AF12040021

# Inhibition of Tumor Necrosis Factor-alpha Release during Endotoxic Shock by Ozone Oxidative Preconditioning in Mice

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## Summary

**Ozone oxidative preconditioning is a prophylactic approach, which favors the antioxidant-prooxidant balance for preservation of the cell redox state by increasing antioxidant endogenous systems in both in vivo and in vitro experimental models. The aim of this study was to analyze the effect of ozone oxidative preconditioning on the level of tumor necrosis factor-alpha (TNF-α) in the serum of mice treated with lipopolysaccaride (LPS). Pretreatment with an ozone/oxygen gaseous mixture was administered intraperitoneally (0.2, 0.4 and 1.2 mg/kg) or by rectal application (0.2 and 0.4 mg/kg) once daily during five days before LPS (0.1 mg/kg, intraperitoneal). TNF-α was measured by cytotoxicity on L-929 cells. One hour after LPS injection, a significant mean increase of TNF-α in mouse serum was observed. Ozone/oxygen gaseous mixture reduced serum TNF-α levels in a dose-dependent manner. Statistically significant decreases in TNF-α levels after LPS injection were observed either with ozone intraperitoneal applications at 0.2 (78 %), 0.4 (98.5 %) and 1.2 (98.6 %) mg/kg or by rectal application at 0.2 (46.2 %) and 0.4 (97.4 %) mg/kg. These results in-**

dicate that ozone oxidative preconditioning inhibits TNF-α production.

## Zusammenfassung

**Hemmung der Freisetzung von Tumor-Nekrose-Faktor-alpha während des endotoxischen Schocks durch Vorbehandlung mit einem Ozon/Sauerstoff-Gasgemisch**

Die oxidative Vorbehandlung mit Ozon ist ein prophylaktisches Verfahren, welches das antioxidante, bzw. prooxidante Gleichgewicht zur Erhaltung des Redoxstatus der Zellen beeinflussen kann und weil auch die antioxidativen endogenen Systeme sowohl in In-vivo- als auch In-vitro-Modellen aktiviert werden können.

Das Ziel dieser Studie war die Untersuchung der Wirksamkeit nach oxidativer Vorbehandlung mit dem Ozon/Sauerstoff-Gasgemisch auf den Lipopolysaccharid (LPS)-induzierten TNF-α-Wert im Serum von Mäusen. Das Ozon/Sauerstoff-Gemisch wurde täglich über einen Zeitraum von 5 Tagen intraperitoneal (0.2, 0.4 und 1.2 mg Ozon/kg) oder rektal insuffliert (0.2, 0.4 mg Ozon/kg). Danach wurde LPS (0.1 mg/kg) intraperitoneal appliziert. Daraufhin wurden die TNF-α-Spiegel anhand der Zytotoxizität an L 929-Zellen gemessen. Eine Stunde nach der LPS-Injektion wurde bei Kontrolltieren ein signifikanter Anstieg des TNF-α-Spiegels im Serum der Maus beobachtet. Das Gasgemisch von Ozon/Sauerstoff reduzierte dosisabhängig die TNF-α-Spiegel im Serum. Statistisch gesehen konnte eine deutliche Reduktion der TNF-α-Spiegel (%) nach der LPS-Applikation beobachtet werden, sowohl in den Fällen nach intraperitonealer Ozon-Insufflation (0.2 mg/kg: 78 %), 0.4 mg/kg: 98.5 %) und 1.2 mg/kg: 98.6 %) als auch nach rektaler Insufflation (0.2 mg/kg: 46.2 %, 0.4 mg/kg: 97.4 %). Diese Ergebnisse haben gezeigt, dass durch eine oxidative Vorbehandlung mit Ozon eine signifikante Reduktion auf die TNF-α-Serumspiegel ausgeübt werden kann.

## Key words

- Endotoxic shock, mouse
- Ozone oxidative preconditioning
- Tumor necrosis factor-alpha

Arzneim.-Forsch./Drug Res. 54, No. ■, ■–■ (2004)

## 1. Introduction

Sepsis continues to be the major cause of death in most surgical intensive care units, in spite of the use of specific antibiotics, careful monitoring, aggressive operative intervention and intravenous hyperalimentation. Therefore, new experimental models are being developed to determine the mechanisms that underlie this complex pathology and to find out novel therapeutic methods. Septic shock is a complex disease state characterized by significant hemodynamic, cardiovascular and metabolic disturbances, which may result in multi-organ failure and death [1]. Experimental and clinical studies have demonstrated that exposure to endotoxin (lipopolysaccharide, LPS) results in the release of various inflammatory mediators. Particular attention has been paid to the pro-inflammatory cytokines, e.g. tumor necrosis factor-alpha (TNF-α), reactive oxygen species (ROS) and nitrogen species, such as nitric oxide (NO) and peroxinitrite which currently are considered key mediators of tissue injury and mortality in septic shock [2–4]. In agreement with these findings some antioxidants and ROS scavengers exert a protective action against endotoxic shock in rodents by inhibiting TNF-α [5].

Ozone/oxygen mixture (OOM) has a strong microbicide activity in vitro comparable to the potent bactericidal activity of NO [6] and might therefore act as a regulator of many inflammatory processes in vivo. It exerts various effects on the immune system [7], such as the modulation of phagocytic activity on peritoneal [8] and alveolar [9] macrophages, which generates the first line of defense against bacteria and its toxins. Therefore, it can be hypothesized that OOM modulates the production/release of pro-inflammatory cytokines in different abdominal organs (e.g. spleen, liver) and may be able to influence the outcome of a severe infection.

Repeated rectal applications of ozone induced cross-tolerance to free radicals released after hepatic and renal ischemia-reperfusion [10–13]. Also it has been demonstrated that low doses of ozone increased the antioxidant endogenous systems such as glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT), preparing the host to face physiopathological conditions mediated by ROS [10–14] and demonstrating that ozone, probably by means of an oxidative preconditioning mechanism similar to ischemic preconditioning, protected those organs against the damage produced by ROS, improving the antioxidant-pro-oxidant balance and the concomitant preservation of the cell redox state [15]. Ozone therapy has also been proved on treatment of various diseases and beneficial effects have been observed with it [13–17]. Ozone oxidative preconditioning also showed a beneficial effect in lethal polimicrobial sepsis in rats because increased animals survival and protected them against death [18].

Taking into account the former results, we decided

to study the potential effects of ozone oxidative preconditioning on TNF-α in blood serum, using two different routes of ozone application: intraperitoneal injection and rectal insufflation.

## 2. Material and methods

### 2.1. Animals and treatments

Adult male Balb/c mice weighing 18–20 g and the laboratory chow were obtained from the National Center for Laboratory Animal Production (Havana, Cuba), in order to be used in this study. Mice were housed in plexiglass cages, maintained in an air-filtered and temperature-conditioned (20–22 °C) room with a relative humidity of 50–52 % and under an artificial light/dark cycle of 12 h.

Animals were fed with standard laboratory chow and water ad libitum. LPS from *Escherichia coli*, serotype 055:$B_5$ (Sigma-Aldrich Chemie, Steinheim, Germany), was given i. p. at 0.1 mg/kg, dissolved in sterile pyrogen-free saline solution.

Ozone ($O_3$) was generated by OZOMED 01 equipment manufactured by the Ozone Research Center (Cuba). Ozone obtained from medicinal grade oxygen was used immediately. Ozone concentration was measured by using an UV spectrophotometer at 254 nm.

OOM was administered intraperitoneally at doses of 0.2, 0.4 and 1.2 mg/kg and by rectal insufflation at 0.2 and 0.4 mg/kg. The volume of OOM administered to each animal was approximately 1 mL. Oxidative preconditioning was performed with five applications (one daily) of the OOM. LPS was injected 24 h after the last ozone/oxygen administration.

A control group receiving LPS and two other groups receiving saline or OOM alone were also included. Dexamethasone (CAS 50-02-2, DEX) at doses of 30 mg/kg, used as a reference drug, was administered i. p. in saline solution 30 min before LPS. The mice were bled from the retro-orbital plexus under light ether anesthesia and serum TNF-α was measured 1 h after administration of LPS.

The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research, Havana (Cuba).

### 2.2. TNF-α assay

TNF-α was measured by cytotoxicity on L929 cells (kindly donated by Dr Marina Sironil, Immunopharmacology Laboratory, Institute of Pharmacology, Research "Mario Negri", Milan, Italy) [19]. TNF-α levels were determined using recombinant human TNF-α (BASF/Knoll, Ludwigshafen, Germany; specific activity $10^7$ U/mg) and expressed as pg/ml. Five mice per group were used and results were compared by Student's t-test. Results are expressed as percent inhibition of TNF-α production. All experiments were repeated at least twice.

## 3. Results

### 3.1 Clinical status of the animals after treatment with ozone/oxygen (i. p. and i. r.) and LPS

The animals did not show any relevant clinical signs because the low dose of LPS (0,1 mg/kg) that was used

of OOM, first with the rectal mucous membrane and later with the peritoneal cavity and the organs it contains, whereas by the intraperitoneal route the gas interacts directly and rapidly with cells of the immune system, and therefore, the inhibitory effect on TNF-α release is greater than by rectal administration. Thus, in order to obtain levels of inhibition of TNF-α release similar to those obtained by the intraperitoneal route, we must use higher doses of ozone by intrarectal insufflation. However, this route of ozone application is a very simple and safe procedure, when intravenous administration is not recommendable for the patient.

To our knowledge, it is the first report on the inhibitory effect of ozone oxidative preconditioning on the TNF-α release in serum in an experimental model of endotoxic shock, although a significant increase in the survival rate of rats was found which may be due to the role of other mediators.

In our opinion, the inhibitory effects of OOM on TNF-α levels in the serum of mice treated with endotoxin are a consequence of the stimulation of the antioxidant defenses induced by the ozone therapy. This point of view is scientifically supported by the fact that ROS are strongly involved in the induction and development of the inflammatory process and in the pathogenesis of endotoxic shock [4].

On the other hand, various antioxidants, with ROS scavenging properties, protected mice against endotoxin-mediated organ injury and reduced TNF-α levels in blood serum [4]. The effects of antioxidant agents have been ascribed by some authors to the inhibition of the nuclear transcription factor kappa B (NF-kB) activation. NF-kB is activated by ROS, with the subsequent induction of various cytokines and enzymes, which are involved in the induction and development of endotoxic shock. [26].

Taking into account our results and the former findings of other authors described above it is conceivable that, as occurs with other antioxidants and ROS scavengers, ozone oxidative preconditioning might exert its effects on endotoxic shock by inhibition of NF-kB activation. This may explain its inhibitory effects on TNF-α. However, further studies will be needed to elucidate the mechanisms underlying the beneficial effect of ozone/oxidative preconditioning.

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**Aknowledgements**

We are grateful to Dr. Ricardo González, Dr. Cheyla Romay and Dr. Carlos Hernández (Ozone Research Center, P.B. 6412, Havana City, Cuba) for their help in the writing of this article.

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**Table 1: Effects of ozone oxidative preconditioning on serum TNF-α levels in mice treated with LPS.**

| Treatment (mg/kg) | TNF-α (pg/ml) | |
|---|---|---|
| | Mean ± SE | Inhibition (%) |
| Saline | 0 | 0 |
| Ozone /oxygen (1.2), i. p. | 0 | 0 |
| LPS (0.1 mg/kg) | 2200 ± 780 | – |
| Ozone /oxygen i. p. (0.2) + LPS | 485 ± 120* | 78 |
| Ozone /oxygen i. p. (0.4) + LPS | 33 ± 17** | 98.5 |
| Ozone /oxygen i. p. (1.2) + LPS | 30.6 ± 5** | 98.6 |
| Ozone /oxygen i. r. (0.2), + LPS | 1182 ± 643* | 46.2 |
| Ozone /oxygen i. r. (0.4), + LPS | 56 ± 22** | 97.4 |
| Dexamethasone (30 mg/kg) + LPS | 29 ± 10** | 98.7 |

* $p < 0.05$, ** $p < 0.01$ vs. control (LPS) by Student's t-test (n = 5). The animals were treated with ozone (0.2, 0.4 and 1.2 mg/kg i. p.) and oxygen ?■ (0.2 and 0.4 mg/kg i. r.); 24 h after the last ozone application LPS was administered (0.1 mg/kg). 1 h later, blood samples were taken to measure TNF-α levels in serum. Dexamethasone (30 mg/kg i.p., 30 min before LPS) was used as control drug.