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Repetitive Pneumoperitoneum with Ozonized Oxygen as a Preventive in Lethal Polymicrobial Sepsis in Rats

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Key Words
Cytokines · Oxygen · Pneumoperitoneum · Rats · Sepsis · Survival

Abstract
The aim of this study was to test whether repetitive pretreatments of rats with ozonized oxygen at relatively low gas volumes into the abdomen (20 ml per rat per day) have any beneficial or detrimental effects on the course of a polymicrobial-induced lethal peritonitis. Peritonitis was induced in a surgical or a nonsurgical model by usage of fecal material from the cecum. As the biological read out we used the mortality analysis. To include possible mechanisms by which ozone might influence the septic outcome, we characterized the gene expression of the pro-inflammatory cytokines IL-1β, IL-2, and TNF-α mRNA in lymphoid organs. In both models, we found a significant beneficial influence of a dose-dependent O_2/O_3 pneumoperitoneum on the survival rate when compared to control animals or to room air. The ozone-enhanced survival seems to be independent from altered cytokine expression because there were no differences noticed in the levels of bacterial-induced gene expression of IL-1β and TNF-α in septic animals pretreated with ozonized oxygen when compared to control animals.

Introduction

In humans and animals gas insufflations into the peritoneum (pneumoperitoneum) are only used to extend the abdominal cavity for adequate visualization in laparoscopic surgery, but little is known about its effects on prevention and/or therapy in different diseases. A theoretical risk of morbidity and mortality on healthy and diseased subjects depends on the amount and quality of gas, on increased intraperitoneal pressure of >10 mm Hg duration, and the repetition of insufflations with CO_2 [1, 2]. Repetitive gas insufflations with CO_2 and increases of abdominal pressure can spread infectious secretions within the abdominal cavity, aggravating peritonitis and causing bacteremia, endotoxemia, and ultimately septic shock [3, 4]. Thus, the risk of applying conventional gases such as CO_2 on abdominal infections during laparoscopy [5–8] and the still unknown mechanisms on the complex peri-

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toneal immune system has encouraged us to look for ozonized oxygen (O_2/O_3) gas mixture, as an 'oxidative stressor'.

Ozonized oxygen is a cytotoxic gas [9], like excessive oxygen, with a strong microbiocidal activity in vitro [10] comparable to the potent bactericidal activity of NO [11] and might therefore act as a regulator or modulator of many inflammatory processes in vivo. Ozonized oxygen exhibits various effects on the immune system [12], such as the modulation of phagocytic activity on peritoneal [13] and alveolar [14] macrophages, which generates the first line of defense against bacteria and or its toxins. It can be hypothesized that ozonized oxygen enhances the production/release of pro-inflammatory cytokine in different abdominal organs (e.g. spleen, liver) and thus may be able to influence the outcome of a severe infection. Hitherto, no studies exist about the effects of repetitive intraperitoneal insufflation with this gas mixture (O_2/O_3 pneumoperitoneum) on healthy and/or experimentally diseased animals.

The aim of this study was to first determine the concentration of ozonized oxygen exhibiting the most detrimental or beneficial effects on the mortality rate of a polymicrobial-induced peritonitis. For this we used the surgical rat model because of its clinical relevance. In a second experimental design we wanted to characterize the effects of O_2/O_3 pneumoperitoneum on the immune system by analyzing the production of pro-inflammatory cytokines in several lymphoid organs within the abdomen. For this, we used a more reductionistic animal model (nonsurgical model) to reduce all possible immunotraumatic influences on infection caused by anesthesia and surgical trauma [15, 16]. In this model we only analyzed the ozone concentrations, which had shown a highly significant biological effect in the surgical model.

Material and Methods

Healthy (as given by FELASA recommendation) male Wistar rats (209–215 g) were kept in rooms with a standardized air conditioning 20–22°C, 50–57% relative humidity, and a 12-hour artificial day/night rhythm. Postoperatively, all animals were kept singly on clean paper sheets in type III Macrolon cages for recovery.

Application of Ozonized Oxygen and Room Air

Ozonized oxygen was generated from medical oxygen by an ozone gas processor (Ozonosan, PTN 60, Dr. Hänsler GmbH, Iffezheim, Germany) or from an Ozomed machine, manufactured at the Ozone Research Center in Havana, Cuba. Ozone concentrations were monitored by using an Ozonosan photometer (Dr. Hänsler GmbH). The gas mixture (5% volume ozone and 95% medical oxygen) was insufflated with a standardized volume of 80 ml/kg rat by injection (needle size: 21 G) into the right lower abdomen of the rats by daily injections for 5 days. The infection with cecal material was started 24 h after the last ozonized oxygen insufflation.

Surgical Model. In the surgical model different ozone concentrations (10 μg/ml, x^{mean} = 0.93 mg; 50 μg/ml x^{mean} = 4.20 mg, and 100 μg/ml, x^{mean} = 7.97 mg ozone/rat; n = 18 rats per group) were used. A sham group (n = 15) was treated with injections of nonfiltered room air of equal volumes. Untreated animals (n = 20) which received no injection of any gas were used as a control.

Nonsurgical Model. In the nonsurgical model we used 10 μg/ml ozone and similar controls (n = 20 rats per group), as described above. The gas with ozone and/or oxygen injected bolus-like into the abdomen was not desufflated, as in laparoscopic surgery.

Preparation of Fecal Suspension

Surgical Model. Under a deep surgical narcosis with 100 mg/kg ketamine and 10 mg/kg xylazine (i.m.) and after skin disinfecting, the abdomen was opened electrosurgically via a 1-cm midline incision to expose the cecum. The right cecal side was opened electrosurgically by a round incision (3 mm Ø) in an area free of blood vessels between the base and apex of the cecum, some fresh autochthonous material was removed and directly used for inoculation.

Nonsurgical Model. A donor rat was narcotized and cecal material (approximately 4 g) was removed from the large cecum into an air-evacuated tube of a syringe and cecal material was immediately used for inoculation.

Microbiological Spectrum of Cecal Material

The mean cecal bacteria from donor rats (n = 10) contained the following organisms (given in colony-forming units (CFU)/g): *Escherichia coli* (1×10^2 to 1×10^7), *Bacteroides distasonis* (0.5×10^7 to 1×10^8), *Prevotella oralis* (0.5×10^7 to 1×10^8), *Proteus mirabilis* (1×10^5 to 1×10^7) and *Enterococcus faecalis* (1×10^6 to 1×10^7) with highest frequencies and *Streptococcus* sp. (1×10^7), *Staphylococcus aureus* (1×10^4), *Bacillus* sp. (1×10^1) and *Micrococcus* (3×10^1) with lesser frequency. This spectrum of aerobes and anaerobes mimics the postoperative clinically relevant situation necessary in order to initiate a secondary peritonitis with inflammation, severe sepsis, and lethal shock, which is dependent on the inoculated amount of fecal material (g/kg body weight rat) into the abdomen.

Experimental Design

Surgical Model. To cause infection fresh unfiltered cecal material was diluted 1:2 with sterile saline and a dosage of 0.65 g/kg rat was inoculated with a syringe into the right lower quadrant of the abdomen. This dosage was known to induce a high mortality of >90% within the period of observation (120 h) in male adult Wistar rats. The cecum was closed by continuous stitches (6.0 nonabsorbable suture material), and then replaced into the abdomen. The abdomen was closed with two layers of sutures using Vicryl. To prevent a possible leakage of the diluted material, the rats were laid on their backs during the postoperative sleeping period of around 2 h after the narcosis. The time between the start of a deep anesthesia and the closure of the abdomen was around 8 min. After operation, the rats were kept in cages (Macrolon type IV) with 5 animals per cage and food and water was given ad libitum. Animals were observed for 120 h (end of experiment). Surviving animals were briefly narcotized with forene and euthanized with intracardially injected embutramide and mebenonium iodide (T 61^R ad. us. vet., Hoechst Roussel Vet).

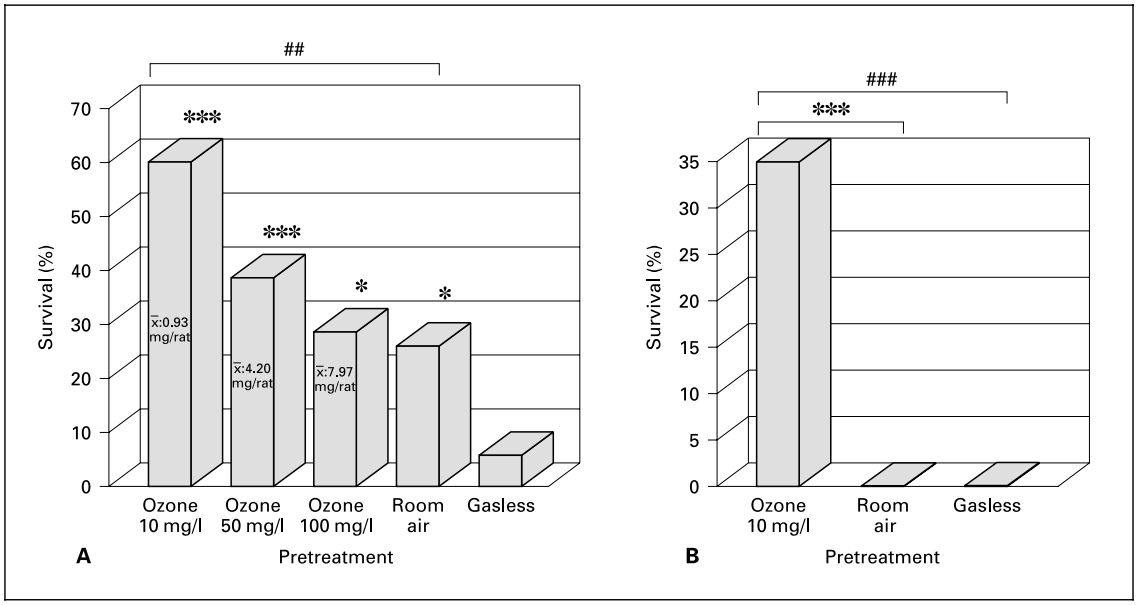

Fig. 1. Mortality analysis of rats infected with polymicrobial bacteria in the surgical model (A) and nonsurgical model (B) showed the survival rates after ozonized oxygen pretreatment and also after pre-treatment with room air. Animals receiving no pretreatment (gasless) were used as controls. To calculate levels of significance, the χ^2 test was performed (ozone vs. gassless: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ozone vs. room air: $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$).

Nonsurgical Model. The fresh cecal material was diluted 1:10 with sterile saline and was injected with a 14-gauge needle into the right lower abdomen. For mortality analysis animals were observed for 120 h. For analysis of cytokine mRNA expression profile, rats were killed at 2, 4, 12, and 24 h after the infection (n = 3 rats per group).

Statistical Analysis

For statistical analysis we used the χ^2 test. $p < 0.05$ was considered significant.

In situ Hybridization

Rat specific cDNA fragments for IL-1β, IL-2 and TNF-α were generated by reverse transcription PCR of total RNA from rat spleen and inserted into the pGEM-T vector (Promega, Germany). For in vitro transcription cDNAs were linearized with the appropriate restriction enzymes and ^{35}S-labeled sense and antisense ribonucleotide probes were generated by in vitro transcription using SP6 or T7 polymerases (Boehringer Mannheim, Germany) as appropriate in the presence of ^{35}S-UTP (Amersham Life Science, Germany). All labeled cRNAs were purified over Micro Bio-Spin® Chromatography columns (Bio Rad, Marburg, Germany) and diluted in hybridization buffer (100 m*M* Tris pH 7.5, 600 m*M* NaCl, 1 m*M* EDTA, 0.5 mg/ml t-RNA, 0.1 mg/ml sonicated salmon sperm DNA, 1 × Denhardt's, 10% dextrane sulfate, 50% formamide) to 50,000 cpm/μl. In situ hybridization was performed on 14-μm-thick serial cryostat sections of rat spleen as described previously [17]. Autoradiograms were taken by exposing the sections to an autoradiography film (Hyperfilm-βmax, Amersham, Dreieich, Germany) for 1–3 days.

Results

In all experiments no case of morbidity (e.g. weight loss) or mortality by repeated gas insufflations with ozonized oxygen or room air was observed until the inoculation with bacterial material was performed.

Surgical Model

In the gasless control group, 19 from 20 animals died, showing a rate of 5% survival (fig. 1A). Pretreatment of rats with air resulted in an enhanced survival rate of up to 26% ($p < 0.03$). When ozonized oxygen was used as the gas, the survival rate was positively influenced, when compared to the gasless control group, and enhanced above the survival rate measured in the air group. There was an inverse relationship between the dose of ozonized oxygen and the survival rate with a significant survival rate of 61.5% for 10 μg O_3/ml, 39% for 50 mg O_3/l and 25.8% survival for 100 mg O_3/l. A highly significant effect was only seen in animals pretreated with 10 μg O_3/ml. Therefore, we exclusively used this dosage in the nonsurgical model.

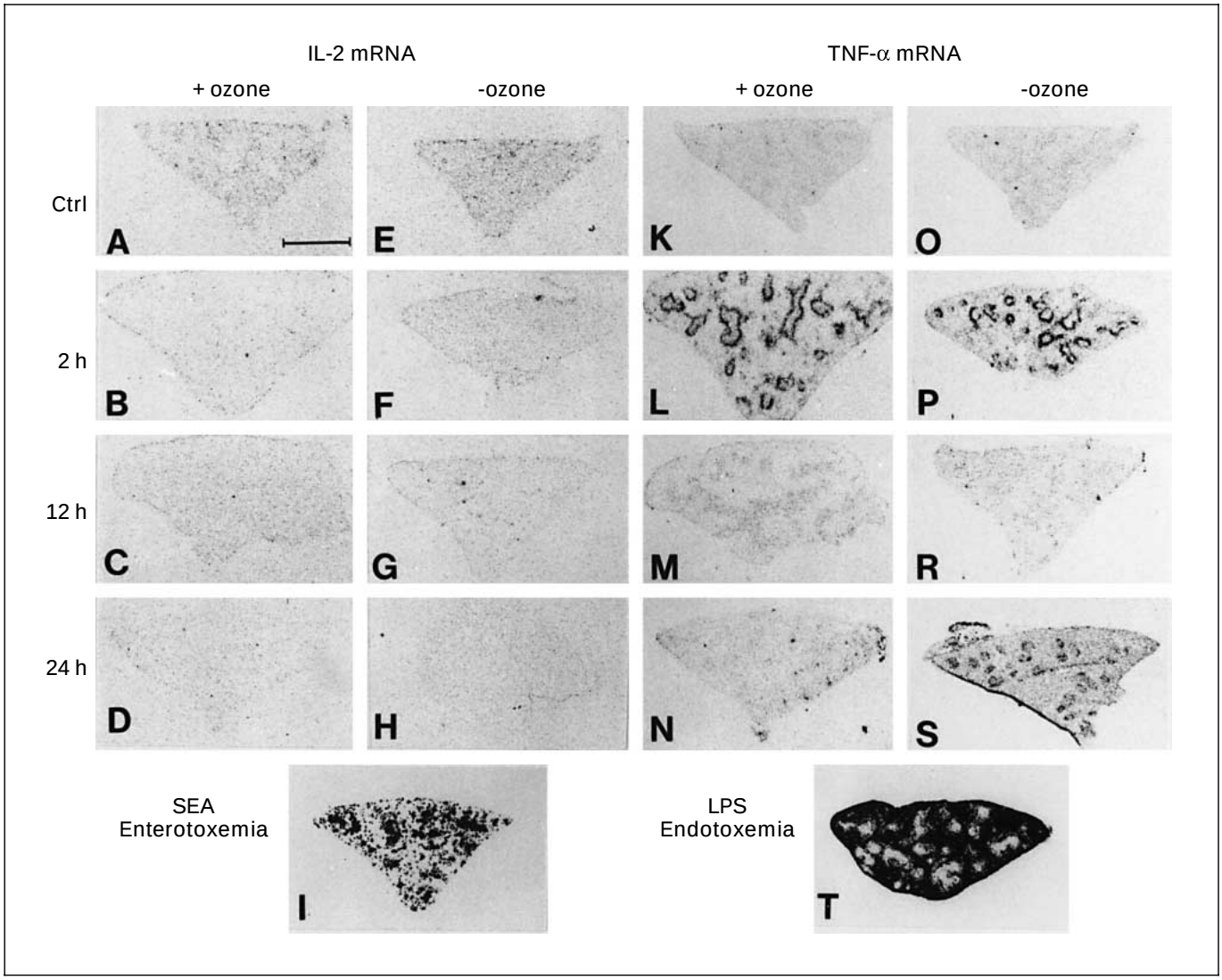

Fig. 2. Autoradiographic detection of in situ hybridization of IL-2 mRNA (A–I) and TNF-α mRNA (K–T) was performed in rat spleen in the course of polymicrobial infection (nonsurgical model). Animals were pretreated with or without repetitive ozonized oxygen (lettering of the groups and treatment is given in the figure). Control animals (Ctrl) received no bacterial suspension. As a positive control for in situ detection of IL-2 mRNA, a spleen from a rat stimulated with staphylococcal enterotoxin A (SEA) was used (I). To detect TNF-α mRNA, a spleen from a LPS-stimulated rat was used (T). Bar in A = 2 mm.

Nonsurgical Model

As seen in the surgical model, 10 mg/l ozonized oxygen enhanced highly significant the survival rate. In the O_2/O_3 pneumoperitoneum group 35% animals survived, whereas all animals of the non-pneumoperitoneum group and the filtered air-pneumoperitoneum group died after infection (fig. 1B).

Analysis of the Immune Status in situ

To unfold the biological mechanisms leading to the ozone-driven effect on survival, we analyzed the expression of pro-inflammatory cytokine mRNAs in spleen, liver and Peyer's patches. In the first step we analyzed the activation of the specific immune system, represented by the presence of IL-2 mRNA expressing T lymphocytes. The polymicrobial infection did not lead to an induction of IL-2 mRNA within the first 24 h after infection, which

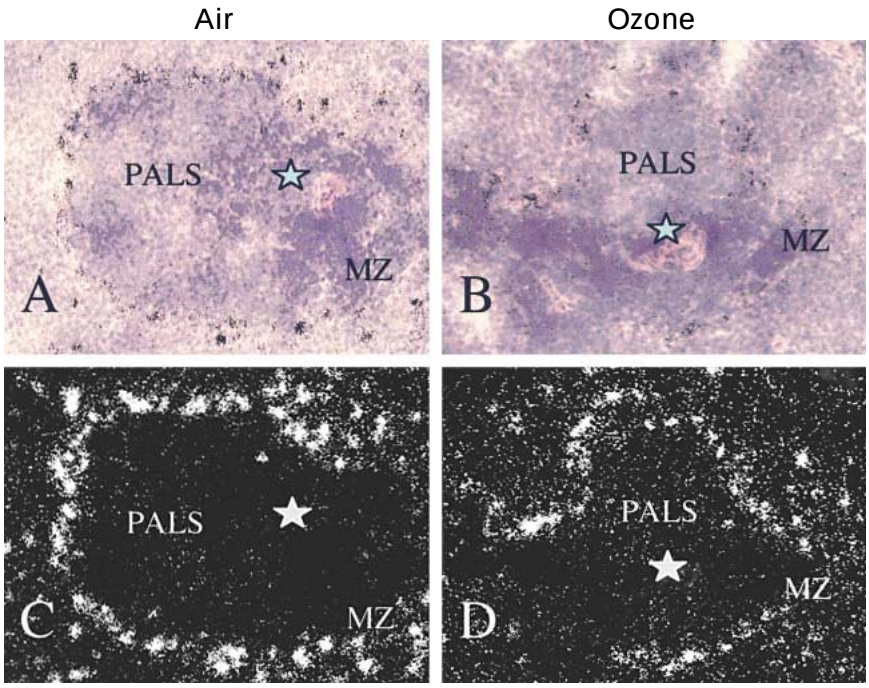

Fig. 3. High-power analysis of emulsion-coated slices from rat spleen hybridized for TNF-α mRNA in nonsurgical polymicrobial sepsis pretreated with air (A, C) or with ozonized oxygen (B, D). Localization of TNF-α mRNA was performed 2 h after infection. For regional analysis of TNF-α mRNA, each tissue slice became emulsion-coated and was analyzed by bright field (A, B) and dark field (C, D). MZ = Marginal zone; PALS = periarteriolar lymphatic sheaths. Bar = 50 μm, counterstaining by cresyl violet.

was the critical time window for survival (fig. 2). An influence of ozonized oxygen on IL-2 mRNA expression could not be detected neither in spleens of non-infected rats nor at any time after infection (fig. 2). In confirmation no other organ (liver, Peyer's patches) showed any signal for IL-2 mRNA (data not shown). These data demonstrate that the polymicrobial infection, despite the presence of gram-positive *Streptococcus* sp. and *S. aureus* did not activate the specific immune system in abdominal organs at least on the level of T cells, as seen by the gram-positive infection induced by SEA in the spleen. Furthermore, repetitive ozonized oxygen without infection did not exhibit any stimulatory influence on the T-cell-dependent immune system.

In contrast to IL-2, monocytically derived TNF-α mRNA expression was transiently increased after polymicrobial infection in the lymph node and spleen. In the spleen, TNF-α mRNA was enhanced 2 h after infection (fig. 2). TNF-α mRNA expressing cells were restricted to the marginal zone of the splenic white matter (fig. 3). Ozonized oxygen treatment resulted in a similar spatial-temporal expression of TNF-α mRNA in the early phase of sepsis (within the first 12 h) in spleen (fig. 2, 3) and also in liver and Peyer's patches (data not shown) as seen after air inoculation. Therefore, no obvious influence of ozonized oxygen pretreatment was observed for TNF-α mRNA in the spleen. Interestingly, in the late phase of sepsis (24 h after infection), we observed a slight secondary increase in TNF-α mRNA in gasless control animals (fig. 2S), which was not present in the ozonized oxygen pretreated animals (fig. 2N). The enhancement of TNF-α mRNA correlated with the lethal outcome of rats within that time schedule.

Also, IL-1β mRNA was strongly enhanced above basal expression in liver, spleen and Peyer's patches after inoculation with cecal material (fig. 4). Interleukin-1β mRNA was seen in single cells within the liver, in the red pulp and the marginal zone in the spleen, and in the outer zone of the folliculi lymphatici of the Peyer's patches. No difference in the strength and the spatial expression of IL-1β mRNA between air-treated or ozonized oxygen-treated animals was seen.

Because of the absence of any visible modulatory effect of repetitive doses of ozonized oxygen on the expression of pro-inflammatory cytokines during sepsis, as opposed to the beneficial influence of repetitive ozonized oxygen pre-treatment on the survival rate, we wanted to find out the pure effect of ozonized oxygen on the immune system of intact animals. For this we applied a single dose of highly concentrated ozone (100 mg/l) to detect possible acute effects of ozonized oxygen present 1 h after ozonized oxygen application on cytokine expression in normal nonsep-

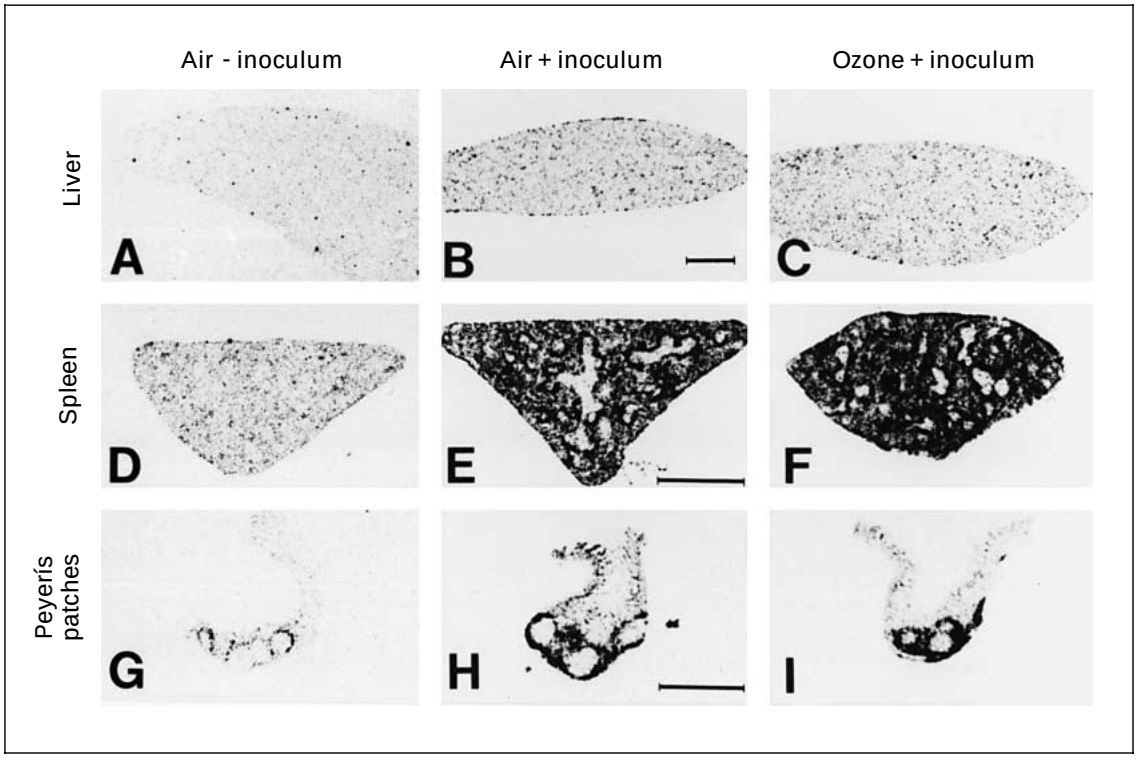

Fig. 4. In situ hybridization of IL-1β mRNA was performed in liver (A–C), spleen (D–F) and Peyer's patches (G–I) of uninfected control animals (control; left column) or in septic animals pretreated with air (+ air + inoculum; middle column) or pretreated with repetitive doses of ozone (+ ozone + inoculum; right column). Abdominal organs were removed 6 h after inoculation of cecal material. Bars: B = 250 μm, E = 200 μm, H = 200 μm.

tic control animals. A bolus-like injection of highly concentrated ozonized oxygen did not lead to any enhanced or decreased cytokine expressions of IL-1β and TNF-α in liver, spleen, Peyer's patches and mesenteric lymph nodes (fig. 5), when compared to gasless control animals (see also fig. 2 and 4, for lymph node data not shown). Therefore, an acute stimulatory as well as an acute inhibitory effect of ozonized oxygen on the abdominal immune system could be ruled out.

Discussion

There are numerous toxicological inhalation studies with ozonized oxygen on the organ lung as the final target [18] with extrapulmonary effects on spleen [19] and on liver [20]. Inhaled ozonized oxygen exhibits strong immune modulatory effects on the innate immune system especially on alveolar macrophages, but also on extrapulmonary macrophages [21–24]. Furthermore, ozonized blood in vitro causes a release of different cytokines such as TNF-α, GM-CSF, IL-2 or IFN-γ [25], but hitherto, no further data exist from in vivo experiments to find out if this gas mixture of ozonized oxygen has any detrimental or beneficial influence on the cytokine release by leucocytes. The described immune modulatory effects of inhaled ozonized oxygen let us assume that intraperitoneally applied ozonized oxygen might have also some effects on the general or to the abdominal restricted immune status. Thus, under septic conditions, O_2/O_3 pneumoperitoneum might have some influence on the biological outcome. There are various interventions which can mimic the clinical and nonclinical situation of polymicrobial sepsis in rats.

In our surgical animal model, which is closely orientated to surgical interventions used in human medicine, we observed a beneficial effect of ozonized oxygen. We measured an inverse relationship between the dose of ozonized oxygen and survival rate with the lowest dose of ozone exhibiting the most effective on survival. The bio-

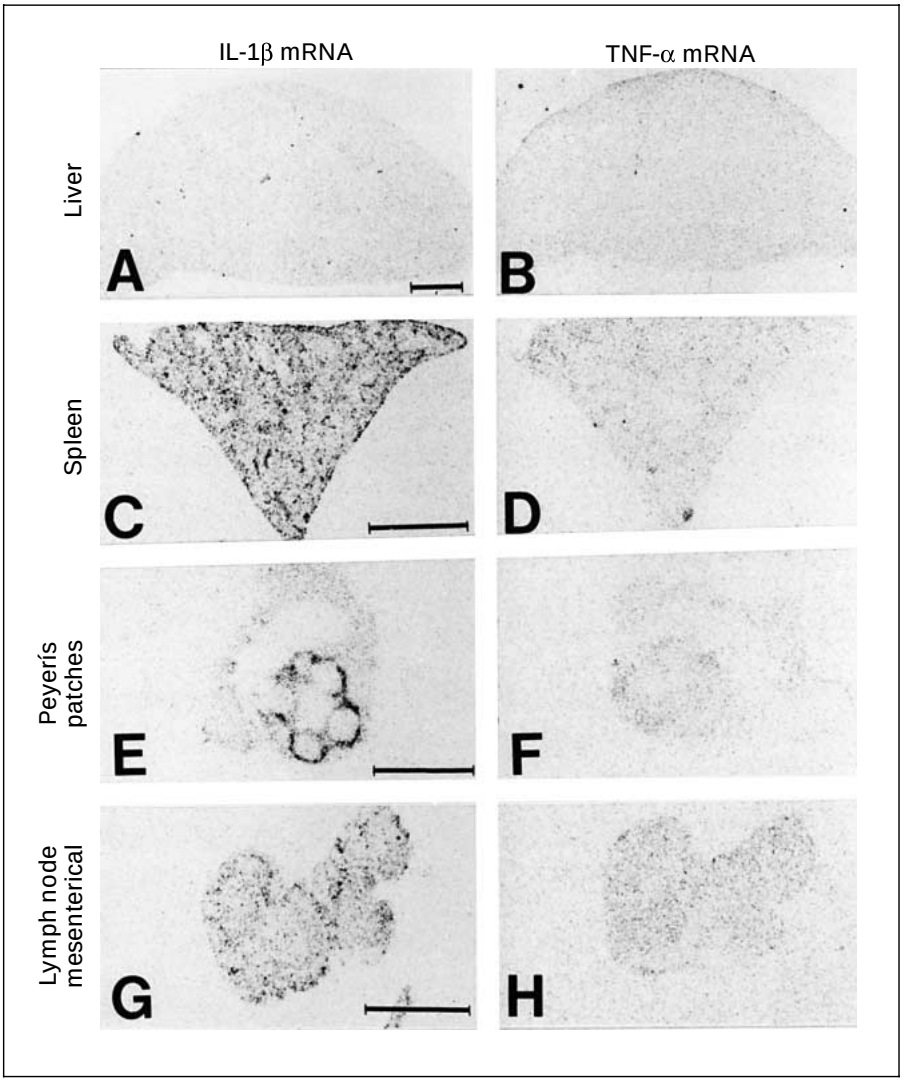

Fig. 5. Expression of IL-1β mRNA (left column) and TNF-α mRNA (right column) in liver (A, B), spleen (C, D), Peyer's patches (E, F) and mesenteric lymph node (G, H) of normal noninfected rats, which received a single dose of high concentration of ozone (100 mg/l) 1 h before tissue removal is shown by in situ hybridization. Bars: A = 250 μm, C, E, G = 200 μm.

logical mechanism for this is still unknown, but it might be due to an altered immune status generated by ozone-mediated priming effects.

It is well known that different gases insufflated into the peritoneum can influence the intraperitoneal immunity [26, 27]. Chekan et al. [28] showed that a CO_2 pneumoperitoneum has been implicated as a possible factor in depressed intraperitoneal immunity with detrimental effects on impaired peritoneal clearance of bacteria. These authors showed that macrophages incubated under CO_2 produced significantly less TNF-α and IL-1 in response to LPS as compared to incubation of air or the inert gas helium [29]. In this context, West et al. [29] suggested that after laparoscopy the activation of macrophages might be due to LPS present in the insufflated air. We also found an enhanced survival in our surgical model using unfiltered room air in comparison to gasless control rats. In contrast, however, we did not find an enhanced survival after insufflation with filtered room air compared to gasless control rats from the nonsurgical model. These data are in line with observations made by Watson et al. [1] who suggested that unknown substances (e.g. LPS) in nonfiltered room air are responsible for the enhanced survival rate by changing the immune status. They reported that factors in the room air might play a role in changes in the immune response. They concluded that factors in the circulating air can induce translocation of mitogenic substances, e.g. LPS, and subsequently can stimulate the postoperative immune response.

A direct effect of cytokine expression by insufflated gases had been described by Evard et al. [30]. Here, CO_2 pneumoperitoneum decreased IL-1 mRNA and TNF-α gene transcriptions persistently in the postoperative course [30]. Also CO_2 pneumoperitoneum significantly decreased the number of IL-2 receptor α chains expressing CD4+ and CD8+ T cells [31]. In contrast to these CO_2-dependent effects on the immune system, we could not find any influence of repetitive O_2/O_3 pneumoperitoneum on the expression of these cytokines. These data suggest that different mechanisms might exist for ozonized oxygen and the CO_2-induced inhibition of IL-1 and TNF-α.

The analysis of a short-term administration of a bolus-like injection showed that ozonized oxygen exhibits no direct effect on the cytokine expression of the local immune system. Therefore, a priming effect of ozone on the abdominal immune system seems not to be the mechanism for the beneficial effect on polymicrobial-induced mortality. This is in contrast to direct effects of ozonized oxygen on the cytokine/chemokine expression [32, 33] and on the adherence of alveolar macrophages [34] pointing out a possible different effect of ozonized oxygen in lung or abdomen. Also, the well-known direct biocidal effects [10], or indirect effects via enhanced microbiocidal cell activation (e.g. eosinophilic granulocytes) caused by ozonized oxygen exposure might also be taken into account. Other effects such as enhanced prostacylin synthesis may play a role in this complex process of lethal infection/inflammation. Therefore, more investigations with this gas mixture are necessary, especially in combination with antibiotics, which will lead to a promising new approach in prevention and/or therapy to overcome severe infectious diseases.

In conclusion, the survival rate of polymicrobial infected rats was enhanced significantly when ozonized oxygen was given in low doses. However, the beneficial effects of repetitive ozonized oxygen treatment could not be explained by an acute modulation of pro-inflammatory cytokines.

Acknowledgments

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