

# Ozone Prophylactic Effect and Antibiotics as a Modulator of Inflammatory Septic process in Rats.

Zullyt B. Zamora<sup>1</sup>, S. Menéndez<sup>1</sup>, M. Bette<sup>2</sup>, R. Mutters<sup>3</sup>, S.Hoffmann<sup>4</sup> and S. Schulz<sup>4</sup>.

<sup>1</sup> Ozone Research Center, Havana, Cuba.

E-mail : ozono@infomed.sld.cu

<sup>2</sup> Institute of anatomy celular and molecular biology, University od Marburg, Germany.

<sup>3</sup> Intitute of microbiology, University od Marburg, Germany.

<sup>4</sup> Institute of animals laboratory, University od Marburg, Germany.

## Abstract

192 Wistar male rats (180- 200 g) were used to evaluate the prophylactic effect of ozone combined with antibiotics in septic shock model. The proinflammatory cytokines (IL-1 $\beta$ , TNF $\alpha$ , IL-6 and IL-2) expression were also determined in different haemathopoyetic organs. The animals were taken at random and divided into 16 groups, 12 animals each: Group A, positive control (sepsis without treatment); groups B and C treated with ozone (200 ug / 250 g b. w) and Cefotaxim (25mg/kg), (0h, +1h) and (+1 h) respectively; groups D and E, ozone and Cefodizim (25mg/kg (0h, +1h) and (+1 h) respectively; groups F and G ozone and levofloxacin (12.5 mg/kg), (0h, +1h) and (+1 h) respectively; groups H and I ozone and Piperacillin /Tazobactam (65mg/kg), (0h, +1h) and (+1 h) respectively. The septic shock was carried out by single intraperitoneal injection of fecal material (0.65 g/kg b/w) from a donor rat, diluted in saline solution 0.9%. The other groups (J-P) of animals were treated only with antibiotics.

The group pretreated with ozone and antibiotics showed a significant increase in the survival rate and showed also a decrease of IL-1 $\beta$  mRNA expression in liver. Ozone pretreatment might prevent lethal peritonitis by controlling inflammatory process generated physiologically into the body against specific damage or specific organism.

## Key Words

Ozone, Ozone treatment, Septic shock, animal's model, rats, Cytokines, Interleukines.

## Introduction

Septic shock is the commonest cause of death in intensive care units. Shock resulting from a systemic response to serious infection, a syndrome usually termed "septic shock", has been increasing since the 1930s (Finland M., 1970), and all estimates suggest that this rise will continue. There are approximately 500,000 cases

each year in the United States (Wenzel et al., 1996) and about 75,000 cases in Germany. The reasons underlying this rapid increase and high incidence are many: increased use of cytotoxic and immunosuppressive drug therapies, increasing frequency of invasive devices, increasing longevity of patients prone to develop sepsis, and an increase in infection caused by antibiotics-resistant organism (Parrillo, 1989).

The pathogenesis of septic shock is extraordinarily complex. Diverse microorganism can generate toxins, stimulating release of potent mediators that act on vasculature and myocardium (Joseph et al., 1990).

Therapy has emphasized early use of antibiotics, critical care monitoring, aggressive volume resuscitation, and, if shock continues, use of inotropic agents and vasopressors (Keith et al., 1980). Pharmacologic or immunologic antagonism of endotoxin or other mediators may prove to enhance survival in this highly lethal syndrome, But clear benefits of these new immunotherapies could not be demonstrated (Klosterhalfen et al., 1998)

Antibiotics appear to have little immediate effect on the course and outcome of septic shock, although they often rapidly inhibit or lyse the causative bacteria (or do both) and sterilize the bloodstream. This severe, acute toxicity that is unresponsive to antibiotic therapy is presumably due to systemic or circulating toxins and mediators (Danner et al., 1989). In fact, it has been hypothesized that the endotoxin of gram-negative bacteria may be released by the action of antibiotics, resulting in clinical deterioration (Healy et al., 1997 and Healy et al., 1996).

In recent decades, numerous experimental animal models septic shock were designed to study the underlying pathophysiological aspect of the disease and to find a new therapeutical strategy. Research focused both on hemodynamic alterations due to septic-shock-like states (Hinshaw and Beller, 1988) and on particular biochemical alterations.

The underlying pathobiochemical alterations in experimental septic-shock-like states consist of a dramatic increase in eicosanoid production (Lefer, 1989) and platelet activation factor acether (Dobrowsky et al., 1991), a release of cytokines in particular interleukin (IL)-1, IL-6, IL-10 and tumor necrosis factor (TNF)  $\alpha$  (Cannon et al., 1990; Flohe et al., 1991; Howard et al., 1993; Klosterhalfen et al., 1992).

Ozone is a potent oxidant agent, which has bactericidal properties. (Bocci, 1992), reported that ozone acts as cytokine inductor. Concentrations between 10-80  $\mu\text{g/ml}$  are able to release such as: interferon (IFN -  $\gamma$  y  $\beta$ ), (TNF- $\alpha$ ), interleukins (IL)1 $\beta$ , 2, 4, 6, 8 y 10, granulocytes-macrophages colony stimulator factor (GM-CSF) and transforming growth factor (TGF-  $\beta$ 1). (Bocci, et al., 1993a, 1993b, 1990 and 1994).

Treatment with ozone may be effective in clinical use in defferent dis\orders, including ischemic conditions (Bocci, 1996) Recently, the effectiveness of ozone treatment in an experimental model of hepatotoxicity induced by  $\text{CCL}_4$  in rat has been reported (León et al., 1998) On the basis of the oxidant properties of ozone and on the possibility that specific cell sensor activated by lipid oxidation products (LOP) may downregulate the immune system.. We postulate that ozone may induce a resistant to septic shock (inflammatory response).

The aim to evaluate the effect of ozone applied as prophylactic in combination with antibiotics on a relevant experimental model of peritonitis in rats.

## **Materials and Methods**

### **Animals and Materials**

Adult male Wistar rats (180-200g) were obtained from the National Center for Laboratory Animals Production, and used for this study. Rats were maintained in an air filtered and temperature conditioned (20-22 °C) room with a relative humidity of 50-52%. Animals were fed with standard commercial pellets and water *ad libitum*.

Ozone was generated by OZOMED equipment manufactured by the Ozone Research Center (Cuba) and was administrated by intraperitoneal route.

Different kind of antibiotics, Claforan<sup>®</sup> 1,0 Cefotaxim –Natrium, (Hoechst, Germany); Opticel<sup>®</sup> 1,0 Cefodizim-Dinatrium, (Hoechst, Germany); Tavanic 500 mg, Levofloxacin, (Hoechst, Germany); Tazobac 4,5 g, (Lederle , Germany) were used.

### **Experimental Design.**

The following experimental groups were used:

The animals were taken at random and divided into 16 groups, 12 animals each other: Group A, positive control (sepsis without treatment).

Groups B and C treated with ozone and Cefodizine (25mg/kg), (0h, +1h) and (+1 h) respectively.

Groups D and E, ozone and Cefodizine (25mg/kg (0h, +1h) and (+1 h) respectively.

Groups F and G ozone and levofloxacin (12.5 mg/kg), (0h, +1h) and (+1 h) respectively.

Groups H and I ozone and Piperacillin /Tazobactam (65mg/kg), (0h, +1h) and (+1 h) respectively.

The other groups (J-P) of animals were treated only with antibiotics with the same schedule like before group described but without ozone pretreatment.

Ozone pretreatment by intraperitoneal injection of a low concentration (10 µg/mL) and a volume of 80 mL/kg of body weight was administrated.

### **Induction of peritonitis**

The peritonitis by a single intraperitoneal injection with fecal material (0,55g/kg) was induced. The fecal material from the cecal of a donor rats was diluted in Ringer solution 1: 1. The animals were observed during five days and the rate of mortality was measured. At five days the survival animals were sacrificed by lethal intracardial injection of T 61 Hoechst Roussel Vet, Germany); and all organs were macroscopically analyzed.

### **Immunohistochemical determinations**

The animals from each group were sacrificed at 6 h after peritonitis induction and pieces from Liver, spleen, mesenteric lymph nodes and plaques Peyer were taken

and frozen in Tissue –Tek (Embedding Medium for frozen tissue specimens) Sakura. U.S.A until the determination of cytokines

Rats specific cDNA fragments for IL-1 $\beta$ , IL-2, IL-6 and TNF $\alpha$  were generated by reverse transcription PCR of total RNA from spleen of a LPS-stimulated rat or in case of IL-2 an animal stimulated with *Staphylococcal* enterotoxin A (SEA). The clonal cDNA fragments are the following: IL-1 $\beta$  cDNA is a 589 bp fragment ranging from bp 206 to 795 bp of IL-1 $\beta$  cRNA (Accession number M98820); IL-2 cDNA (a gift from Richt, Giessen) is a 505 bp fragment ranging from base 138 to base 542 (Accession number M22899); IL-6 cDNA is a 206 bp fragment ranging from bp 232 to bp 437 (Accession number M26744); TNF $\alpha$  cDNA is a 291 bp fragment ranging from bp 4432 within Exon 1 to bp 5348 within Exon 3 of the TNF $\alpha$  gene (Accession number L00981). All cDNA-fragments became inserted into pGEM-T vector (Promega, Germany) and were transfected into *E. coli* bacteria from XL1-blue strain. To linearize the vectors containing the different cytokine cDNA fragments the following restriction enzymes (Boehringer Mannheim, Germany) were used:

IL-1 $\beta$ : Not I (antisense) or Nco I (sense); IL-2: Apa I (antisense) or Sal I (sense); IL-6: Not I (sense) or Nco I (antisense); TNF $\alpha$ : Apa I (sense) or Pst I (antisense) restriction enzymes.

<sup>35</sup>S-labeled sense and antisense riboprobes were generated by in vitro-transcription using SP6 or T7 polymerases (Boehringer Mannheim, Germany) as appropriate in the presence of <sup>35</sup>S-UTP (Amersham life Science, Germany). All labeled cRNAs were purified over Micro Bio-Spin<sup>®</sup> Chromatography

columns (Bio Rad, Germany) and diluted in hybridization buffer (100mM Tris pH 7.5, 600mM NaCl, 1mM EDTA, 0.5 mg/mL t-RNA, 0.1 mg/mL sonicated salmon sperm DNA, 1x Denhardt's, 10% dextrane sulfate, 50% formamide) to 50.000 cpm/ $\mu$ L. Labeled cRNA was stored for no longer time than three weeks at – 75 °C.

In situ hybridization was performed on 14  $\mu$ m thick serial cryostat sections of rat spleen, liver, Peyer plaques and lymph nodes. Tissue sections were fixed in 4% paraformaldehyde in PBS at 4 °C for 1h, washed 3 times in PBS, penetrated by 0.4 % Triton X-100 in PBS for 5 min. and acetylated for 10 min. in 0.1M triethanolamine pH 8.0 with 0.25 % acetic anhydride. Tissues were washed in 2x SSC, Dehydrated in ethanol and stored at – 20 °C until hybridization. Hybridization with cRNA, prepared by in vitro transcription. The incubation of 35  $\mu$  L of cRNA on tissue sections for 16-18 h at 56°C in a moist chamber was performed. The slides in 2x SSC and 1x SSC for 10 min were washed and single stranded RNA was digested by 10  $\mu$ g/mL RNase, 1U/ml T1 RNase Boehringer Mannheim in Tris/EDTA pH 8.0, 150 mM NaCl for 1h at 37°C. Afterwards, slides were desalted by passing them through 1x SSC, 0.5x SSC, 0.2x SSC for 10 min. And washed in 0.2x SSC at 60°C for 1h. Then the tissue sections were washed in H<sub>2</sub>O for 10 min., dehydrated by ethanol and became airdried. Autoradiograms were taken by exposing the sections to an autoradiography film (Hyperfilm- $\beta$ max, Amersham, Dreieich, Germany) for 1-3 days.

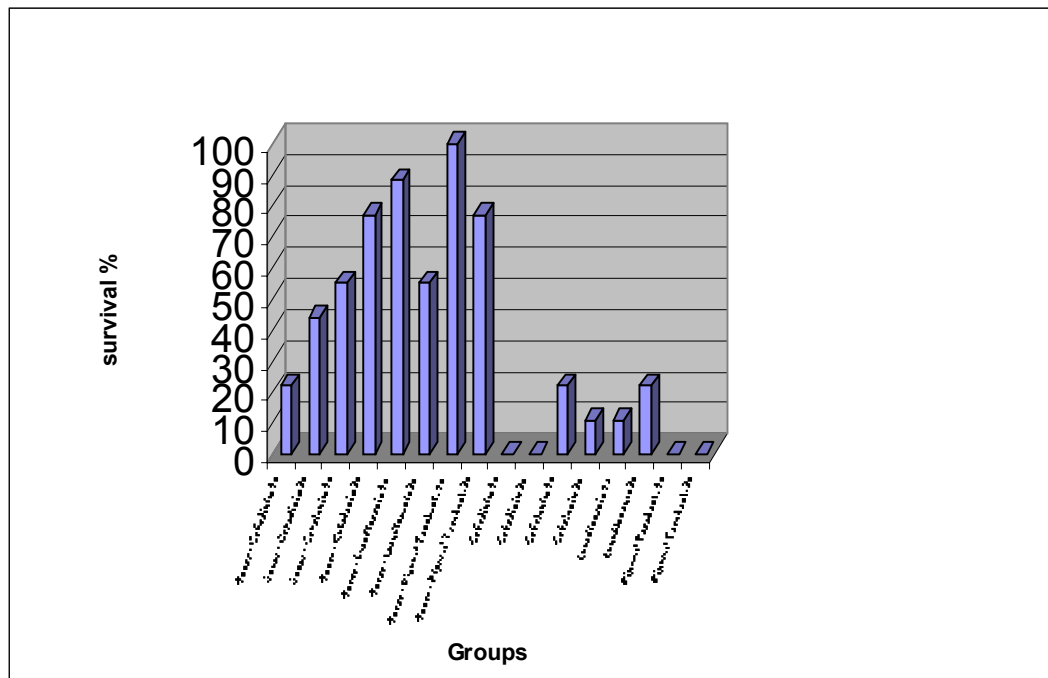
## Results and Discussion

### ***Survivals in animals pretreated with ozone and antibiotics***

Groups pretreated with ozone and antibiotics in combination showed a significant increase of survival in comparison with the groups treated only with antibiotics.

In the groups pretreated with ozone and antibiotics, were found no differences in the time of antibiotics injection except for the group treated with Tazobac with 100% of survival in the subgroup treated two times and only 22% for the subgroup treated one time, it is unknown why it occurs. The survival rate for the groups treated only with antibiotics have less than 25 %. These results show clearly that ozone had a mean key in the inflammatory response. Ozone pretreatment in combination to antibiotics were capable to reduce the mortality (**Graf I**)

**.Graf.I Rate of survival in rats pretreated with ozone plus antibiotics and**



**antibiotics alone.**

The microorganisms have developed resistance to antibiotics, so that in the pharmaceutical field new germicidal products such as cephalosporine and quinolones are being continuously developed. In this study is demonstrated that ozone applied prophylactically was able to increase or support the antibiotics action

Antibiotics appear to have little immediate effect on the course and outcome of septic shock, although they often rapidly inhibit or lyse the causative bacteria (or do both) and sterilize the bloodstream. This severe, acute toxicity that is unresponsive to antibiotic therapy is presumably due to systemic or circulating toxins and mediators (Danner et al., 1989). In fact, it has been hypothesized that the endotoxins of gram-negative bacteria may be released by action of antibiotics, resulting in clinical detrimental (Shenep et al., 1984).

While antibiotics remain the cornerstone of treatment for patient with bacterial sepsis, extensive in vitro data indicate that significant differences exist among antibiotics with respect to their ability to liberate bacterial endotoxin, (Jackson et al 1992; Crosb et al., 1994; Evans et al., 1993; Buijs et al., 1994) these differences may have biological or clinical consequences in patients treated with different antibiotics (Hurley, 1992; Prins et al., 1994). Dofferhoff et al., 1995 compared three  $\beta$ -lactamis with respect to their release of endotoxin and production of TNF  $\alpha$  and IL-6 during treatment using an experimental model of *E. coli* peritonitis in rats. Healy et al., 1997 has demonstrated that antibiotics such as Tazobac, Cefazidime, Imipenem, Ciprofloxacin, Gentamicin are capable to increased endotoxin released and IL-6 production but it depend on the antibiotics doses and the organism bacterial.

According to these results we could suggest one of the mechanism by which ozone pretreatment could potency the antibiotics action in ours peritonitis model compared with the survival of the group treated with antibiotics alone. Firstly the antibiotics increased the endotoxin release and one of the proinflammatory cytokine (IL-6) so that the animals treated only with antibiotics and specifically Tazobac had 0% of survival unlike the animals pretreated with ozone getting 100% of survival. The ozone might act as an inhibitor of the release of this kind of cytokines.

### ***Regulatory effect of ozone pretreatment on the liver expression of IL-1 $\beta$***

There was a significant decrease on the liver IL-1  $\beta$  expression on animals pretreated with ozone and antibiotics (**Fig 1**). But there was no difference among each antibiotic. The groups pretreated with ozone and antibiotics showed a decrease of IL-1  $\beta$  expression whereas in the groups pretreated with antibiotics without ozone there was a significant increase. Therefore, ozone may inhibit the expression of one of the most important cytokine expressed during septic shock. But there have not differences in the expression of the other cytokines (TNF- $\alpha$ , IL-2 and IL-6). These cytokines have a specific time of detection. It has been reported that TNF- $\alpha$  was detectable in serum, spleen, liver and lung during the first 4h, with a peak 2h after cecal ligation and puncture (CLP). IL-6 levels increased significantly in serum throughout the first 16 h after CLP and IL-1 $\beta$  was measurable in serum after 24 h, and levels increased significantly in spleen and liver 4 and 8 h after CLP (Villa et al., 1995). In this study the organs samples from the animals to determine cytokine expression were taken at 6h after induced peritonitis. This time coincides with the period of detection to IL-1 $\beta$  reported, so that, there it is showed only variability in the expression of IL-1 $\beta$  in liver.

We could postulate that ozone as oxidative agent produces a controlled oxidative stress by which is activated the protective intracellular mechanism and inhibits the most important proinflammatory cytokines (IL-1  $\beta$ ) in this case.

## Conclusions

Ozone may be applied in combination with antibiotics, it potentials the antibiotic's activity. It is a very important result nowadays with the development of bacterial resistance to antibiotics. The prophylactic application of ozone may downregulate the inflammatory response and inhibits the IL-1  $\beta$  expression in the liver.

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