Analytical Methods

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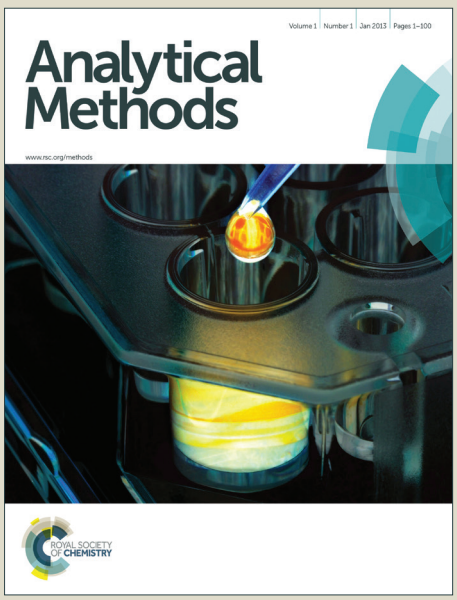

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Ozone dosage effect on C6 cell growth, *in vitro* and *in vivo* tests: Double bond index for characterizing

Arizbeth Pérez[a], Clara L. Santos Cuevas[b], Isaac Chairez[c] , Tatyana Poznyak[a] ,

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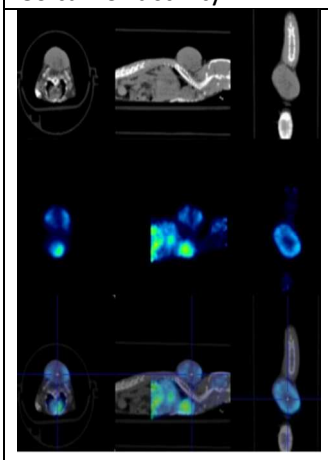
The ozone dissolved in saline solution applied as a medical therapy promoted a decrement of 85% C6 tumor activity.



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ABSTRACT

Due to its chemical properties, ozone gas has being used in the medical field for the treatment of many diseases. In general, all therapies based on ozone reported regulatory clinical effects. However, ozone dose has not been clearly defined and connected with the specific stage of the illness evolution. Double bond index has been used to identify or characterize some therapy's effects on patients. The aim of this article was explore the possibility of using DB-index as a simple and fast biochemical test to characterize the effect of ozone therapy for cancer. In this study, this claim was confirmed with an in vitro and in vivo test. Proliferation analysis of C6 rat glioma cells was correlated with the DB-index variation. The in vivo test used athymic mice with inducted tumors of the same cell line (C6). Tumor's volume and its activity,cholesterol/triglycerides variations and hydroperoxides quantification were correlated with the DB-index variation in plasma, erythrocytes and tissues. In all cases, there was a specific and a plausible relationship between the DB-index behavior in the clinical and imaging results obtained by micro PET studies.

Keywords: Ozone dose, DB-index, C6 growth cells, Tumor activity.

INTRODUCTION

Even when the oxygen is considered as a fundamental element for living organisms; this gas is one of the main oxidative compounds that affect the organism metabolism. The cellular breath promotes the accumulation of reactive oxygen species (ROS) trigger a negative effect over the cell (1), (2), (3) Among others, hydroxyl radicals (.OH) are compounds recognized as toxic for enzymes and Desoxirribonucleic Acid (DNA). The aging process and metabolic disorders (atherosclerosis, diabetes, cellular degeneration) are directly associated with oxygen metabolism and may be aggraviated by the presence of reactive oxygen species (ROS) (1), (4), (5). ROS have been associated with tumor development; however, their roles have not been clearly exposed (6), (7), (8).

ROS are mediators, triggers or executioners of essential protective mechanisms such as apoptosis, phagocytosis and detoxification reactions. Among these mechanisms, apoptosis which eliminates precancerous and cancerous, virus-infected and otherwise damaged cells is particularly important. Increasing of ROS concentration by depletion of antioxidants enhances apoptosis and thereby inhibits tumor growth. Excessive antioxidants decrease ROS level inhibit apoptosis and suppress the elimination of cancer cells induced by anticancer drugs (9), (10). Numerous data demonstrate that ROS are capable of oxidizing cell constituents such as DNA, proteins and lipids, thereby incurring oxidative damage to cell structures.

Even when there are a lot of studies dealing with the oxidative reaction with lipids, proteins and glycosides independently, there are no clear explanations about the interaction between them as a mix and the oxidative compounds (11). However, it is well known that lipids are particularly susceptible to be oxidized. This is the reason to use the forced lipid oxidation (lipid peroxidation or LPO for short) as an indicator of the metabolic equilibrium between oxidants and antioxidants (7), (6), (4), (1), (2), (8). Then, the effect produced by ROS can be followed by the LPO. Moreover, LPO has been recognized as a key factor to monitor the positive or negative effects produced by oxidative compounds. Indeed, it has been shown that ROS and LPO are associated with some multiple oxidative/reduction biochemical reactions that occur in human cells (4), (2), (12), improved the antioxidant regulations and the release of stem cells. This is the theoretical basement of many oxidative medical therapies as hyperbaric treatment and ozone therapy (1), (13), (7), (3), (11), (10).

Several methods have been developed for monitoring *in vivo* the LPO (and oxidative stress indirectly), such as: direct quantification of reactive species by electron spin resonance, as well as indirect methods such as determination of antioxidants and total antioxidant capacity (TAC) and detection of oxidized biological markers (malondialdehyde, 4-hydroxynonenal, isoprostanes, oxidized LDL, etc.) and the measurement of DNA damage by a high performance liquid chromatography (HPLC) or a gas chromatograph (GC). However, all those methods have limitations regarding sensitivity, specificity and timing analysis (3), (7), (14), (11), (2).

The present article introduced the method of the Double Bond index (DB-index) based on the patented total unsaturation analyzer (TUA) equipment analog of the double bonds analyzer (DBA) (8), (15), (16). By this methodology, it is possible to determine in a short time (1-3 min) and with high precision (± 1%) the total lipids unsaturation (TLU) in biological substrates. In general, the DB-index determination is a promising method to evaluate the lipid peroxidation (LPO). This method consists in the quantification of ozone that reacts with the double bonds presents in the lipid fractions of plasma and cells (8).

The TUA equipment operation is based on the fast reaction between the ozone and the organic unsaturated compounds. The general TUA operation involves the next steps [(8)].

1. Oxygen gas is transformed into ozone by a crown discharge generator
2. Controlled ozone concentration is obtained in order to carry out the reaction with a sample of lipids extracted from plasma or cells by a simple modified Folch method.
3. The measure of non-reacting ozone (in the gas phase) is realized by an ozone sensor (UV detection), the values are registered and plotted on a computer, obtained as a result a curve called ozonogram.
4. The area under the curve from the ozonogram is proportional to the double bond quantity in the sample.

The application of the ozone in cancer therapy, one of the most important topics to study is the reaction dynamics between the ozone and the biological tissue. In general terms, cancer treatment based on ozone applications has showed many positive and negative biological effects. According to some authors (6), (17), (18), (19), (20), some of those observations are:

- Improves the blood circulation and oxygenation of ischemic and neoplastic tissues
- Improves the metabolism
- Corrects chronic oxidative stress due to regulate the antioxidant system.
- Induces activation of the immune system.

- Induces activation of the neuroendocrine system.
- Decreases the size of tumors through the treatment and prevents the metastasis.
- Induces the tumor necrosis.

Oxidative therapies have been studied for many years, but there are some open questions about their action mechanisms such as the influence of the stage of the disease when the proper dosage of ozone is looking.

From a chemical point of view, there are some researches try to explain the kinetics of those reactions, such as the consideration that the first step of the reaction mechanisms is the interaction between the ozone and the double bonds of the lipids, following the Criegee's mechanism (8), (21). The main products of that reaction are the ROS and the lipid peroxidation products (LPOP) that according with some theories cause the clinical effect reported in the medical field (11), (3), (9), (2). This is the reason that motivates study the reaction mechanisms that will have the potential application in the development to regulate the ozone dosage for any disease treatment, in this case applied for cancer treatment.

In the present study, the viability of the DB-index as an indicator of the ozone dose effect on the tumor evolution was explored, and additionally, as the measure of the LPO, which also can be used as an indirect indicator of the tumor activity.

MATERIALS AND METHODS

Physiological solution (NaCl 0.09%) ozonation

Physiological solution of NaCl 0.9% (saline solution) was used as carrier media for the oxidant agent (ozone or oxygen). Figure 1 shows the laboratory scheme of the ozonation process, in which the gas flow at 0.3±0.1 L/min of ultra-dry oxygen was fed into an Ultra-Violet ozone generator, resulting in a mixed stream of ozone/oxygen with an ozone concentration of 4.6 ± 0.2 mg/L. This gaseous mixture was injected into a glass micro reactor (5 ml) with saline solution (the dissolved ozone concentration was 1.2 mg/L). The ozone that did not react with the saline solution was measured by a gas phase ozone sensor (BMT-930). The registered graphical representation of the ozone concentration at the reactor output is called ozonogram.

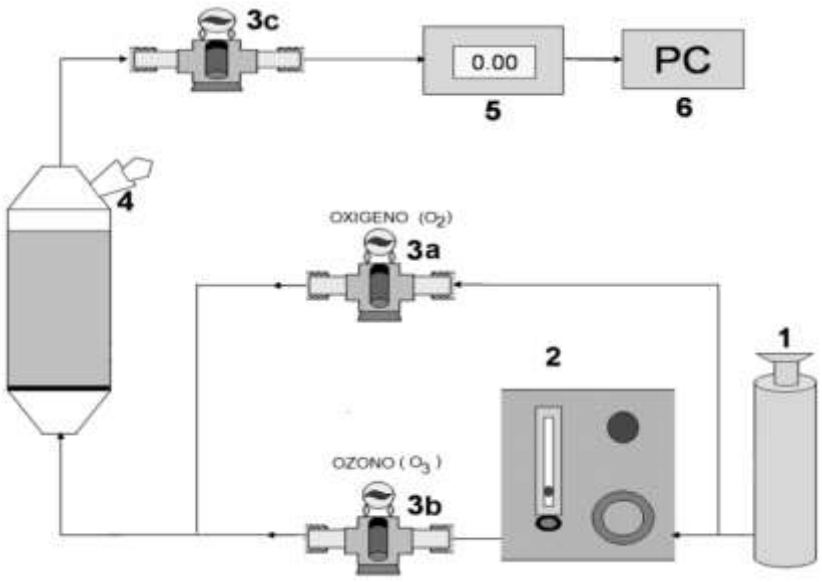

Figure 1. Simplified scheme of the ozonation process developed to prepare the ozonated saline solution. 1- the ultra-dried oxygen tank, 2-the ozone generator, 3a-c - electro valves, 4 - the ozonation reactor, 5 -the ozone sensor and 6 - the computer where the ozonogram is registered.

Determination of kinetics of ozone decomposition in aqueous phase was made in order to confirm that there was no kind of reaction between the NaCl and the ozone that could interfere with all the experimentation. Those results are not presented in this paper because that is beyond of its scope.

The methodology followed in this paper was divided in two stages, the first one considers the analysis of the cell line C6 rat glioma, which was exposed to pure oxygen, and a mixture of oxygen / ozone (94/6 %) dissolved in saline solution. These experiments were used to observe the effect that oxidants have on cell proliferation and its relationship to the ozone dose. The DB-index determination was correlated with the C6 cell count and by an indirect method based on DNA quantification.

The second stage considered the implantation of C6 cells in an animal model. The oxidants dissolved in the saline solution were dosed by intraperithoneal pathway in athymic mice (Balb/CNu/Nu). The effects on the animal's metabolism and tumor growth were correlated with the DB-index too.

In vitro test

Cell line

Cell line C6 rat glioma was originally obtained from ATCC (USA). The cells were routinely grown at 37°C, with 5% CO_2 atmosphere and 100% humidity in minimum essential medium eagle (MEM, Sigma-Aldrich Co., Saint Louis, Missouri, USA) supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). All chemical reactants were analytical degree.

Oxygen, Ozone/oxygen dosage

Evaluation of the ozone dosage over cell proliferation was done with three different dosing schemes: the Group 1 the ozone was dosage only at the beginning of the experiment. The Group 2 implies a daily ozone dosage and finally, the Group 3 the ozone dosage was dosage every second day. The volume of saline solution with dissolved ozone was 100 µL per 100 µL of culture medium. The effect of the ozone dosage on the C6 proliferation dynamics was also followed by the DB-index analysis of cellular lipids.

Proliferation test

The effect of the ozone on the cell proliferation was evaluated by the Cy-Quant® cell proliferation assay kit (Molecular Probes, Invitrogen). This method uses a proprietary green fluorescent dye that exhibits strong fluorescence improves when it is bounded to cellular nuclear acids.

Approximately $1x10^3$ C6 cells were dispensed into wells (96-well culture plate; n=6). The cells were cultured for 24h, and the growth medium was replaced with a flesh medium containing O_2 and O_3. The cells were cultured for 6 days at 37°C, and the growth medium with or without the O_2/O_3 treatment was replaced each time that the ozone was dossed.

On the 6^{th} day, the cells were frozen for 30 min (-70°C), thawed and lysed by the addition of the buffer containing CyQuant® green fluorescent dye. Fluorescence was measured directly at excitation/emission maxima of 480/520nm.

The absorbance of the wells was measured in a micro plate reader (Bio-Tek, SinergyHT, Nova Biotech, El Cajon, California, USA). Finally, the wells absorbance were correlated with DNA concentrations (ng/mL) using a standard curve.

In vivo test

Animal Model

Tumor studies in mice were carried out to evaluate the effect of ozone dosage on the lipid fraction of samples of blood, tumor and some organs such as kidney and liver. These measurements were made by the DB-index quantification and correlated with the hydroperoxides concentration.

Athymic male mice (20-22g) were kept in sterile cages with wood-shavings bedding, constant temperature (25 °C), humidity (60 %), noise and 12:12 light periods. Water and feed (standard PMI 5001 feed) were given *ad libitum*.

Tumor induction in athymic mice

Tumor uptake studies in mice were performed according to the rules and regulations of the Official Mexican Norm 062-ZOO-1999. The study was approved by the Institutional Committee for the Care and Use of Laboratory Animals ("Instituto Nacional de Ciencias Médicas y Nutrición de Salvador Zuribán"). Glioma tumors were induced by subcutaneous injection of C6 cells (1.5×10^6) suspended in 0.2 mL of phosphate-buffered saline into the upper back of twenty 6-7-week-old nude mice. Injection sites were observed at regular intervals for tumor formation and progression.

Therapeutic protocol: Ozone/oxygen dosing strategy

Four groups (n=6) of athymic nude mice bearing C6 gliomas (tumor size 74.60 ± 21 mm^3) were used. Three dosing strategies were considered. The first one considered an ozone dosage every 2^{nd} day (7x times), the second group contemplated ozone dosage every 5^{th} day (3x times), while the third group was dosed with oxygen dissolved in saline solution every 2^{nd} day (7x times). A control group was considered to observe the differences with the dosed ones. Tumor growth was monitored daily, the length (L) and width (a) were measured with calipers and the volume was determined as $V= (a^2*L)/2$. Whence, 90 µL of saline solution with oxygen or ozone dissolved ($9.5*10^{-5}$ mg of O_3) was intraperithoneal injected into the mice when all the tumors have approximately the same volume (70 mm^3) to initialize the treatment (19), (20) and this was considered as the initial day. After 15 days of oxidant application, the mice were sacrificed, and samples of blood and tissues were recollected to make the DB-index studies, hydroperoxides, cholesterol and triglycerides quantification.

[18F]FDG in tumors with PET/CT: tumor metabolic activity

[^{18}F]FDG (2-deoxy-2-[^{18}F]-fluoro-D-glucose)-positron emission tomography (PET) and X-ray CT imaging were performed using a micro-PET/CT scanner (Albira, ONCOVISION, Spain). The

images were acquired at the end of the treatments. The micro-PET field of view was 60 mm. Mice were injected into the lateral tail vein with 9 MB of [^{18}F] FDG in 100 µL PBS under 2% isoflurane anesthesia. After a resting period of 1 h the mice were transferred to the scanning room and placed in a prone position, and whole body imaging was performed. The PET acquisition time was 7.5 min. The CT parameters were 35 kV sure voltage, 700 µA current and 600 micro-CT projections.

Clinical analysis

After the complete treatment, blood samples were obtained to make the determination of total cholesterol and triglycerides. This part of the study was conducted considering that main reaction of the ozone is with the double bonds of the lipids, particularly with cholesterol and triglycerides that were presented in the cell membrane. Besides, triglycerides were one of the main energy resources of the organism, it was decided to observe the effect that presence of tumor and the oxidants have on the concentration of these compounds.

Hydroperoxides quantification

Quantification of lipid peroxidation was essential to assess the role of oxidative injury in pathophysiological disorders. Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. Sensitive colorimetric assays were developed to measure these aldehydes. Cayman's Lipid Hydroperoxide assay kit ® measure the hydroperoxides directly utilizing the redox reaction with ferrous iones. Hydroperoxides were highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions were detected using thiocyante ion as the chromogen.

DB-index analysis

The plasma and erythrocyte samples were previously treated in order to make the lipid extraction according to the modified Folch method. The organic phase in chloroform was used to determine the DB-index of the sample (22).

A prototype of the DBA equipment was fed with a volume of 0.1 L/min of ultra-dry oxygen. The UV ozone generator produced a mixed stream of ozone/oxygen with an ozone concentration of 6.4 ± 0.2 mg/L. This mixed gas is injected into a glass micro reactor (5 mL), which contains 4 mL of CCl_4 (diffusion media). A volume of 10 µL of lipids extract in chloroform was injected into the micro reactor. The ozone that did not react with the lipids was measured by a phase gas ozone sensor (BMT). Ozonogram was an adopted term for describing the monitored ozone concentration [(8)]. The DB - index was derived from the next mathematical expression:

$$DB\text{-}index = \frac{C_{st} V_{st} S_s V_{sol}}{S_{st} V_s W_m}$$

In this equation, $C_{st}$is the concentration of the standard solution (mol/mL); V_{st} and V_s are the volumes of the standard sample and the analyzed sample, respectively (mL); S_{st} and S_s are the areas of the standard ozonograms and the analyzed sample; V_{sol} is the volume of the analyzed sample solution (mL), W_m is the volume or the weight of the analyzed sample (mL or mg). The standard solution is stilbene ($5x10^{-5}$mol/L) and the injection volume is 10 µL, which is used for the method calibration.

Statistical analysis

Differences between the treatment groups were evaluated with simple one-way Anova analysis. (Significance was defined as $p<0.05$).

RESULTS AND DISCUSSION

In vitro test

Proliferation cell test

Figure 2 shows the effect of oxidant dosage on the cell culture proliferation. These measurements were done by DNA concentration followed by CyQuant® assay and its correlation with the aforementioned calibration curve. As expected, the control group where the saline solution was injected did not show any effect on the dose strategy.

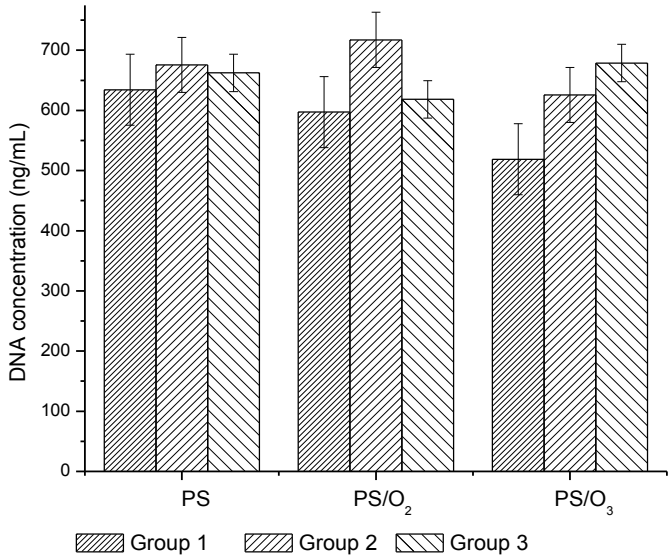

Figure2. DNA concentration measured in the different experimental groups n= 6

Pure oxygen dosed on C6 cells showed a promoting effect over their concentration, when the gas was dose every 2nd day. However, no statistically significant outcome was observed between Group 1 and 3. When oxygen was dosage just at the beginning of the study, was not enough to promote the cell growing. On the other hand, when oxygen was supplied every day, a possible oxidative effect modified the cells growing evolution.

The presence of ozone showed a direct relationship between the total amount of applied ozone and DNA concentration. Indeed, this condition was directly associated with the recognized stressing effect of ozone over cells. Therefore, less inhibition of cell growing by increase the ozone concentrations, but never beyond the concentration achieved when pure oxygen was in contact with the tumor cells.

Ozone has ambivalent effects that increased the cell proliferation when the dose was given every 2nd day or inhibited it when a single dose was used. In these cases, the DNA concentration decreased indicating that the inhibitory effect prevails. The single dose was more effective for reducing the C6 cell concentration. This reduction yield to the lowest cell concentration among all others experimental conditions considered in this study.

DB-index determination

Recalling that ozone reacts primarily with the double bonds of lipids, the counting cell was proportional to DB-index and the relationship should be valid with the DNA variation presented above. Figure 3 shows the DB-index values measured in cell samples obtained from the experiment describing the ozone dose on C6 growing dynamics for groups 1 and 2, the group 3 was not considered for this study due to the huge effect that the ozone has over the cell growth.

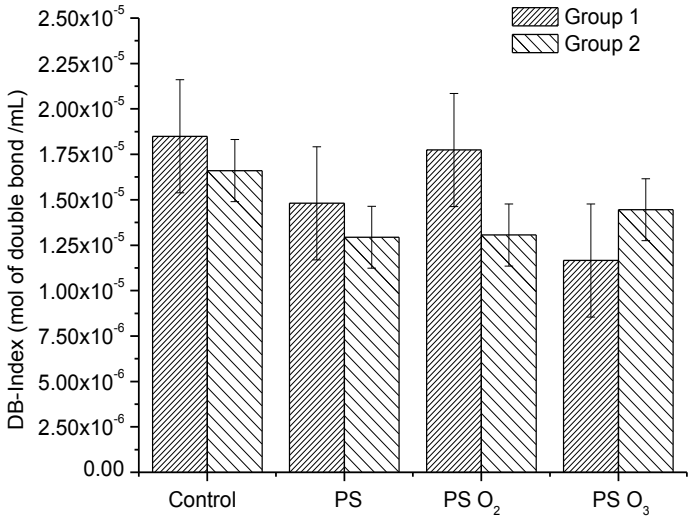

Figure 3. DB-index variation of lipids extracted from the C6 cells n=6

When the ozone was applied in the second grous, it promoted the increase of the DB-index (about 10%) compared to the exposition of pure oxygen under the same dose strategy. When only saline solution was supplied to C6 cells, there was no difference with the case when oxygen was injected. Because the DB-index was determined only by the lipid fraction, the slight difference between DB-index and their corresponding DNA variation was not statistically significant.

The figures 2 and 3 show the effect that the oxidants have on the cell grown. When the ozone was applied every second day (Group 2) was observed that the DNA concentration and the DB-Index presented an increase compared with the control group, this can be explained by the promoting effect that the ozone has over the cell reproduction.

In vivo test

Tumor volumetric evolution

The tumor volume variation was the simplest way to characterize the effect of the ozone dose over the tumor cells. The same *in vitro* dose strategies were followed in this case (control, oxygen every 2nd day, ozone every 2nd day and every 5th day). As demonstrated the figure 4, in comparison with the control group, in general, ozone promoted the tumor volume growing.

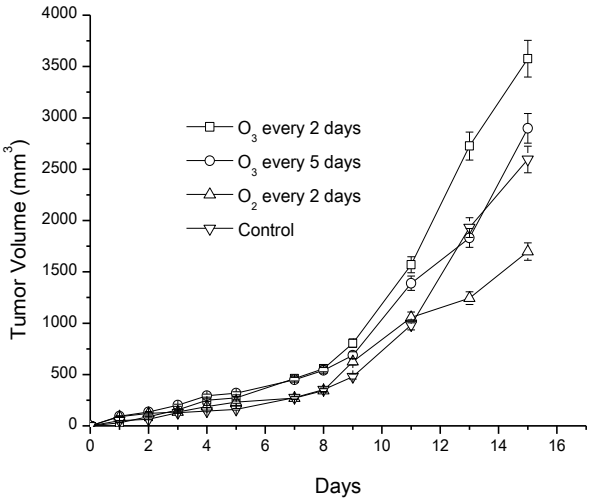

Figure 4. Tumor volume increase associated with the dose strategy showing the control group, pure oxygen and ozone dosed every 2nd day and every 5th day.n=6

This was more evident when the ozone was dosage $3.024*10^{-3}$ mg every two days instead of $1.104*10^{-3}$ mg every five days. It must have observed that the smaller tumor volume was observed when pure oxygen was injected. It has been observed (2), (3), (23) that oxygen can inhibit the angiogenesis associated with the tumor. This was also preventing the nutrient availability. These two factors explained why the tumor growth slowly compared to the control group. Additionally, ROS concentration (hydroxyl and superoxide radicals as well as hydrogen peroxide) promoted by oxygen can obstruct the tumor growing (9), (23). This can be explained by a feasible metabolic acceleration promoted by ozone. In other words, the stressing effect of ozone was reflected by the initial tumor accelerated growing. However, in both ozone doses, the tissue showed a higher degree of necrosis appearing by the higher ROS accumulation than the case when oxygen was supplied. However, when ozone was dosed every 5th day, the necrotic effect was more evident. Additionally, the slope of tumor volume growing was lower than the ozone dose every 2nd day. So, ozone dose is playing a relevant role in controlling the tumor volume. This increment was less than 10%, but with

a smaller tumor activity measured by PET image analysis. Despite the tumor volume increment its metabolic activity was reduced by the ozone dosage.

Clinical analysis (Cholesterol and Total Triglycerides)

Tumor growing has an effect on the nutrient balance within the organism. Triglycerides and cholesterol in the blood were identified as a potential energy source for the C6 tumor growing (24). No controlled increment of these lipids in the blood can be promoted by the C6 tumor activity (24).Figure 5 shows how the total triglycerides and total cholesterol concentrations were reduced when the ozone dosage was also reduced. The control group is showing an increase of 25 % for cholesterol and 15 % for triglycerides with respect to a healthy subject.

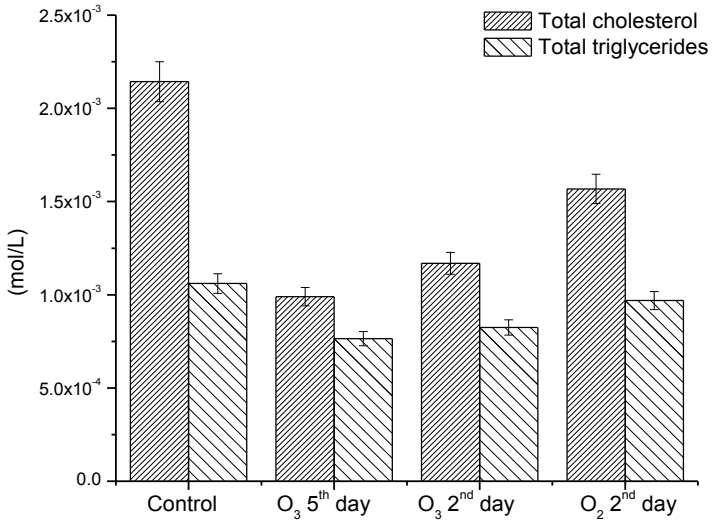

Figure 5.Concentration of cholesterol and total triglycerides in a plasma sample. n=6

Recently, a study has claimed that ozone is produced by some organism antibodies (1) (4) (6). Moreover, its effect on cholesterol concentration has been followed by some biomarkers. These results also can be used to explain the most evident effect on cholesterol than on triglycerides.

All the groups where any oxidant gas was injected showed a reduction of both lipids. The variation of triglyceride concentration was smaller than the cholesterol one in all cases, where the oxidant gas was supplied to the subject. Oxygen dosed every 2nd day showed a decrease of 25% with respect to the control group for cholesterol and 20% for triglycerides. Moreover, the effect of ozone is obtained in both dose strategies (every 2nd and 5th day). When the ozone dose was higher, the

cholesterol concentration was reduced 40%, but when the smaller dose was considered, this reduction was increased up to 50 %. For triglycerides, the higher ozone dose generated a 25% concentration reduction, while the low ozone dose increased this factor up to 30%. The smaller ozone dose can have this remarked effect by the regulated ROS concentration achieved with such low gas dose. Therefore, the equilibrated ozone dose can have a deeper effect on the tumor activity than higher ozone doses.

Determination of DB-index of plasma, erythrocytes and mice organs (kidney, liver and tumor)

To correlate the variation in the tumor growth with other factors: the cholesterol/total triglycerides variations and the changes in the concentration of the reactive sites of the ozone, the DB-index determination in plasma, erythrocytes and tissue samples from mice (tumor, kidney and liver) were carried out. Ozone dosage effect on the DB-index was characterized and its relationship with the clinical analysis was established.

Figure 6 shows the differences of the DB-index obtained from the plasma and erythrocyte samples.

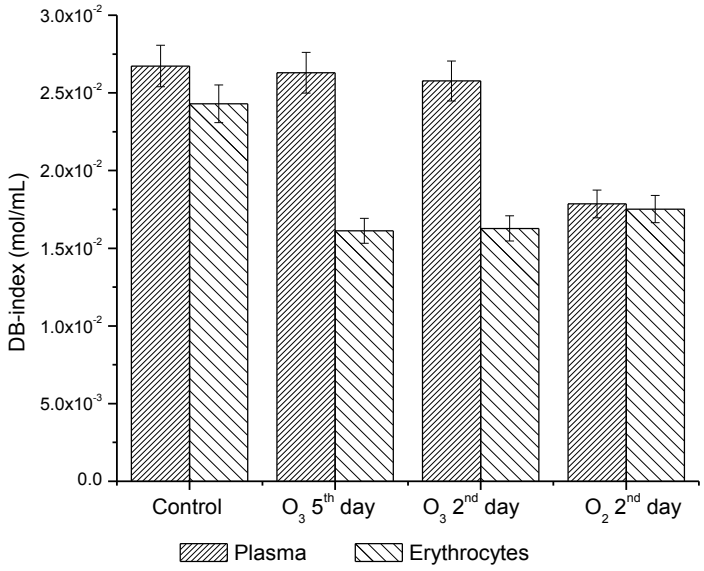

Figure 6.DB-index values obtained from lipid samples extracted from plasma and erythrocytes. n=6

As observed, the plasma samples had no statistically significant variation in the groups dosed with ozone compared with the control group. This particular behavior may come from the regulatory clinical effect showed by ozone. Then, the DB-index variation indirectly characterizes the ozone

action on the tumor's capacity for using lipids as an energy source. However, the ozone dosage in all groups was not enough to have some kind of effect over the erythrocytes membrane.

A complementary fact was the well described promoting influence of tumor activity for increasing the concentration of some specific lipids in blood. The variation of lipids also can be monitored by the DB-index measurements. However, this methodology cannot separate both effects.

The complementary DB-index analysis of tumor, liver and kidney tissue samples was used to clarify what is the prevailing factor. As it is shown in the figure 7, the DB-index values decreases 98% compared with the control group when the ozone was dosed every 5th day , this may be due to the inhibition in cell metabolism. Accordingly to some previous researches, the presence of a cancer cell inhibited the presence of TIGAR enzyme (which keep low levels of ROS) so the ROS concentration increase. Perhaps, the presence of ozone improves, even more, the ROS production, inducing the cellular apoptosis [9]

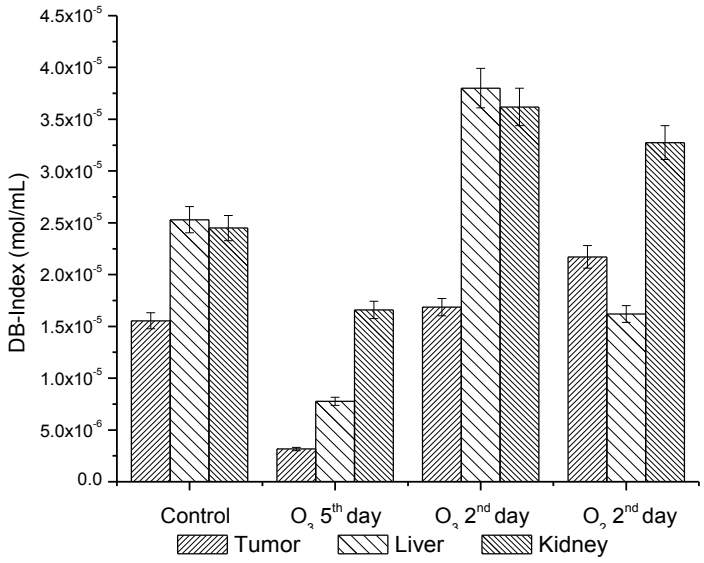

Figure 7. DB-index values observed in different tissues: tumor, liver and kidney. n=6

However, in the system when the ozone was injected every second day was no observed a significant statistical variation, due to the oxidant did not affect the cell metabolism under those conditions. On the other hand, in the pure oxygen system, the DB-Index increased 33% compared with the control group. It may owe the increase in the double bond availability in the medium, due

to the overexpression of some enzymes (such as FAS), that improved the cell proliferation by the excess of oxygen presence (20), (9).

When pure oxygen was supplied, the DB-index in liver samples decreased 40% compared with the control, due to the decrease of the energetic consumption (caused by the decrease in the tumor volume). Therefore, the fatty acids, phospholipids and cholesterol in serum accumulation and production were inhibited (25).

The aforementioned effect could be explained due to there are two possible metabolic pathways to synthetize fatty acids in the organism. The first one is from energetic reserves (glucagon) in liver and the second one is by the fatty acids cycle regulated by the liver also. The first process is regulated by the lipids' reduction in blood, which is a faster process happening in the liver. The second one is regulated by the liver but there is not any energetic transformation in it.

When the ozone dosage was made every second day, the lipids unbalance in blood was not evident (Figure 6), that means that the liver made the regulation in order to compensate the oxidize fatty acids. Due to the high ozone's quantity present in the system, the fatty acids synthesis should be carried out by the glucagon pathway, so the lipids can be accumulated in the liver. That possible additional lipids source explains the increase in the DB-Index observed in Figure 7.

On the other hand, when the ozone was dosage less frequently, no lipids accumulation effect was observed. This fact can be a consequence of regulatory process carried on out of the liver. This fact can explain the DB-Index reduction in this case.

Therefore, the glycogenesis could be stimulated by the intermediate cell metabolisms, caused by the ozone presence dosage every 2^{nd} day, because that could improve a 33% the DB-index value. Due to the tumor volume in this group was the highest; the organic system needs produce a highest quantity of fatty acids as an energy resource to improve the tumor growth. A key factor associated with the liver samples was the reported accumulation of conjugated linoleic acid. This compound has been detected (26) in the liver when tumor cells were stressed by any possible therapy. This condition was observed in this group.

However, DB-index value decreased 50% in the group where the ozone was dosed every 5^{th} day group. Besides it was not an evident variation in the tumor volume. The fatty acid production and its accumulation in the liver were lower in this group due to the cellular death process.

This means that the ozone seems to have a promoting effect on the cell proliferation and tumor metabolism. This condition was confirmed by the increased in the tumor volume as well as the enhanced cell proliferation observed in the in-vitro experiment. However, the increase in the tumor size was not correlated with the tumor activity observed by microPET analysis, because all the tumor activities were determinate at the ending of the experiment.

We assume that after 15 days of treatment, the cell cycle was in the death stage. On the other hand, the control group, the cells continued its reproduction in a normal way.

Kidney samples showed a similar behavior to the liver samples when any of both oxidant gases were supplied to mice. However, the variation between the oxygen dose and the higher ozone dose were not as big as the liver case. This can be explained due to the indirect relationship between kidney and metabolism modification by the tumor presence.

In addition, for the comparison purposes a complementary analysis of the hydroperoxides (HP) concentrations was performed by a LPO assay kit ®. This analysis was carried out for all tissue samples described above under the different dose strategies.

Figure 8 shows the correlation between the hydroperoxides quantification and the DB-index value for the tumor tissue sample; this result confirmed the hypothesis about the inverse relationship between the HP concentrations and the corresponding DBI behavior. When the tumor metabolic activity was lowest, the DB-index decreased due to the lower disposition of double bonds in the biological substrate, because they were oxidized to form hydroperoxides. Therefore the increase of its concentrations was observed. This condition appeared in the group where the ozone was applied every 5^{th} day.

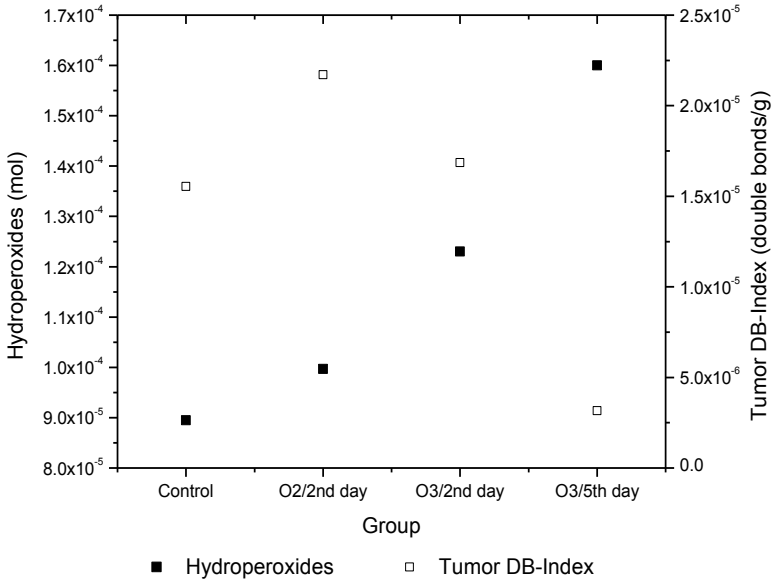

Figure 8. Comparison of hydroperoxides concentrations against the DBI for tumor tissue. n=6

Imaging studies

The figure 9 shows an example of the images obtained by microPET analysis embedded over the tomographic image. This figure is showing the signal of FDG into the mouse body. The figure demonstrates the computerized tomographic image obtained in the three different planes of exposition (top of the image). In the middle, the PET images acquired with a gamma-camera in the same planes are showed. On the bottom, the superposition of both set of images (tomographic and PET) is showed to correlate the anatomical position of the tumor activity.

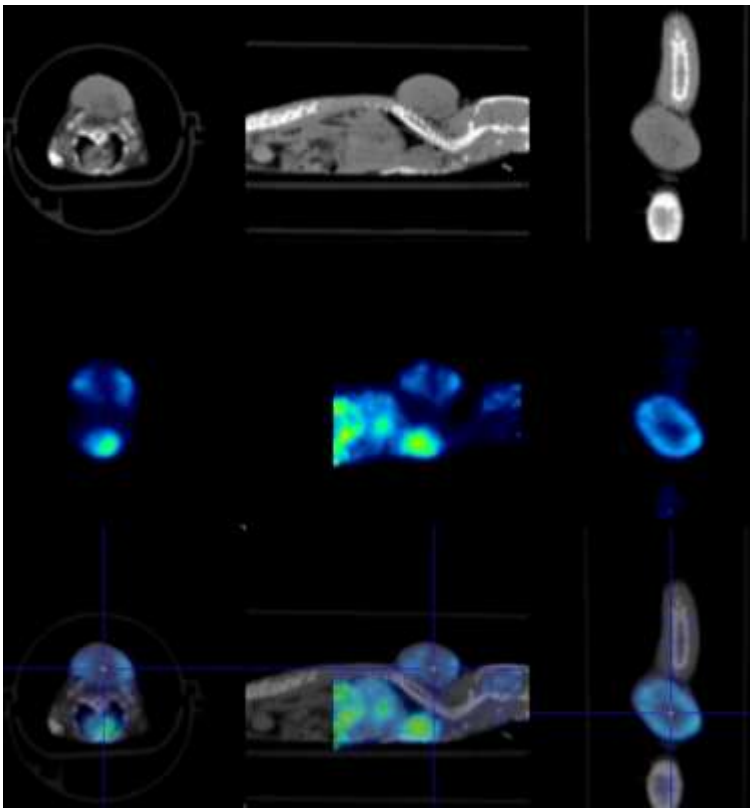

Figure 9. Example of microPET image obtained when the ozone gas dosage every 5th day

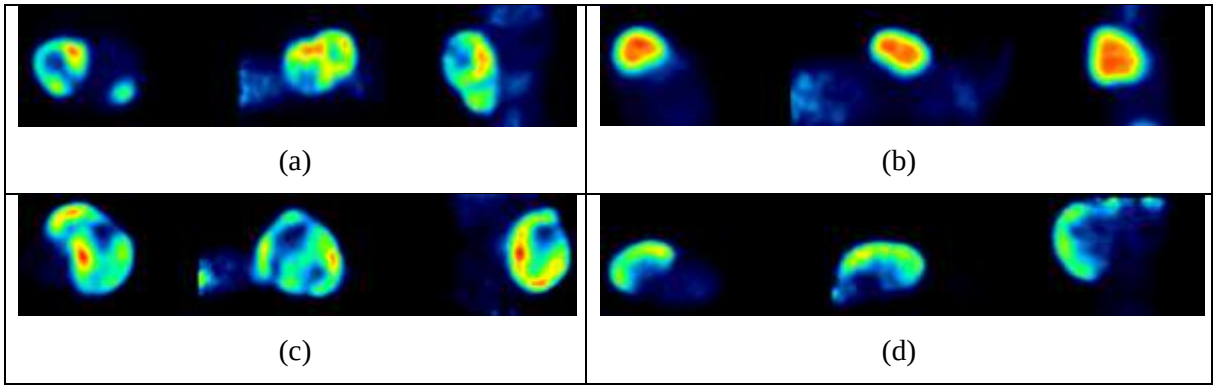

Figure 10.^{18}FDGTumor activity of the considered studied groups: control (a), only oxygen every 2nd day (b), ozone every 2ndday (c) and 5th day (d).

Specific zones within each image where tumor cell activity is higher are in light red color. On the opposite, blue regions are labeling the regions with low or null activity. In this sense, the figure 10.b, which corresponds to the oxygen dose, is showing a larger area with intense red color. This behavior corresponds to the characterization offered by the DB-index. This area is clearly larger

than the one gotten from the control group. Figures 10 c and d correspond to the subjects that were ozone dosed every 2nd and 5th day. Interestingly, the DB-index variation correlates with the tumor activity. Indeed, when lower ozone dose was supplied to mice, an important diminish of cell activity is observed (more than 80%).

All these results confirm the observation made by the DB-index analysis. Therefore, one may say that DB-index can be used to characterize the ozone dose effect on the tumor cell growing dynamics. Moreover, there was no need to increase the ozone dose to get a better effect in controlling the tumor activity. A simple analysis was done over the graphical results associated with the tumor cell activity. The ratio between the red zones (high activity) and the whole highlighted area (colored section) provided a standard and normalized way compare the activity of tumor cells in different subjects. The corresponding activity of tumor cells is shown in Figure 11 that was correlated with the DB-index variation for tumor tissue. This figure shows an evident relationship between both parameters. Actually, when the activity was lowest, the DB-index value was also decreasing as well. It confirmed, in certain way, that the DB-index could be used as a control method for treatments that involves any kind of oxidant reagent.

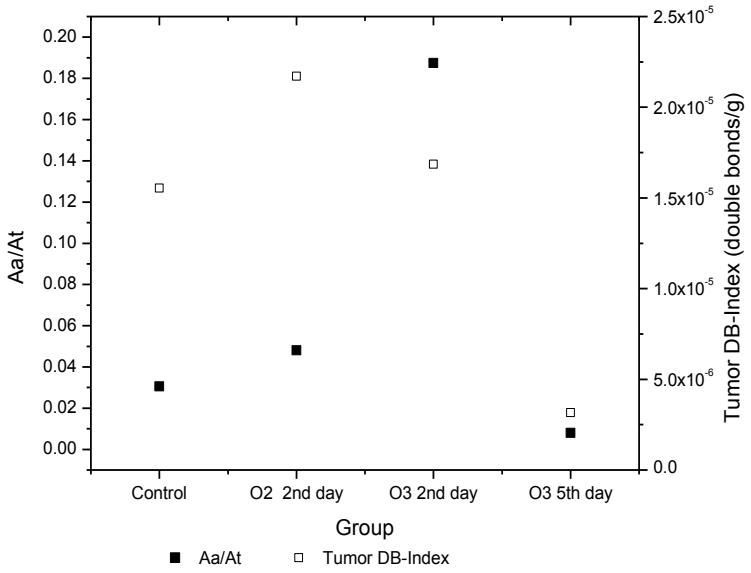

Figure 11. Relative tumor activity calculated from the MicroPet studies accordingly in the considered studied dose groups. The ordinate axis is showing the tumor activity versus DB-Index.

Glial tumors constitutes the most common group of intracranial tumors, they have fast evolution and resistance to conventional therapeutic methods such as chemotherapy, radiotherapy and

surgery. This kind of tumors is distinguished from some others by its fast mitosis, high neovascularization with endothelial hyperplasia that may cause vascular obstruction. Therefore many necrotic tissue areas can appear depending on the tumor development. These areas act as a hypoxic stimulus that induces angiogenesis (27) (28) (29).

When a continuous oxidative stress was induced by the presence of any oxidant agent (ozone), a constant ROS production was originated. That condition contributes to developing all those biologic phenomena's mentioned above. Besides of the angiogenesis stimulation, the adhesion of some integrins ($\alpha_5\beta_3$ y $\alpha_5\beta_5$) occur that improving cell proliferation. All these facts can explain why the tumor volume increases when more ozone is injected to the mice. The presence of ROS derived from the ozone injection, yields to an increment on the tumor volume by the angiogenesis and cell proliferation in the proximal region inside the tumor. However, at the same time, a faster cell necrosis may appear in the distal section of the tumor (27) (28) (29).

CONCLUSIONS

The DB-index technique has shown to be adequate as an indicator of the ozone effect on cell dynamics growth. The relationship between DB-index with cell activity and hydroperoxides quantification confirmed the possibility of characterizing the ozone dose effects. As an indirect result, the considered low ozone dose (every 5^{th} day) was more effective than higher dose (every 2^{nd} day), because improves the cell metabolism, which is confirmed by the decrease of DB-Index value for tumor and the correlation with the tumor activity index, in the case of in vivo test; and, with the DB-Index for C6 rat glioma cell culture and the DNA quantification. Noteworthy that, the chemical reaction between the NaCl and the ozone, does not generate toxic reagents [(21)] that could interfere with the results obtained.

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