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# Ozone Treatment Does Not Induce Toxicity in Human NIH3T3 Fibroblast Cells

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**SUMMARY** - *Ozone ($O_2$-$O_3$) has recently been subjected to criticism and emphasis in relation to clinical efficacy and toxicity. It is well-established that ozone is one of the most potent oxidants but its toxicity is due to the cumulative ozone dose that, day by day, elicits the formation of noxious products. This fact has established the dogma that ozone is always toxic and its medical application should be proscribed. However, ozone therapy is becoming very useful both on its own or applied in combination with orthodox medicine in a broad range of pathologies, so it is important to clarify this controversial issue. In order to understand the problem of the toxicity induced by ozone, the aim of the present study was to evaluate whether $O_2$-$O_3$ treatment could have any toxic effect on the human NIH3T3 fibroblast cell line. We found that $O_2$-$O_3$ did not induce toxicity by using the appropriate dose in contrast with the established dogma that ozone is always toxic and its medical application should be proscribed.*

## Introduction

Ozone ($O_2$-$O_3$) has recently been subjected to criticism and emphasis in relation to its clinical efficacy and toxicity, respectively. It is well-established that ozone is one of the most potent oxidants [1], but its toxicity is due to the cumulative ozone dose that, day by day, elicits the formation of noxious products. This fact has established the dogma that ozone is always toxic and its medical application should be proscribed.

This dogma has been reinforced by prejudice, pharmaceutical interests and a lack of knowledge of the mechanisms of its action [2]. Ozone, tenfold more soluble than oxygen, reacts immediately with a number of biomolecules (antioxidants and polyunsaturated fatty acids) present in plasma and within a few minutes disappears thus generating a calculated amount of hydrogen peroxide (ROS) and lipid oxidation products (LOPS). By binding with or diffusing into cells, these products activate well-defined biochemical pathways [3-7] such as the activation of specific lipases, which trigger the release of endogenous mediators [8].

However, ozone therapy is becoming very useful both on its own or applied in combination with orthodox medicine in a broad range of pathologies, and thus it appears important to clarify this controversial issue [2]. It has been shown that ozone therapy is very useful in the following diseases: chronic osteomyelitis, pleural empyema, abscesses with intractable fistulae, infected wounds, bed sores, chronic ulcers and initial gangrene, necrotizing fasciitis, diabetic foot, skin, mouth, vaginal and rectal bacterial and viral infections and burns [4,5,7]; advanced ischemic diseases (peripheral obstructive arterial disease and heart ischemia) [9]; age-related macular degeneration (atrophic form only) [10]; lumbar and cervical herniated discs as well as localized osteoarthrosis [11]; dentistry, regarding primary root carious lesions, particularly in children [3].

In spite of being a very reactive molecule, ozone is not always as toxic as it has been supposed. Clinical experiences have demonstrated that an appropriate ozone dose in contact with blood for a few minutes ex vivo activates several biochemical pathways in erythrocytes, leukocytes and platelets without eliciting any acute or chronic toxicity [2]. In fact it has been reported that $O_2$-$O_3$-induced toxicity is overcome by using the appropriate dose. Indeed, the range of the therapeutic window has been determined between 20 and 80 µg/ml per ml of blood cells which did not produce any toxic effect. In order to understand the problem of the toxicity induced by ozone, the aim of the present study was to evaluate whether $O_2$-$O_3$ treatment could have any toxic effect on the human NIH3T3 fibroblast cell line.

## Materials and Methods

### *Cell culture*

Human NIH3T3 fibroblast cell line was maintained as a monolayer culture in DMEM supplemented with 10% heat-inactivated FCS (Life Technologies, Scotland, United Kingdom), antibiotics and L-glutamine (2 mM) at 37°C in a 5% $CO_2$/95% air atmosphere in a humidified incubator.

### *Ozone generation*

Ozone therapy was administrated by using clinical grade $O_2$, the $O_2$-$O_3$ gas mixture was prepared with an ozonosan α plus photonic device 1014/10 (Dr. Hansler, Germany) and sterilized by passage though a sterile 0.2 μm filter.

### *Treatment and viability*

Cells were seeded at a density of $1.5\times10^5$ cells *per* T25 flask and after 24 h of cell culture were treated at room temperature using an ozonosan α plus photonic device. Different $O_2$-$O_3$ doses (10 μg/ml, 25 μg/ml and 50 μg/ml) were tested as a single-dose regimen. After $O_2$-$O_3$, the flasks were immediately put back into the incubator and cell viability, evaluated as trypan blue exclusion test, was determined daily from day 2 (24 h after treatment) to day 6 (120 h after treatment) of culture. Data were evaluated as percentages of control (i.e., absolute treated cell number/absolute control sample). All the experiments were repeated four times and each experimental sample was seeded in triplicate.

### *Cell cycle analysis*

The cell cycle was studied using Propidium Iodide (PI) staining. PI stained treated and untreated cells were harvested, washed in cold PBS, fixed in 70% ethanol for at least one hour, and, after removing alcoholic fixative, stained with a solution containing 50 μg/ml PI (Sigma Chemical) and 75 KU/ml RNase (Sigma Chemical) in BPS for 30 minutes at room temperature in the dark. Samples were then measured using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA, USA).

## Results

### *Effects on cell growth after treatment on the NIH3T3 fibroblast cell line*

In a previous article[12] we investigated the role of $O_2$-$O_3$ as an antineoplastic drug in neuroblastoma cell lines. The present work examined the effects produced on cell growth and cell cycle at different times after $O_2O_3$ exposure (24, 48, 72, 96 and 120 h) on the NIH3T3 fibroblast cell line. We exposed cells to 10, 25 and 50 μg/ml $O_2$-$O_3$ doses. At each time, cells were harvested and counted using the trypan blue dye exclusion test.

Cells exposed to the lowest $O_2$-$O_3$ dose of 10 μg/ml did not show any significant effect on cellular growth: the growth inhibition was about 10% at each time, whereas growth inhibition of 40% was obtained after 24 h (day 2) of exposure to 25 μg/ml $O_2$-$O_3$. This latter effect was partially lost during the following days, with a cell growth inhibition value of 24% evaluated 120 h (day 6) after treatment. Twenty-four hours after exposure of cells to 50 μg/ml $O_2$-$O_3$ dosage, a cell growth inhibition of 50% was observed. The inhibitory effect became 77% after 120 h (figure 1).

### *Cell cycle profile after treatment*

To evaluate the effect of $O_2$-$O_3$ on the cell cycle profile, PI staining and fluorescence-activated cell sorting analysis were done at different times on fibroblast cells exposed to 10, 25 and 50 μg/ml $O_2$-$O_3$. The relative number of cells in each phase of the cell cycle was estimated by CELL Quest software analysis. As shown in figure 2, no detectable changes were evident in the cell cycle distribution in the samples treated with the 10 μg/ml $O_2$-$O_3$ dose with respect to control cells. This finding was consistent with cell growth number evaluation, which failed to disclose significant cell growth inhibition. On the contrary, the dosage of 25 μg/ml $O_2$-$O_3$ induced an accumulation of the cells (43%) in the G2 phase of the cell cycle, compared with control cells (14%) 24 h after treatment. As shown in figure 2, the G2 accumulation was overcome during the following hours (19% at 120 h), suggesting that cells treated with the 25 μg/ml $O_2$-$O_3$ dosage were able to recover from the $O_2$-$O_3$-induced G2 block. On the contrary, after 50 μg/ml $O_2$-$O_3$ treatment, we observed a G2 accumulation of about 46% at 24 h but this accumulation was still evident on the following time points (25% at 120 h versus 12% of the control), indicating that this dosage delayed NIH3T3 cell proliferation.

### *$O_2O_3$ does not have a toxic effect on the NIH3T3 fibroblast cell line*

As shown in figure 3, the 10 μg/ml $O_2$-$O_3$ dosage did not show any toxicity together with the absence of any effects on cell growth and the cell cycle. Moreover, we did not observe any toxicity in the cells treated with the 25 μg/ml $O_2$-$O_3$ dosage. On the other hand, in spite of the antiproliferative effect observed after 50 μg/ml $O_2$-$O_3$ dose, this higher dose did not produce any toxicity: in fact a high viability ranging from 96% to 99% was detected at all time points.

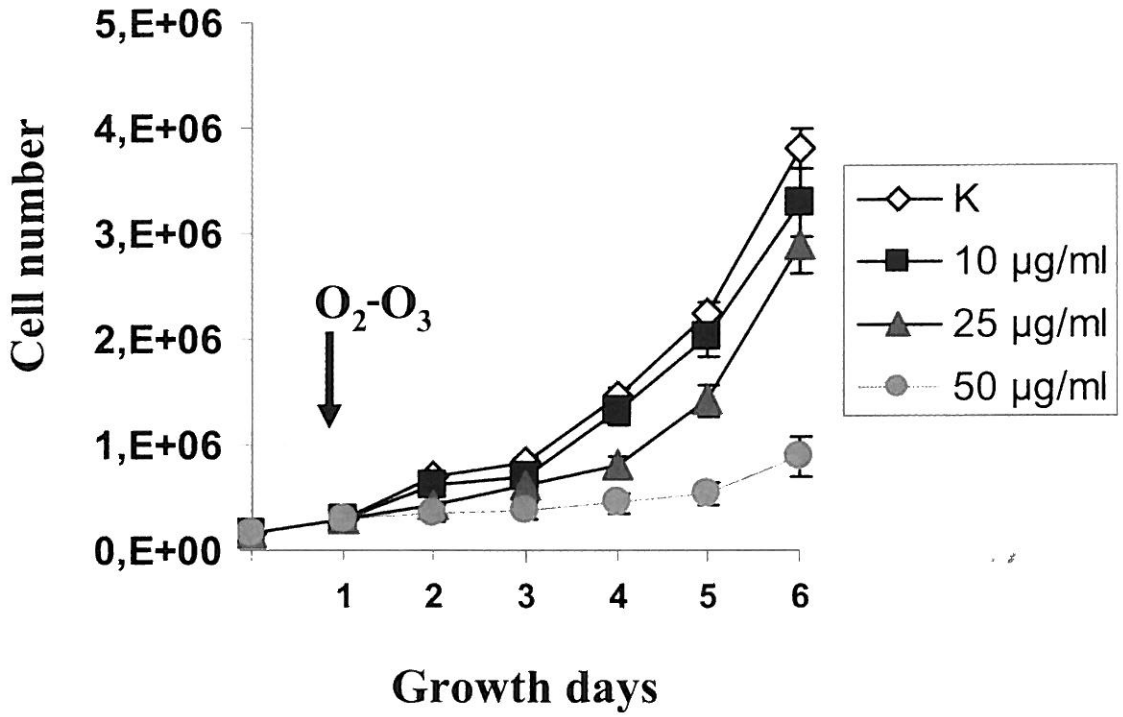


Figure 1 Cell growth number in NIH3T3 cells. At the indicated time after treatment, cells were harvested and counted using a trypan blue dye exclusion test. The arrow indicates the start of $O_{2/3}$ treatment. Statistical significance of the data was evaluated by two-way analysis of variance (ANOVA) followed by Tunnet post hoc test.

## Discussion

Ozone is the allotropic form of oxygen with three atoms and two unpaired electrons, which has more oxidizing capacity than oxygen [13]. A review on the potential role of ozone therapy as a biological response modifier in oncology has been published by Bocci [14]. In a previous work, we demonstrated that $O_2$-$O_3$ affects cell growth and the cell cycle in the neuroblastoma SK-N-SH cell line, suggesting that ozone can act as a possible antineoplastic drug because of its capacity to inhibit the growth of human cancer cells [15]. However, there have been several arguments for prohibiting the use of ozone in medicine: the first is that ozone is a strong oxidant and a toxic gas that should never be inhaled. The second is the fact that several diseases are perpetuated by chronic oxidative stress, and therefore a gas generating free radicals should be proscribed. The third is the fault of unscrupulous quacks who, without any medical qualification, have injected the $O_2$-$O_3$ gas mixture intravenously causing lung embolism. Thus, it has been easy to label ozone therapy as dangerous quackery [16].

Nowadays ozone is generally used to treat disc herniation and the so-called nerve-root conflict, and its action is bound to the effect on the biohumoral environment. Today the success of ozone therapy depends on using low ozone dosages able to stimulate a number of biochemical pathways, ultimately responsible for the activation of the natural healing capacity without inducing toxic effects. The application of ozone in disc herniation is considered scientific and successful thanks to the possibility of statistically estimating the reduction or disappearance of the anatomic protrusion. However, an adequate follow-up considering the status of the patient following treatment could better indicate the efficacy of the ozone treatment compared to different methodologies such as paravertebral, intradiscal, epidural or intraforaminal injections. Moreover, the huge variability of the clinical responsiveness of patients introduces further difficulties in the establishment of standardized studies [1].

Since $O_2$-$O_3$ has been used in such diverse specialties as neurology, orthopaedics, internal medicine, sports medicine, endocrinology, and others, it is difficult to categorize ozone as a therapeutic agent. In the present study we used $O_2$-$O_3$ treatment on normal human NIH3T3 fibroblast cell line to investigate the possible toxic effect of this gas mixture on healthy cells. The data reported herein provide experimental evidence that no significant cell growth inhibition was revealed after 10 µg/ml and 25 µg/ml $O_2$-$O_3$ dosages. By contrast, we observed consistent cell growth inhibition values after the 50

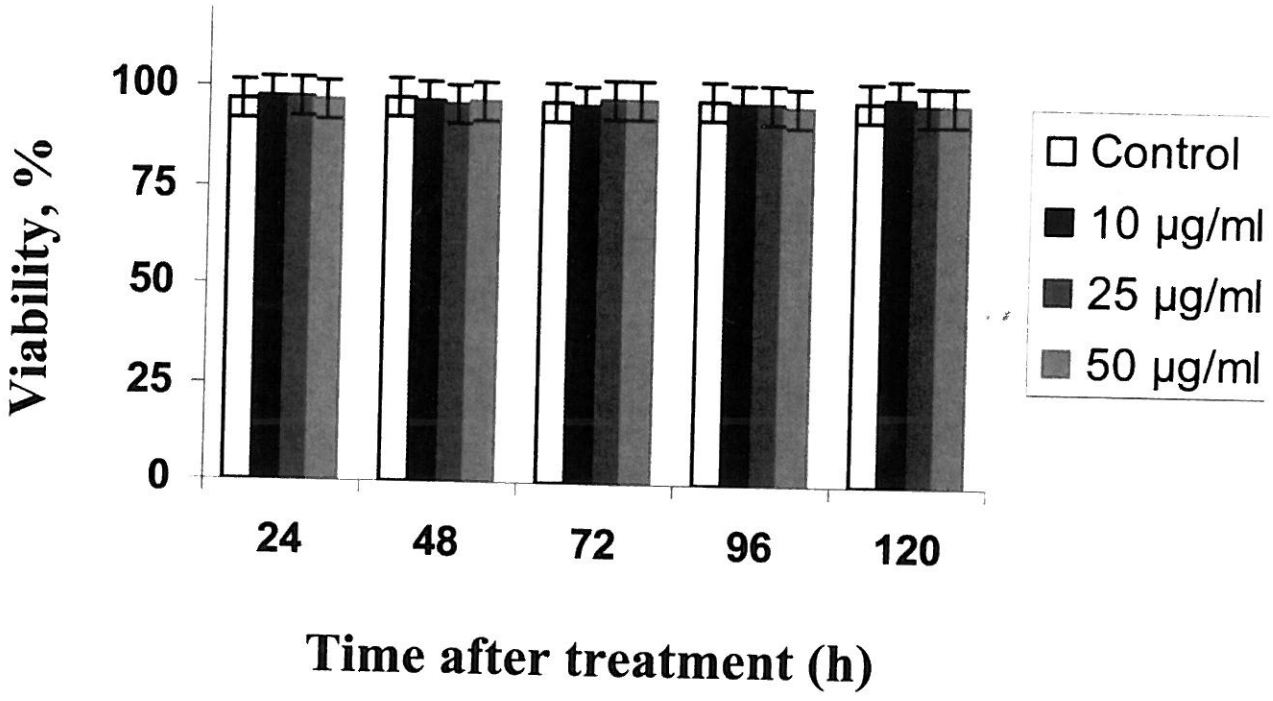


Figure 3 Toxicity after each treatment was determined by trypan blue exclusion test. Statistical significance between the different groups was evaluated by Student's t-test ($P< 0.05$).

in normal signal-transduction processes so that there is a mayor entry of $O_2$-$O_3$ into neoplastic cells with respect to normal ones. On the other hand, Matsuoka et Al [19] also showed the different response between neoplastic and normal cells after paclitaxel treatment. In accordance with Bocci et Al [12] we did not observe any toxic effect after $O_2$-$O_3$ treatment *in vitro* on the NIH3T3 cell line. On the other hand, Re et Al [1] showed that low ozone doses did not produce any risk of toxicity on an *in vivo* Parkinson's model. Clinical experience has demonstrated that an appropriate ozone dose (therapeutic range 10-50 µg/ml) in contact with blood for a few minutes ex vivo activates several biochemical pathways in erythrocytes, leukocytes and platelets without eliciting any acute or chronic toxicity [2]. We conclude that $O_2$-$O_3$ did not induce toxicity by using the appropriate dose in contrast with the established dogma that ozone is always toxic and its medical application should be proscribed.

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