Free Radical Research, Volume 37 Number 11 (November 2003), pp. 1163–1168

Taylor & Francis
healthsciences

The Ozone Tolerance: I) Enhancement of Antioxidant Enzymes is Ozone Dose-dependent in Jurkat Cells

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Accepted by Professor A. Azzi

(Received 28 April 2003; In revised form 9 June 2003)

We have begun to examine the biological and toxic effects of ozone on Jurkat T cells incubated thereafter for 24, 48 and 72 h. Tissue culture medium was strengthened by adding 20% fetal calf serum with an albumin content of about 6 mg/ml. Ozonization was performed by exposing for 10 min a volume of cell suspension (4×10^5/ml) to an equal volume of a gas mixture composed of oxygen–ozone with precise ozone concentrations ranging from 1.5 up to 72 μg/ml (31.5–1512 μM). The proliferation index declined progressively and was ozone dose-dependent. The response of enzymatic activities varied depending upon the enzyme and ozone concentrations: glucose-6-phosphate dehydrogenase begins to increase at an ozone dose of 6 μg/ml (126 μM), reached a peak at 12 μg/ml (252 μM) and rapidly declined thereafter. On the other hand activities of superoxide dismutase, glutathione peroxidase and glutathione reductase increased progressively from the ozone concentration of 12 μg/ml. Thus, as we have observed in blood, the biological response is linked to the ozone dose that must reach a threshold to be effective.

Keywords: Jurkat T cells; Ozone; Antioxidants; Ozonetherapy

INTRODUCTION

The toxicity of ozone has originated a real dilemma in biology and medicine: on one side there is the dogma that ozone is always toxic[1] and on the other that precise and judicious use of ozone can be therapeutically useful.[2,3] What is the evidence supporting the dogma?

(a) There is no doubt that the bronchial-pulmonary system is very sensitive to ozone and this gas should never be inhaled.[4]

(b) Saline-washed erythrocytes resuspended in saline undergo hemolysis after ozone exposure.[5]

(c) Cells cultured in antioxidant-poor media, exposed for days even at very low ozone concentrations, owing to a cumulative toxic effect, undergo apoptosis.[6]

(d) Similarly, leukocytes present in saline-diluted blood exposed to 5 mM ozone for 1 h display some genetic damage.[7]

All of these data are important but they show that ozone toxicity is exerted when cells are incubated in antioxidant-free or poor culture media. Obviously this fact deeply limits their physiological and practical significance. The only exception is the pulmonary system that for anatomic, biochemical and metabolic reasons is always at a loss against ozone. In contrast blood is a far more ozone-resistant tissue and we have proved that hundreds of human blood samples exposed for 5 min to ozone concentrations ranging from 20 μg/ml (0.42 mM) up to 60 μg/ml (1.26 mM) do no show any damage.[3] Moreover, the total antioxidant status (TAS), normally in the range of 1.3–1.8 mM decreases only of no more than 20% during the first 5 min and returns to the original value after 30 min clarifying that exposure to ozone causes only a transitory and partial depletion of antioxidants.[3]

The last but not least important fact is that, during the last three decades, millions of ozonized autohemotherapy (O_3-AHT) have been performed in patients without yielding neither acute, nor chronic toxic effects.

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000160417O

Dogmas can be double-edged swords and, if wrong, delay scientific progress. Thus, the question is if ozone is always toxic? After a decade of experimental and clinical work we have come to regard ozone as a real drug able to activate several biological effects (reviewed in Ref. [3]). As any other chemical drug, ozone displays a fairly wide therapeutic window below, which there is no activity and above which there are toxic effects. Moreover, a very interesting property is that ozone can slowly induce the upregulation of antioxidant enzymes and the expression of oxidative stress proteins.

In this paper we have begun to examine this paradoxical phenomenon in cultured Jurkat cells by briefly exposing them to different ozone doses and examining antioxidant enzymatic activities up to 72 h after ozonation.

MATERIALS AND METHODS

Ozone Generation and Measurement

O_3 was generated from medical-grade O_2 using electrical corona arc discharge, by the O_3 generator (Model Ozonosan PM 100K, Hansler GmbH, Iffezheim, Germany), which allows the gas flow rate and O_3 concentration to be controlled in real time by photometric determination, as recommended by the Standardisation Committee of the International O_3 Association. Tygon polymer tubing and single-use silicon treated polypropylene syringes (ozone resistant) were used throughout the reaction procedure to ensure containment of O_3 and consistency in concentrations.

Cell Culture

Jurkat human T lymphoma cells (ECACC, UK) were cultured in RPMI 1640 medium containing 20% of heat-inactivated FCS, supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Sigma Co.). Cells were incubated at 37°C in air, 5% CO_2 atmosphere at concentrations between 2×10^5 and 1×10^6 cells/ml and adjusted to a final concentration of 4×10^5 cells for ozonization.

O_3 Delivery to Biological Samples

Predetermined volumes of O_2—O_3, at various concentrations, were collected with a syringe and immediately introduced into the second syringe containing the samples *via* a multidirectional stopcock. We always use a cell suspension/gas volume at a 1:1 ratio. In order to obtain reproducible results, it needs to be emphasized that O_3 is a very reactive gas so that extremely rapid and precise handling is required. The final gas pressure remained at normal atmospheric pressure. Samples were gently but continuously mixed with the gas mixture at different O_3 concentrations (1.5, 3, 6, 12, 24, 36 and 72 μg/ml per ml of cells corresponding to 31.5, 63, 126, 252, 504, 1008 and 1512 μM, respectively) for 10 min. We have previously ascertained that during this period of time ozone reacts completely with substrates implying that cell samples receiving ozone react with the ozone dose totally. Control samples were either not treated or mixed with an equal volume of pure medical O_2. It is worth mentioning that O_2 represents at least 96% of the O_2—O_3 mixture. After ozonization cells were again cultured at 37°C in air, 5% CO_2 atmosphere for 24, 48 and 72 h.

Cell Proliferation

Cell proliferation was evaluated by a colorimetric immunoassay (Boehringer Mannheim, Mannheim, Germany) based on BrdU incorporation. Briefly, after 24, 48 and 72 h of incubation at 37°C in air-CO_2 (5%) and 100% humidity, the cells were labeled with BrdU for 3 h (10 UI/well). The cells were then fixed, anti-BrdU-POD antibody was added and the immune complexes were detected by the subsequent substrate reaction. The proliferative index (PI) was obtained by calculating the ratio between either O_2 or O_2—O_3 treated cells and the unstimulated ones, after subtraction of the corresponding blanks.

The cells viability was assayed by the Trypan blue exclusion technique and light microscope observation. Viable cells were counted with a hemocytometer.

Biochemical Determinations

Glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd) and superoxide dismutase (SOD) were measured with spectrophotometric Randox test kits (Randox Laboratories Ltd., UK) and reported as U/10^6 cells. Briefly, GSH-Px was measured according to Paglia and Valentine's method;[8] GSH-Rd according to Melissinos' method;[9] SOD activity was determined by the inhibition of *p*-iodonitrotetrazolium (INT) reduction due to the O_2^- generated by the combination of xanthine and xanthine oxidase. Glucose-6-phosphate dehydrogenase (G6PDH) was detected with a Sigma spectrophotometric kit (Sigma Diagnostic), according to a modification of the spectrophotometric method of Kornberg and Horecker and Lohr and Waller,[10,11] and expressed as U/10^{12} cells.

Protein thiol groups (PTG) were measured according to Hu[12] using procedure 1 with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) dissolved in absolute methanol. Values are expressed as mM.

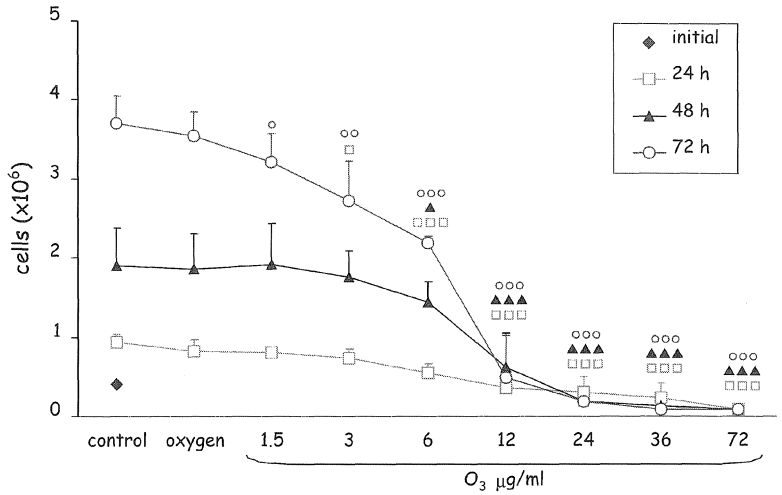

FIGURE 1 The diagram shows that the number of Jurkat T cells during incubation times of 24, 48 and 72 h varies in relation to the ozone concentrations (from 1.5 up to 72 µg/ml) to which cells were exposed for 10 min at time zero. Control (untreated cells), oxygen (oxygenated cells) and increasing ozone concentrations from 1.5 to 72 µg/ml.

Statistical Analyses

Results were expressed as the mean ± the standard deviation of the mean (SD) and the data were analyzed using the Student's *t*-test. *p* Values less than 0.05 (*), 0.01 (**) and 0.001 (***) were considered significant. Either the oxygenated sample or the treated ones (O_2—O_3) were compared to the untreated sample (control).

RESULTS

Our aim was to investigate how Jurkat cells suspended in RPMI medium fortified with 20% FCS reacted after being exposed for 10 min to a gas mixture composed of oxygen–ozone, with ozone concentrations ranging from 1.5 µg/ml of gas (31.5 µM) up to 72 µg/ml (1.51 mM) per ml of cell suspension against two controls of which one was not exposed to any gas (control) and one exposed to oxygen only. After the 10 min period of gas exposure, cells were incubated against air-CO_2 for 24, 48 and 72 h. After each of these periods live cells were counted (Fig. 1): control cells proliferated abundantly and from 4×10^5 cells at zero time, their number increased up to $9.3 \times 10^5 \pm 0.1$, $1.9 \times 10^6 \pm 0.5$ and $3.7 \times 10^6 \pm 0.3$ after 24, 48 and 72 h, respectively. Oxygen-treated cells proliferated slightly less but ozone-treated cells showed a decline after being exposed to 3 (63 µM) and 6 µg/ml (126 µM) that became striking at ozone concentration of 12 µg/ml (252 µM). After ozone exposure of 12 µg/ml (1.51 mM) there was a significant block of proliferation. The proliferation index is reported in Fig. 2 and shows highly significant decrements already after 24 h incubation for cells ozone-exposed at 3 µg/ml. The diagram shows that a cell fraction to remains viable at 72 h even after being exposed at the highest ozone concentration.

Another purpose of this study was to examine if Jurkat cells, after ozone exposure, will modify the activity of typical antioxidant enzymes. Data for

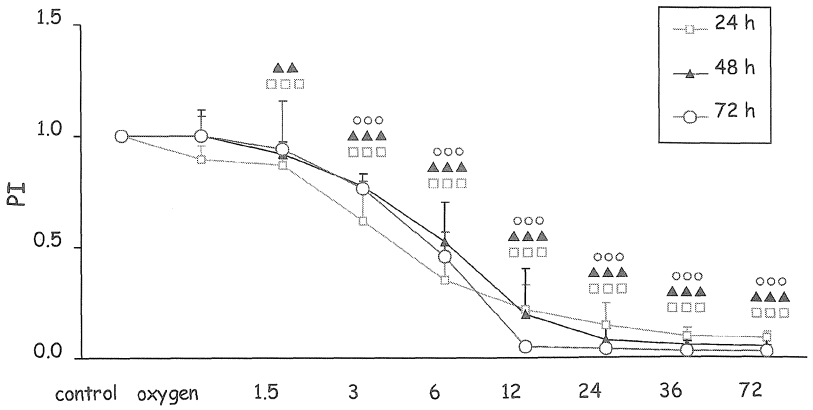

FIGURE 2 The diagram shows the proliferation index (PI, O_2 and O_3 exposed Jurkat T cells/control cells) after 24, 48 and 72 h of incubation in relation to the progressive increase of ozone concentrations (from 1.5 up to 72 µg/ml).

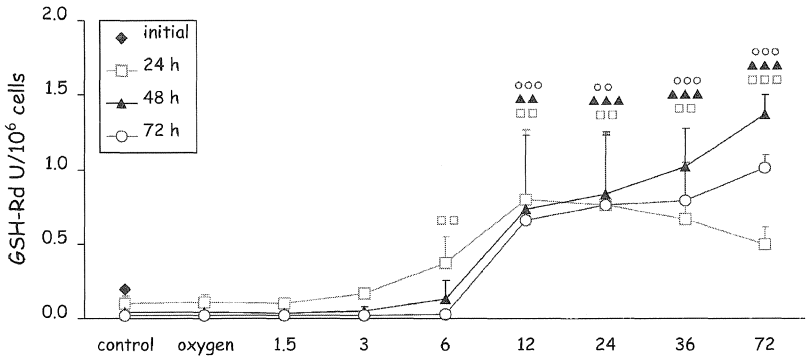

FIGURE 3 The diagram shows that the ozonized Jurkat T cells display an increased SOD activity in relation to the progressive increase of ozonization (from 6 up to 72 μg/ml). The maximum activity is reached after 72 h of incubation. Symbols are the same of Fig. 1.

SOD, GSH-Px and GSH-Rd are reported in Figs. 3–5, respectively. The activity of these three enzymes significantly increases in relation to the increase of ozone concentration from 12 to 72 μg/ml and is particularly evident for SOD and GSH-Px. The pattern for GSH-Rd was somewhat anomalous because, although it rose already after 24 h, a frank upregulation was observed only after 48 and 72 h. This delay was consistently recorded in several experiments.

At variance with these enzymes, the pattern of G6PDH (Fig. 6) did show a statistically significant increase only when cells had been treated with an ozone concentration of 12 μg/ml. Thus, it appears that very low (1.5 and 3 μg/ml) ozone concentrations were ineffective, while the intermediate ones (6 and 12 μg/ml) induced the enzymatic upregulation. Above these levels ozone concentrations were possibly toxic and inhibitory.

DISCUSSION

During the last 7 years at the University Ozonetherapy Center, we have carried out some 6000 O_3-AHT consisting in exposing for 5–10 min, with gentle mixing, 225 ml of blood plus 25 ml Na Citrate 3.8% to 225 ml of O_2–O_3 with an ozone concentration ranging from 20 μg/ml (420 μM) up to 60 μg/ml (1.26 mM) followed by a rapid infusion into the donor.[3] The treatment is particularly useful in age related macular degeneration and in chronic limb ischemia (stage II and III) when orthodox therapies are no longer valid. O_3-AHT consists in a precisely calculated acute oxidative stress that does not procure any cell damage because the blood antioxidant system is able to quench the oxidant activity of ozone. Blood ozonization is performed *ex vivo* and already after 10–15 treatments, most of the patients show the remarkable phenomenon of ozone tolerance, i.e. the ozonized blood stimulates some of the enzymatic antioxidant defenses in the patients.[3,13]

In order to better understand the phenomenon, we thought worth while to evaluate an *in vitro* model using Jurkat T lymphoma cells thus avoiding the great variability of human samples and individual responses as well as the problem of separating a pure lymphocytic cell population. Moreover, Jurkat T cells have shown already to be able to stimulate within 48 h the activity of G6PDH after pretreatment with concentrations of dehydroascorbate ranging from 200 up to 800 μM.[14]

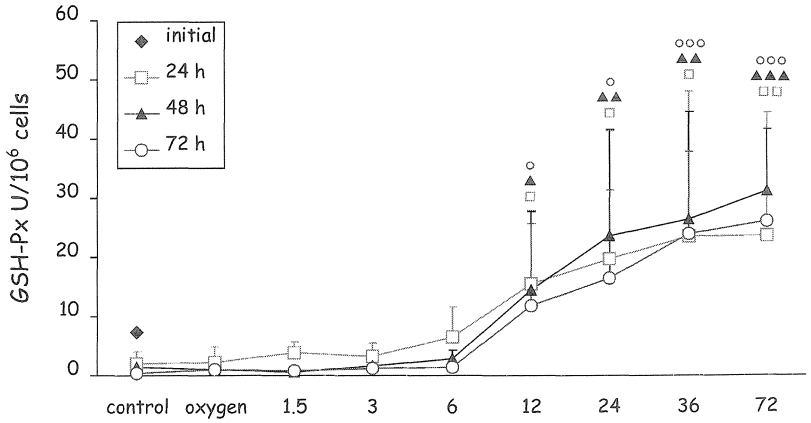

FIGURE 4 The diagram shows that ozonized Jurkat T cells display an increased GSH-Px activity in relation to the progressive increase of ozonization (from 6 up to 72 μg/ml). The maximum activity is reached after 48 h of incubation. Symbols are the same of Fig. 1.

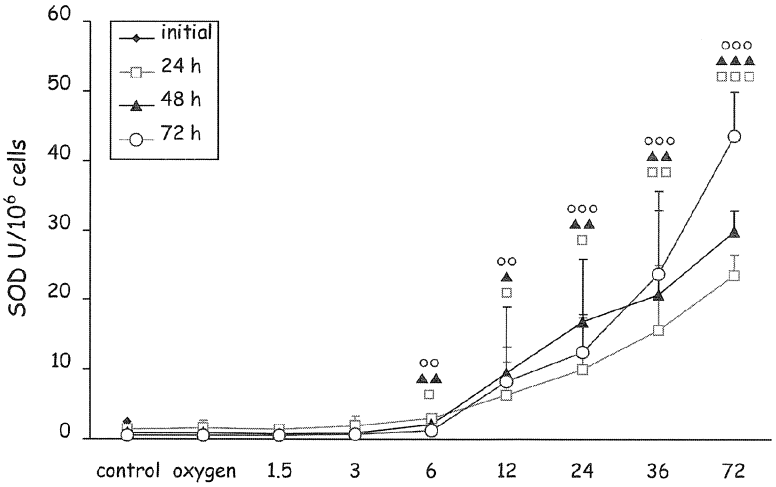

FIGURE 5 The diagram shows that ozonized Jurkat T cells display an increased GSH-Rd activity in relation to the progressive increase of ozonization (from 6 up to 72 μg/ml). The maximum activity is reached after 48 h of incubation. Symbols are the same of Fig. 1.

This interesting result[14] stimulated us to test the reactivity of these cells to ozone which has also acted as an inducer of enzymatic activities.

In comparison to blood having an hematocrit of about 45% and an albumin content of about 20 mg/ml of blood, Jurkat cells are usually cultured in RPMI 1640 medium (hence at a very low hematocrit value) supplemented with 10% fetal calf serum containing only about 3 mg albumin. Furthermore, FCS has a markedly lower antioxidant capacity than fresh human plasma (PTG values are usually as low as 0.07 mM in cell suspension while are about 0.4 mM in human plasma). For these reasons in our study we have increased the FCS concentration to 20% that is still less protective against the ozonation acute stress than human plasma. This consideration can explain why after 72 incubation, Jurkat T cells suspension exposed to 126 μM ozone (6 μg/ml) for 10 min show a highly significant decrease of proliferation (Fig. 2) in comparison to control and it is noteworthy that a fraction of original cells survive after exposure to the maximum ozone concentration (1.51 mM). On the other hand at the same ozone concentration neither hemolysis, nor release of lactic dehydrogenase has been detected in plasma after blood ozonation *ex vivo*.

The evaluation of enzymatic activity of typical antioxidant enzymes has yielded interesting, although unexpected, results. We have observed a fairly linear increase of activity for SOD, GSH-Px and GSH-Rd in relation to the progressive increase of ozone concentration (Figs. 3–5) while for G6PDH there is only a peak of activity at an ozone concentration of 12 μg/ml (252 μM) (Fig. 6).

A reasonable explanation of our data is that, similar to blood, if the Jurkat cell suspensions are not exposed to an ozone concentration just above the total antioxidant threshold, no biological effect ensues. Our previous work[3] has shown that ozone dissolved in the plasmatic water instantaneously reacts with biomolecules (polyunsaturated fatty acids, albumin thiol groups, cysteine, etc.) and disappears generating reactive oxygen species

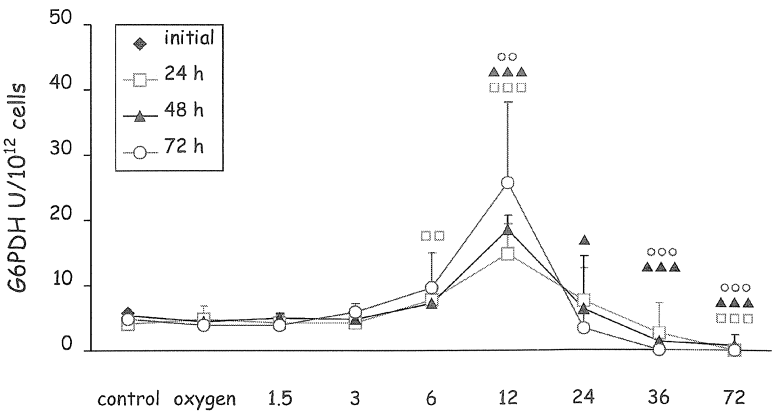

FIGURE 6 The diagram shows that ozonized Jurkat T cells display an increased G6PDH activity after undergoing ozonization at 12 μg/ml. The maximum activity is reached after 72 h of incubation. Symbols are the same of Fig. 1.

(ROS) among which the most relevant is hydrogen peroxide (H_2O_2) and lipid oxidation products (LOP). Only when a concentration of about 10–30 μM H_2O_2 is formed, H_2O_2 freely enters into the cells and activates the glycolytic and the pentose phosphate pathway as well the phosphorylation of IKBs. However, in a matter of seconds, intracellular H_2O_2 is reduced to H_2O by a combination of antioxidants such as reduced glutathione, catalase and GSH-Px.

The increase of the antioxidant enzymatic activities, mostly likely due to similar mechanisms, appears to have reached the threshold value between 6 μg/ml (126 μM O_3) and 12 μg/ml (252 μM O_3). While for G6PDH ozone concentrations from 24 μg/ml (504 μM O_3) upwards are inhibitory, they are stimulatory for the other three enzymes. However, in comparison to blood, these ozone concentrations are low owing to the low antioxidant capacity of the medium even if fortified with 20% FCS.

We do not yet know whether this differential increase of enzymatic activities is due to a cell subfraction constitutively more resistant to the acute oxidative stress or to a prevalent effect of ROS with a half-life of a few seconds (H_2O_2) or to LOP particularly 4-hydroxy-2,3-*trans*-nonenale. Work in progress may clarify this aspect. A final comment is that also the model of Jurkat T cells suspended in fairly poor antioxidant medium has shown that the effect of ozonation is not too negative provided that ozone concentrations do not overwhelm the antioxidant capacity.

Acknowledgements

This research was partially financed by MURST grants (60% local funds). We are grateful to Mrs Helen Carter for linguistic revision and editorial assistance.

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