is the main concept that explains ozone's therapeutic effect over the human body. Ozone's paradoxical effect as a promoter of antioxidant response capable of regulating oxidative stress is common in the animal kingdom. This fact suggests that the adequate ozone's dose, besides the oxidation induced in biological subjects, may enhance the antioxidant response of the living organism. This is a critical factor issued by the immunological system to overcome infections, ischemia, and cellular regeneration [3].

Most of the technical methods employed to control the medical application of ozone are based on the measurement of oxidative stress. Some of the typical methods employed to measure the oxidative stress include the quantification of reactive species by electronic paramagnetic resonance; the analytical determination of antioxidants and measurement of total antioxidant capacity; the detection of oxidized biological markers, such as the lipid peroxidation products (pLPO), malondialdehyde, 4-hydroxynonenal, isoprostane, and oxidized proteins; as well as the measurement of damaged DNA [10]. While these methods measure key species associated with the oxidative stress, they present some inconveniences regarding their own analytical limitations, mainly the sensitivity and the time required to complete the analysis.

Nowadays, ozone's medical application remains empirical. According to the "Madrid Declaration on Ozone Therapy," each patient responds differently to the controlled oxidative stress induced by ozone treatments; thus, ozone's administration must be developed in a progressive way, that is, starting with small doses and progressively increasing them [9].

### 5.1. Determination of the total unsaturation

The determination of the total unsaturation (TU) of organic compounds is a technique developed by Russian researches in the middle of the last century [11]. It is a useful tool based on ozone's reactions, particularly, with the double bonds ( $>C=C<$ ). Ozone reacts selectively with different compounds; one of the most specific reactions of ozone takes place with unsaturated organic compounds [12]. The reaction rate constants of ozone with all the  $>C=C<$  bonds are similar, regardless of the structure of the compounds that contain them [13]. Through the TU technique, it is possible to determine the TU of the lipids in the biological substrates, and the ones contained in vegetable oils, due to their composition, which make TU a suitable technique to control ozone's therapeutic applications.

By this technique, the ozone reactive substrate contained in one sample can be quantified in a precise way ( $\pm 1\%$ ) and in a short time of analysis (1–3 min) [11, 14, 15]. The TU determination consists of the measurements of ozone necessary to react with certain samples diluted in chloroform. Afterwards, this quantity is compared with ozone consumed in its reaction with a standard sample of known concentration, as well as the stoichiometric of its reaction with ozone. Ozone's consumptions are obtained from the area of the characteristic plot of ozone concentration versus time, called ozonogram. The detailed procedure can be reviewed in [15, 16]. The mathematical formula used to calculate TU is:

$$TU = \frac{C_{ST} \times V_{ST} \times S_S \times V_{SOL}}{S_{ST} \times V_S \times W_S} [=] \left[ \frac{\text{mol}_{>C=C<}}{\text{g}_{\text{sample}}} \right] \quad (1)$$

where  $C_{ST}$  is the concentration ( $\text{mol L}^{-1}$ ) of the standard solution,  $V_{ST}$  and  $V_S$  are the volumes (ml) of the standard and the sample, respectively,  $S_{ST}$  and  $S_S$  are the ozonogram areas for the standard and samples, respectively, while  $V_{SOL}$  and  $W_S$  are the solution volume (ml) and weight of the sample (g), respectively.

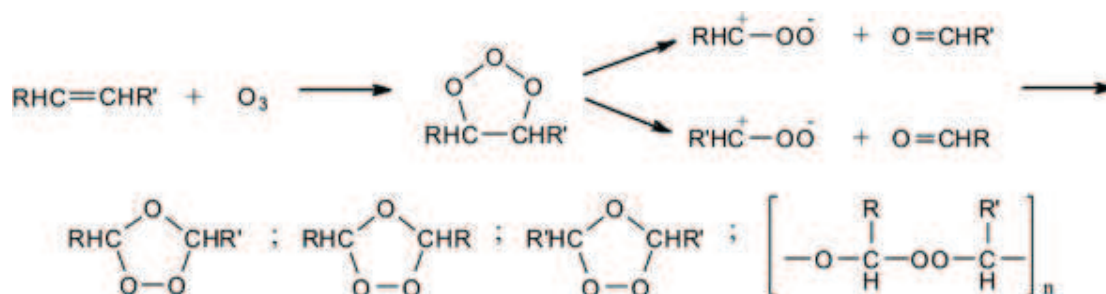
### 5.1.1. Determination of the ozonation degree of vegetable oils by TU

Vegetable oils are composed mainly of fatty acids (free and as esters in triglycerides); the unsaturated ones constitute the principal substrate that reacts with ozone. In addition, vegetable oils contain minor compounds (unsaponifiable matter), which include sterols, polyphenols, pigments, antioxidants, as well as characteristic compounds extracted from seeds. These compounds are also reactive with ozone because they contain double bonds and some other oxidizable elements. The products of ozonated oils constitute a mixture of peroxides (isozonides, hydroperoxides, poly peroxides) with therapeutic action. The formation of these species is described by Criegee mechanism. The type and their yield depend on reaction conditions [12, 13, 17, 18]. **Figure 1** shows the reaction pathway of ozone with the unsaturated compounds of oils.

TU quantifies ozone mass that reacts with an oxidizable sample. Notice that this method considers that the stoichiometry of the reaction of ozone with  $>C=C<$  is 1:1. Then, the TU quantifies the oxidizable substrate by ozone that is contained in the analyzed sample. Thus, the ozonation degree of oils can be easily obtained, as the percentage of all compounds in oils that react with ozone (major and minor compounds). The importance of a reliable determination of the ozonation degree is related to the therapeutic effects of ozonated oils, which, in turn, strongly depend on the type of oil and its ozonation degree.

### 5.1.2. Double bond index (DB index)

The DB index is the term used to extend the TU application to biological substrates. It is obtained from the measurement of ozone that has reacted with the double bonds of lipids, previously extracted from biological fluids or tissues [15]. With the determination of the total unsaturation of lipids in plasma and cell membranes, it is possible to evaluate changes in



**Figure 1.** Reaction pathway of ozone with unsaturated compounds.

lipid metabolism [11, 14]. The DB index is strongly related with the level of oxidative stress in subjects, since the lipids involved in the measurement correspond to those remaining after the oxidative stress mechanism [15].

The DB index determination corresponds to the procedure described for TU measurement. For liquid samples, such as blood plasma, the mathematical expression DB index is calculated according to:

$$\text{DB - index} = \frac{C_{ST} \times V_{ST} \times S_S \times V_{SOL}}{S_{ST} \times V_S \times V_{pi} \times K} [\text{c.u}] \quad (2)$$

where  $C_{ST}$  is the concentration of the standard solution ( $\text{mol L}^{-1}$ ),  $V_{ST}$  and  $V_S$  are the volumes (ml) of the standard and the sample, respectively,  $S_{ST}$  and  $S_S$  are the ozonogram areas for the standard and samples, while  $V_{SOL}$  and  $V_{PL}$  are the volume (ml) of solution and the volume (ml) of blood plasma from sample, respectively;  $K$  is a correlated coefficient equal to  $10^{-7}$  mL/mole. C.u means “conditional unit” ( $1 \text{ c.u} = 1 \times 10^{-5}$  mole D.B./mL) [15].

The DB index, measured in healthy subjects, has been determined showing its impendence of age or sex, excluding children  $\leq 1$  year and aged people  $\geq 60$  years. Apparently, only diseases or pathologies produced changes in this value [14]. The DB index of lipids (blood plasma and erythrocytes) for healthy European and Mexican population are listed in **Table 2**.

Some reported clinical cases illustrated the prognostic and diagnostic criteria of the DB index changes. Among the diseases where the DB index has been successfully used as a preclinical tool, the oxidative process of pneumonia in children was precisely characterized by this method [11]. In this study, and inverse correlation between the DB index and the LPO activity was observed. The authors concluded that the DB index evolution can describe the disease evolution accurately [11].

Another relevant case of the DB index application in medical procedures corresponds to its application to evaluate the therapy effectiveness of burned patients [19]. Depending on the burned magnitude, the reported DB index values of different patients ranged from 34 to 287c.u. The evolution of the DB index correlated for each subject with its personal damage and its treatment effectiveness. The authors concluded that the changes in the DB index were observed before the clinical manifestations, showing its potential as a prognostic tool for clinical practice [19]. Some other diseases where the DB index has been related with the evolution of the illness and the effect of the treatment include cancer [20], diabetes [14], exposition to hexavalent chromium [21], as well as inflammatory processes [22]. In these cases, the DB index resulted in a powerful tool to adjust the therapeutic treatment, according to the individual needs of the subjects under treatment.

Population	DB <sub>plasma</sub> -index (c.u)	DB <sub>erythrocytes</sub> -index (c.u)
European	250 ± 10	50 ± 2
Mexican	160 ± 10	100 ± 2

**Table 2.** DB-index value for European and Mexican healthy population [15].



## 6. In vivo studies of the TU and the DB index application

### 6.1. Direct applications: cancer

The methodology proposed in this section evaluated the implantation of C6 cells in an animal (murine) model [23, 24]. The oxygen and the ozone dissolved in the saline solution were dosed by an intraperitoneal pathway in athymic mice (Balb/CNu/Nu). To evaluate the effect of ozone dosage on the tumor implanted in mice, the measurements of the DB index were carried out on the lipid fraction of plasma of blood, erythrocytes, tumor, and liver.

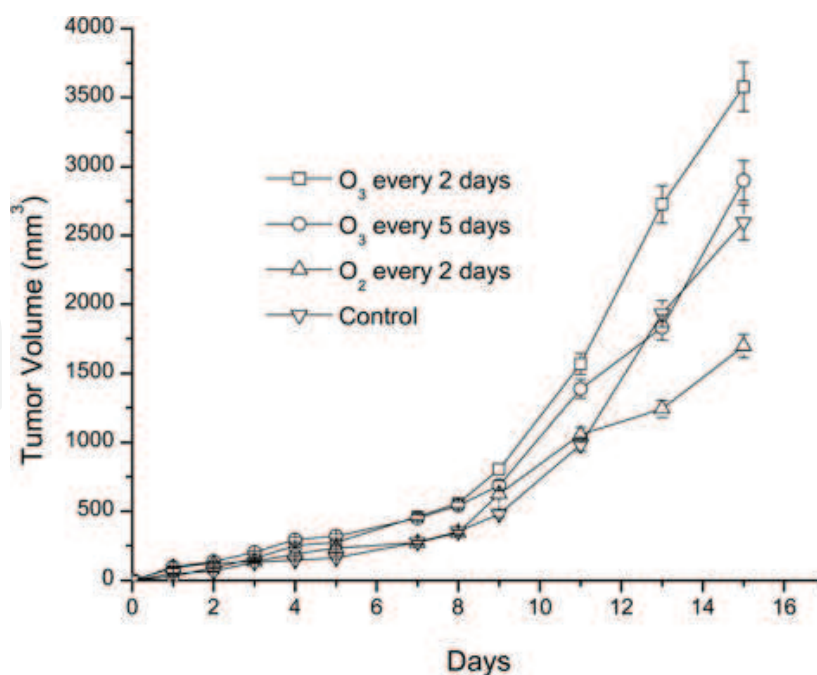
**Ozonated physiological solution (NaCl 0.9%):** Physiological solution (0.9% NaCl) was used as carrier media for the oxidant agent (ozone or oxygen). The ozone concentration in oxygen was  $4.6 \pm 0.2 \text{ mg L}^{-1}$  that corresponds to its concentration of  $1.15 \pm 0.2 \text{ mg L}^{-1}$  of ozone in the saline solution. Considering the volume of the injected physiological solution ( $90 \mu\text{L}$ ), only  $0.103 \mu\text{g}$  of ozone was injected to mice in treatments.

**Therapeutic protocol:** This experiment considered four groups ( $n = 6$ ) of athymic nude mice with C6 glioma that practically have the same tumor size ( $74.60 \pm 21 \text{ mm}^3$ ). The oxidant agents were dissolved in the physiological solution, and they were administrated into the mice by intraperitoneal injection ( $90 \mu\text{L}$ ) [25]. The treatment period length was 15 days. The number and the frequency of injections were different for each treated group. In the first and second groups, the injections (oxygen for the first and ozone for the second) were carried out every second day (7 times); the third group was treated with ozone every fifth day (three times). Then the mice were sacrificed, and the samples of blood and tissues were selected to determine the DB index.

**Evolution of volume and necrosis of tumor:** The variation of tumor volume for 15 days is shown in **Figure 2**. As we can see, ozone promoted the tumor volume growth compared with the control group by 10 and 44% every fifth and second day, respectively, both compared with the control group. On the contrary, oxygen inhibits the tumor growth by 30%. Even when the tumor volume results suggest a better performance in the group treated with oxygen, the tissue necrosis demonstrate a lower activity of tumor cells in groups treated with ozone. Furthermore, the microscopically obtained results showed that the ozone dose influenced the tumor necrosis.

Some studies have shown that oxygen may inhibit the tumor angiogenesis, which limits the nutrient availability [26], and could be related with slower tumor growth observed in the group treated with oxygen. On the other hand, ozone showed a stressing effect, which was reflected by the accelerated tumor growth. Ozone may induce a pronounced influence on tumor metabolism, particularly in the respiratory cycle and glycolysis, showing a positive influence on oxygen utilization in tumor [27]. These facts may explain the increased rate of tumor growth [28, 29].

Since tumor growth was slower when the ozone dose also was applied every fifth day (compared with ozone applied every second day), and a higher necrosis was observed, we may conclude that ozone dose plays a major role in two observed phenomena, in regulating the

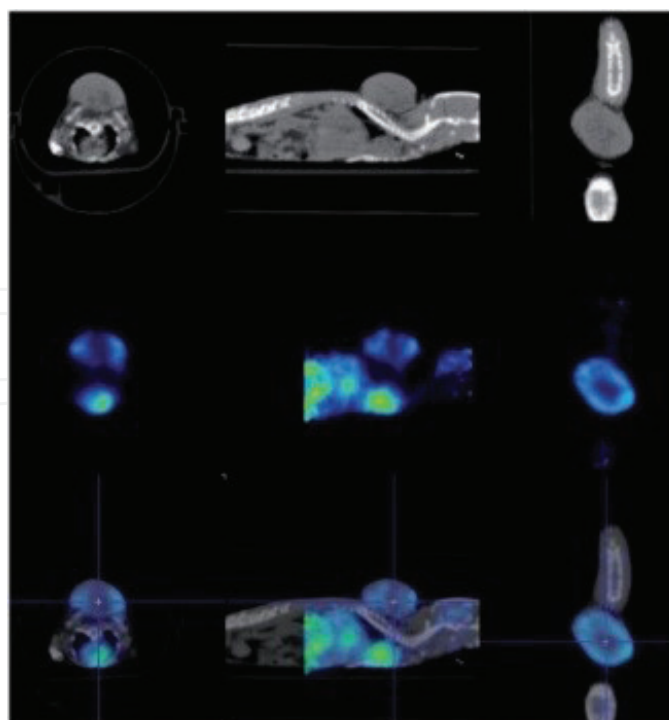


**Figure 2.** Tumor volume increase associated with the dosage strategy showing the control group, pure oxygen and ozone dosed every second and every fifth day (n = 6).

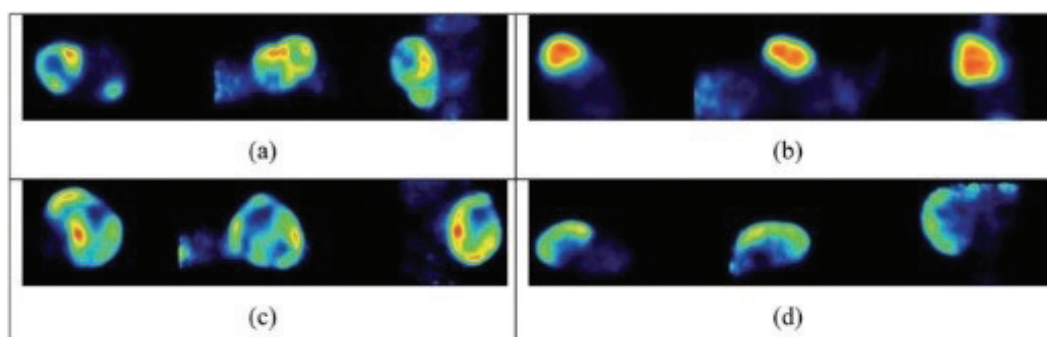
rate of the tumor growth and in the tissue necrosis. It is important to note that both, the tumor cell activity and tumor necrosis, the significant positive effects of the treatment were achieved under the smaller ozone dose. Considering that tumor necrosis is a positive result of the treatment based on ozonated saline solution, the smaller ozone dose (every fifth day) was the better tested strategy to treat this type of tumor.

**[18F] FDG in tumors with PET/CT: Tumor metabolic activity:** [18F] FDG (2-deoxy-2-[18F]-fluoro-D-glucose)-positron emission tomography (PET) and X-ray CT imaging were performed using a micro-PET/CT scanner (Albira, ONCOVISION, Spain). **Figure 3** shows the variation of FDG in the tissue of tumor that was obtained by the image processing analysis corresponding to the set of three planes of exposition (top of the image). In the center of the figure, the acquired PET images (with the gamma camera taken in the same planes) are located. At the bottom, the over-position of both images, tomographic and PET, is shown to correlate the tumor anatomical position with its activity.

**Figure 4** represents the specific areas that demonstrate the tumor activity by color variation. The red color corresponds to larger tumor activity, and, contrary to that, the blue areas describe the regions with smaller or even null activities [30–33]. **Figure 4b** corresponds to the mice dosed with dissolved oxygen, showing an area in red color that is larger than the one detected for the mice of the control group. **Figures 4c** and **d** represent images of the mice dosed with ozone every second and fifth day, respectively. As we can see, under the smaller ozone dose the significant decrease of tumor cell activity is obtained (>80%). It is important to note that in both cases, the tumor cell activity and tumor necrosis, the significant positive effects of the treatment were achieved under the smaller ozone dose.

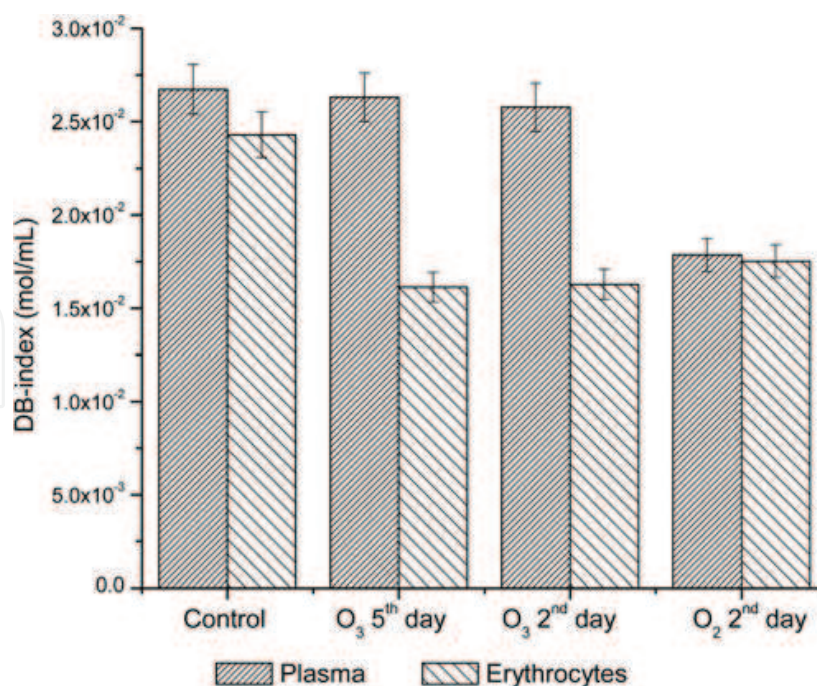


**Figure 3.** Example of microPET image obtained when the ozone gas Dosage is administered every fifth day.



**Figure 4.**  $^{18}\text{F}$ FDG tumor activity of the considered studied group control (a), only oxygen every second day (b), ozone every second day (c) and ozone every fifth day (d).

**The DB index variation of plasma, erythrocytes, tumor, and liver:** The measurement of DB index (reactive sites of biological substrates) of lipids of plasma and erythrocytes as well as of tumor tissue and liver was carried out to find the possible correlation with the ozone dose and its effect on the tumor volume and activity. **Figure 5** depicts the DB index of the plasma and erythrocytes from mice after the ozone and oxygen treatment, both compared with the control group. The first fact to note is the values of the DB index of both plasma and erythrocytes, which are very high in comparison with healthy mice:  $2.7 \times 10^{-2}$  and  $2.35 \times 10^{-2}$  with respect to  $2.0 \times 10^{-5}$  and  $0.57 \times 10^{-5} \text{ mol ml}^{-1}$ . Usually the values of the DB index of lipids of the plasma and erythrocytes are close to each other ( $0.57$  and  $0.43 \times 10^{-5} \text{ mol ml}^{-1}$ ) [15]. These high values of the DB index in mice with this type of cancer indicate that lipid peroxidation was substantially reduced.

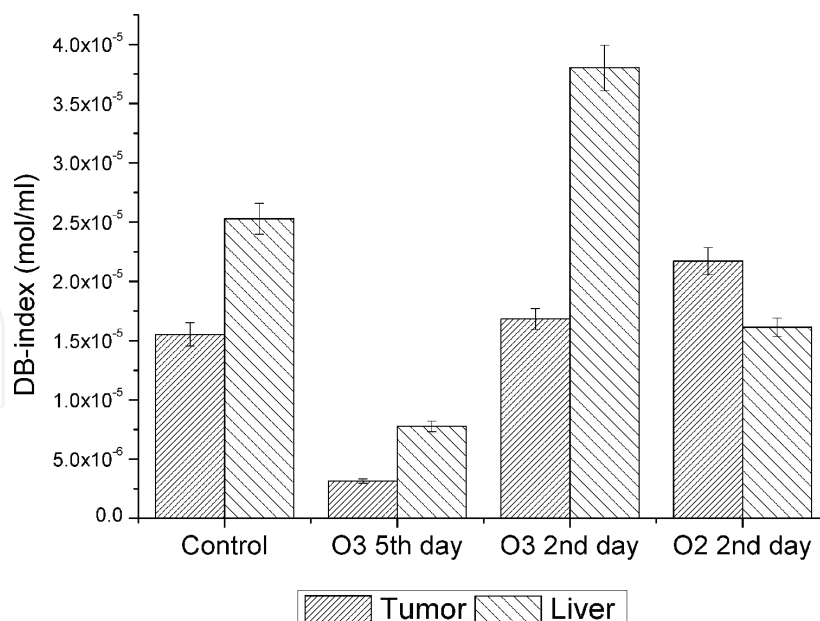


**Figure 5.** DB-index values obtained from lipid samples extracted from plasma and erythrocytes (n = 6).

On the other hand, in mice treated with ozone and oxygen, this index for erythrocytes was lower than the control group (around 32%), which indicates the tendency to normalization of LPO.

**Figure 6** depicts the DB index of lipids extracted from tumor and liver tissues. These were measured in our previous study for the first time in mice with tumors [20]. The DB index of tumors reduces by 83% and of the liver by 70% in the group of mice with smaller doses of ozone compared to the control group and the other groups treated with ozone and oxygen. This phenomenon was observed in both tumor and liver tissues and it seems to be a consequence of the modification in metabolism promoted by ozone. According to the preliminary studies, the cancer cells repressed the TIGAR enzymes. The lower concentrations of these enzymes keep the ROS concentration at an abnormal higher level [8] that caused the apoptosis of cancer cells [34]. The mice treated with ozone every second day and oxygen had higher values for DB index of tumor tissues, which is related with the cell activity observed microscopically. In fact, there was no significant statistic difference of the DB index measured in tumor tissue treated with ozone every second day and the control group. It seems that this dose had no effects over the tumor cell metabolism, contrary to the lower dose (every fifth day), where the mice's system was able to better regulate the oxidative stress induced in the treatment, and this was reflected in both the lower cell activity and the tissue DB index. In the group of mice treated with oxygen, the DB index increased 33% compared with the control group. This may be caused by the overexpression of some enzymes, such as FAS, which improved the cell proliferation by oxygen presence [9, 20].

However, the DB index of liver tissue increased by 50% compared to the control group. This variation points out to the sensibility of this index on the ozone dose, that is, this value decreased by 70% in mice dosed every fifth day and increased by 50% in subjects dosed every



**Figure 6.** DB-index values observed in tumor and liver tissues (n = 6).

second day. In the group treated with oxygen, the DB-index in liver decreased 40% compared with the control, due to the decrease of the consumption of the energy caused by the decrease of tumor volume. The DB index of the liver suggests a modification in the energy consumption associated with the tumor activity because of the treatment. The accumulation and production of lipids appeared to be inhibited in mice treated with ozone (every fifth day) and oxygen [25]. This effect could be explained by two possible metabolic pathways of the fatty acids synthesis in the organism. The first considers that lipids in plasma come from energetic reserves of the liver (glucagon), which is regulated by the lipid reduction in blood indeed. The second assumes that the fatty-acid cycle regulates the lipid concentration in plasma, which is also regulated by the liver, but there is no energetic transformation. Under the higher dose of ozone, the liver regulates the lipid imbalance to compensate the oxidized fatty acids. This additional lipid source may explain the increase of the DB index.

On the other hand, under the smaller ozone dose, the lipid-accumulation effect was not observed. The last can be a result of the regulatory process conducted outside of the liver tissue that seems to justify the DB index reduction. The tumor cell activity correlated with the DB index of the lipids is obtained from the tumor tissue. When their activity was the smallest or almost zero, the DB index value was smaller also. This confirms that the DB index determination can be a reliable method to control the medical treatment efficiency for regulating the tumor growth and its activity as well.

## 6.2. Indirect applications: vegetable oils

The ozonated vegetable oils (OVO) have shown interesting applications in diverse fields, such as food, pharmaceutical, and cosmetic industries, since their applications have resulted in several positive in vitro, in vivo, and clinical effects. In addition to their therapeutic potential,

the OVOs have some advantages over other ozone applications, since they are composed of stable reaction products [35]; thus, it is not necessary to produce them in situ. This is an additional advantage from a commercial point of view.

Among the most reported therapeutic effects of the OVO, one may list bactericidal, fungicidal, as well as inflammation and wound-healing mediators [35–38]. These effects are highly related with the oil type, as well as the ozonation degree.

The determination of the TU and DB index has been useful in the application of vegetable oils. By using these parameters, it is possible to control the ozonation conditions to achieve a certain ozonation degree, as well as observe the treatment's evolution and evidence of the biochemical changes derived from the treatment.

### 6.2.1. Ozonation degree of vegetable oils

The therapeutic action of the OVO depends on the accumulated ozonation products. A lot of techniques have been employed to characterize these compounds, such as the spectroscopic methods (Fourier transform infrared (FT-IR) and hydrogen-1 nuclear magnetic resonance ( $^1\text{H}$  NMR)). The identified products by these techniques corresponding to those described by the Criegee's mechanism (iso-ozonides, poly-peroxydes, hydroxyperoxides) [12, 13, 17, 18, 39, 40].

Different studies have justified that the observed effects of the applied ozonated oils depend on the oil's ozonation degree. For example, the in vitro tests showed that the bactericidal and fungicidal effects increase when the ozonation degree increases [41–43]. Complementary, the in vivo evaluations showed that the adequate ozonation level depends on the treated illness and the vegetable source of oil [44–46].

For example, the inflammation process induced in the mice's skin by 2,4-dinitrofluorobenzene was inhibited after the application of olive oil ozonated 100% (iodine value = 0). However, the repeated applications produced hair losses, hypervisibility and swelling reactions [44]. Another work demonstrated that the ozonated sesame oil showed diverse effectiveness for mice's wound healing, depending on the peroxide index of the applied oil [45]. The authors found that the better peroxide value was  $1631 \pm 64$  mEq/kg. The higher and lower values of oil's peroxide value were less effective.

In our previous work, we studied the ozonation of two oils: sunflower (SFO) and grape seed (GSO) [16]. The different ozonation products (mainly ozonides) were identified by the spectroscopic techniques (FT-IR,  $^1\text{H}$  NMR). Also, the changes in OVO's viscosity were associated with the formation of poly-peroxides. We also determined the dynamics of  $>\text{C}=\text{C}<$  decomposition and product accumulation. We found that the TU decrease was similar in both oils, but the distribution of their ozonation products was different. It was established that the maximum amount of ozonides were formed faster in GSO. This oil accumulated a higher proportion of poly-peroxides related with its viscosity, when compared with the SFO [16].

Since the therapeutic effects of ozonated oils are strongly related to the accumulation of the ozonation products, our previous investigations offer an alternative method for controlling the ozonation degree in the preparation of ozonated oils.

### 6.2.2. Inflammation and wound healing

The effectivity of TU and DB index in ozonated oils' applications was evidenced in our previous work [22], where the anti-inflammatory and wound healing effect of the ozonated grape seed (GS) and sunflower (SF) oils in mice were tested (for wound healing, diabetic mice were tested). The ozonation degree of both oils (determined by TU) was related with the *in vivo* effect of oils. For comparison, the ozonated physiological solution was applied (subcutaneous injection, only for inflammation test), as well as the commercial drugs indomethacin and Furacin® for anti-inflammatory and wound-healing tests were used, respectively.

Some differences on the biochemical effect of different treatments were found, depending on the oil type and their ozonation degree. These differences were revealed by the DB index values of the treated tissues [22].

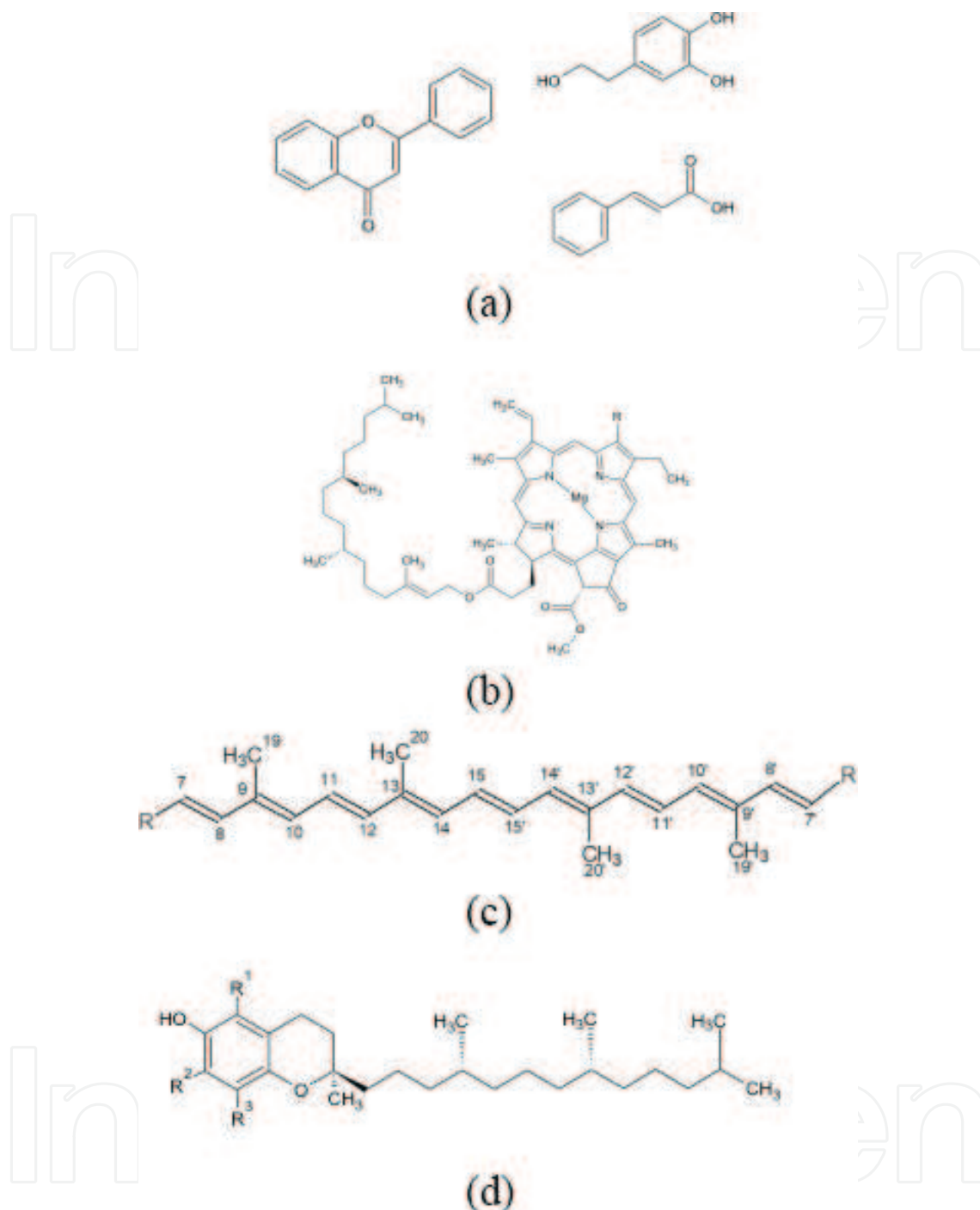
In the case of the SF oil with the ozonation degree of 44%, the DB index of  $2.32 \times 10^{-4} \text{ mol g}^{-1}$  corresponds to the inflammation inhibition (INI) which is about 32%. For the GS oil, the maximum INI was 25% under the ozonation degree of 24% and corresponds to the DB index of  $2.26 \times 10^{-4} \text{ mol g}^{-1}$ . In this last case, the increase of the ozonation degree of GS oil up to 41% decreases the INI down to 23%.

The effect of the vegetable source of oils on both the INI and the DB index suggests an active participation of their minor compounds typically contained in oils, in the response of the immunological system (polyphenols, tocopherols, carotenoids, chlorophylls). **Figure 7a–d** shows the chemical structures of some these compounds. As seen, they are susceptible to reacting with ozone, due to their unsaturated structures. So, they may participate in the therapeutic effects of ozonated oils. Even when the concentration of these compounds is low, in comparison with unsaturated fatty acids, they can consume considerable amounts of ozone. For example, the stoichiometry of the reaction of phenols with ozone is 1:3.5, while  $>C=C<$  with ozone is 1:1.

A lower INI and the DB index of the pavilion of the ear (15% and  $1.57 \times 10^{-4} \text{ mol g}^{-1}$ , respectively) resulted from the application of ozonated physiological solution (PS), when compared with ozonated oils. This fact indicates that the action mechanism of ozone, when applied directly (dissolved in PS) or indirectly (ozonated oils), is different.

In the case of wound-healing evaluation for diabetic mice, we also found promising results, considering that this sickness negatively affects the wound-healing process. We observed that the complete wound healing of diabetic mice treated with ozonated oils was obtained during 12 days [22]. This time is comparable with the one reported for wound healing of non-diabetic mice (14 days) treated with ozonated sesame oil [45]. It is worth mentioning that no infection signs were observed over the mice skin tissue. The glucose content was also monitored throughout the treatment. None of the agents showed a regulatory effect of glucose, as expected. Then, the cutaneous application of ozonated oil did not have a systemic effect which is considered a positive effect because these oils acted locally.

Our results showed that minor compounds presented in oils may have biological activity, which contributes to the effect of the well-known therapeutic ozonation byproducts, namely



**Figure 7.** (a) Structures of polyphenols. (b) Structures of chlorophylls. (c) Structures of carotenoids. (d) Structures of tocopherols.

ozonides, as a product of triglycerides' ozonation. This effect was more pronounced in the case of inflammation. For wound-healing tests, a slight improvement was observed in the implementation of the SF oil, compared to the GS oil (both ozonated up to the same ozonation degree). Based on these data, we may conclude that the positive clinical effect of ozonated oils depends on their ozonation degree and their nature and then the composition of their minor compounds.



## 7. Conclusion(s)

The TUL determination was an adequate parameter to evaluate the effect of ozone on biological substrates. The versatility of this technique allowed the control of the ozonation degree of oils, as well as the correlation of biochemical changes in tissues involved in ozone-based treatments for C6 tumor cells, inflammation, and wound healing, considering direct and indirect applications. The effect of dissolved ozone dosage (direct application) on the tumor evolution was observed, and the main result was that at low doses of ozone (every 5 days), there is a greater effect on the inactivity of C6 glioma cells, decreasing their reproduction and therefore, reducing the DB index of tumor tissues, in comparison with other groups. This effect depends on the type and stage of the disease. Since it has been reported that the application of ozone reduces the size of certain tumors, in this context, it was observed that although ozone had a positive effect with respect to the activity of the tumor quantified by micro PET and the DB index determination, the volumetric growth of the tumor was disproportionated. The results presented in this study demonstrated that the key factor for controlling the tumor activity, inflammation, and wound healing through direct and indirect applications of ozone was precisely ozone's dose. Our results suggest that low doses of ozone may induce a micro-oxidative stress that stimulates the organism to perform a redox regulation, which is reflected as a self-inhibition of the cancer tissue activity. The micro-dose of ozone may have a systemic and prolonged effect on the organism. Therefore, in this study, three injections for 15 days were enough to get a decrease, > 80%, of tumor cell activity in mice. In addition, the DB index values pointed at different reaction mechanisms of direct treatment with ozone (dissolved in the physiological solution) and to ozonation byproducts (ozonated oils). Ozone's administration route influenced the inflammation inhibition, ozonated oils being the best anti-inflammatory agents. We found that the ozone dosage, meaning, the ozonation degree of oils, as well as the frequency of application, is a key factor in the biological effect of ozone-based therapies.

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## Conflict of interest

Authors declare no conflict of interest.

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