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Effects of Different Ozone Doses Added to the Antibiotic Treatment on Cytokine Levels in Experimental Sepsis Model

Deneysel Sepsis Modelinde Antibiyotik Tedavisine Eklenen Farklı Dozlardaki Ozonun Sitokin Düzeylerine Etkisi

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Öz

Bu çalışmada E.coli endotoksini ile oluşturulan deneysel sepsis modelinde antibiyotik tedavisine eklenen ozon tedavisinin proinflamatuvar ve antiinflamatuvar sitokin düzeylerinde yaptığı değişiklikleri incelemeyi amaçladık. Her birinde 10 rat olan 6 deney grubunun 5 tanesinde intraperitoneal E.coli endotoksini injeksiyonuyla sepsis oluşturuldu. İlk 3 grupta antibiyotik tedavisine 0.6 mg/kg, 0.9 mg/kg ve 1.1 mg/kg dozlarında ozon tedavisi eklenirken, 4. gruba sadece antibiyotik tedavisi verildi. 5. gruba tedavi verilmedi. 6. gruba ise diğer gruplarla eş zamanlı olarak intraperitoneal serum fizyolojik enjeksiyonu yapıldı. Tüm tedavilere 5 gün boyunca devam edildi. 6. günde sakrifiye edilen ratlardan alınan kanların serumlarında IL-1, IL-10 ve TNF-alfa düzeylerini çalışıldı. Ozon tedavisi verilen tüm gruplardaki proinflamatuvar sitokin düzeylerinin (IL-1, TNF-alfa) diğer gruplara göre anlamlı olarak düşük olduğunu tespit edildi. Ozon tedavisi verilen grupların kendi aralarında yapılan karşılaştırmada, IL-1 düzeylerinde anlamlı bir fark olmadığı, TNF-alfa düzeylerinin ise daha düşük doz verilen iki grupta (0.6 mg/kg ve 0.9 mg/kg) yüksek doz verilen gruba (1.1 mg/kg) göre anlamlı olarak düşük olduğu gözlemlendi. Antiinflamatuvar bir sitokin olan IL-10'un serum düzeylerinde ise gruplar arasında anlamlı bir farklılık tespit edilmedi. Sonuç olarak, sepsiste antibiyotik tedavisine eklenen ozon tedavisinin inflamatuar süreci baskılayarak sağ kalım oranlarına olumlu etki yapabileceği sonucuna varıldı.

Anahtar Kelimeler: IL-1, IL-10, Sepsis, Ozon, TNF-alfa

Abstract

We aimed to examine the changes caused by ozone therapy added to the antibiotic treatment on proinflammatory and anti-inflammatory cytokine levels in an experimental sepsis model formed with E. coli endotoxin. Rats were divided into 6 groups of 10 rats. Sepsis was formed by dosing 5 groups of rats with intraperitoneal E. coli endotoxin injection. For the first 3 groups, 0.6 mg/kg, 0.9 mg/kg, and 1.1 mg/kg doses of ozone therapy were added to the antibiotic treatment and group-4 only received antibiotic treatment. Group-5 was not treated. Group-6 received intraperitoneal serum physiologic injection simultaneously with the other groups. All treatments were sustained for 5 days. IL-1, IL-10, and TNF-alpha levels were detected in blood serum taken from rats sacrificed on day 6. It was seen that IL-1, TNF-alpha levels are significantly lower than the levels in other groups that received ozone therapy. In the comparisons amongst the groups receiving ozone therapy, it was observed that IL-1 levels do not have a significant difference and TNF-alpha levels are significantly lower in the two groups receiving lower doses than the group receiving a higher dose. There were no significant differences detected between groups at serum levels of IL-10 which is an anti-inflammatory cytokine. It was concluded that ozone added to the antibiotic treatment in sepsis could have a positive effect on survival rates by suppressing inflammatory process.

Keywords: IL-1, IL-10, Sepsis, Ozone, TNF-alpha

Introduction

Sepsis is a systemic inflammatory reaction mediated by endogen mediators that affects all organs and systems (1).

It was seen that release of various metabolites such as tumor necrosis factor (TNF), interleukins, platelet activating factor (PAF), arachidonic acid metabolites in the etiopathogenesis of sepsis. Bacteria or bacterial endotoxin cause the secretion of cytokine from endothelial cells, macrophages and mono cysts. Proinflammatory cytokines; TNF, IL-1, IL-6, IL-8, interferon gamma (IFN-gamma) and anti-inflammatory cytokine IL-10 are the main cytokines that participate in the sepsis pathogenesis of soluble cytokine receptors. It was seen that during sepsis, these compounds reach high numbers in circulation (1, 2). However, these cytokines and their soluble receptors rise also in cases other than infection such as pancreatitis, trauma, burns, surgery and even heart failure. All moderate or severe bacterial infections cause the creation of protein and cytokines specific to the acute phase of inflammation. Changes in the density of acute phase proteins and cytokines may be used as signs of bacterial infections. However, due to the rise of cytokines also in nonspecific inflammation, the progression of sepsis, the fact that they do not indicate changes in the terminal period reliably and

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the high cost of and long duration needed for measurements, they are not used in routine diagnosis (3).

Endotoxemy is the most important reason for systemic inflammatory response (4). Endotoxin circulation activates the complementary system and ensures the secretion of cytokines and secretion of TNF-alpha and IL-6. Inflammatory mediators secreted from leukocytes cause hypotension, metabolic acidosis, damage tissues and cause organ disfunction (5, 6).

Endotoxemy and endotoxic shock are the main problems of intensive care units and have a high rate of mortality. Cardiovascular dysfunction is usually seen with endotoxemy and frequently resistant. Endotoxemy causes changes in proinflammatory and anti-inflammatory cytokine levels as a consequence of a series of events. Not only endotoxins but cytokines are also effective in endotoxic shocks and cardiovascular dysfunctions (7-10).

After being discovered by Christian Friedrich Schönbein in 1840, the medical use of Ozone increased worldwide and healthcare professionals were more interested in how Ozone was effective and what its benefits were. The number of Ozone therapists continues to increase worldwide and more patients benefit from this therapy day by day. On the other hand, it is a fact that it is not easy to be accepted by everyone, that there is a resistance against ozone therapy among the medicine community and that more and coordinated efforts are necessary in order for it to be functional in the legal area (11).

According to the Madrid Declaration on Ozone Therapy published in June 2010, sepsis and multiple organ failures are listed amongst the third category diseases which ozone could be applied. The same declaration also indicates that combined use of ozone could be beneficial for these diseases however there is a lack of factual proof regarding this issue.

In this study, we compared the effects of different doses of ozone therapy added to antibiotic treatment on cytokine levels in the experimental sepsis model.

Material and Method

After obtaining the approval of Marmara University Animal Experiments Ethical Committee (MÜHDEK) numbered 14.2011.mar dated 11.03.2011, the study on "Effects of different doses of ozone therapy added to antibiotic treatment on cytokine levels in the experimental sepsis model" was conducted in Marmara University Experimental Animals Laboratory.

Animals:

12-week old, 250-300g 60 female rats of Wistar albino strain were used in the study. They were housed to include 10 rats in each cage in the same conditions and fed with standard feed in controlled laboratory environment temperature.

Experimental Design:

In line with the study criteria, 60 rats were divided into 6 groups where n=10.

Group 1: Group to receive 0.6 mg/kg ozone therapy + antibiotic

Group 2: Group to receive 0.9 mg/kg ozone therapy + antibiotic

Group 3: Group to receive 1.1 mg/kg ozone therapy + antibiotic

Group 4: Group to receive only Antibiotics.

Group 5: Group that will receive no treatment after sepsis.

Group 6: Group to receive intraperitoneal serum physiologic.

Induction of Sepsis

Sepsis was formed in 50 rats from study groups 1, 2, 3, 4 and 5 with 2.1 x 109 CFU Escherichia (E.) coli (ATCC 25922) in 1 ml saline given intraperitoneally. Rats in Group 6 received 1 ml intraperitoneal serum physiologic.

Anti-biotherapy

Following the skin cleaning of rats 24 hours after creating sepsis with E.coli, implementation of the treatments that were planned according to the groups were started. Treatments were given daily and in one dose. 50 mg/kg cefepime was given as an antibiotic treatment (12). Ozone generator (Dr. Hansler Ozonasan Photonic) was used to produce ozone/oxygen mixture for ozone therapy. Medical ozone was applied intraperitoneally with 20 mcg/ml for five days. Every day we gave all doses of ozone by a single shot (0.6 mg/kg, 0.9 mg/kg, or 1.1 mg/kg)

Experimental procedure:

Treatments were given at the same time daily for 5 days. The application of only intraperitoneal serum physiologic was continued for Group 6 concomitantly. At the end of day 5, 2 ml blood was taken by tracheotomy from rats sacrificed by applying 100 mg/kg intraperitoneal thiopental.

In the serum of blood taken, IL1, IL10 and TNF-alpha levels were studied by using ELISA method. RayBio®Rat TNF-alpha (Catalog #: ELR-TNFa) and IL1 beta (Catalog #: ELM-IL1b) ELISA kits and eBioscience®Rat IL10 (Catalog #: E-EL-R0016) ELISA kit were used during this study.

Simultaneously, E. coli endotoxin was analyzed in the related serum by using endotoxin kit (ToxinSensor™ Endotoxin Detection System) in the spectrophotometer device (Beckman-Coulter DU 800).

Statistical analyses:

Data were analyzed statistically to determine the differences between groups. "Statistical Package for Social Sciences for Windows 17.0" (SPSS17inc) program was used to analyze the data obtained from the study. Descriptive statistical method (Mean, Standard Deviation) was used to analyze the study data. Whether the distribution was normal was tested by using Kolmogorov Smirnov test. Since the range of IL- 1 and TNF-Alpha values were not normal,

Kruskal-Wallis test was applied. As the range of IL-10 values were normal, One-Way Anova test was used to evaluate. Results were evaluated at 95% reliability interval and $p<0.05$ significance level.

Results

Firstly, a study on E.coli endotoxin was conducted on the serum of all experiment groups by spectrophotometric method. Values other than zero in concentration measurement were considered to indicate the presence of endotoxins. It was determined that Group 1, 2, 3, 4 and 5 had E. coli and absence of E.coli endotoxin in rats in Group 6 was confirmed. Then, interleukin levels in related serums were analyzed (Table 1).

Comparison of IL-1 Values

Median values of Group-1 (0.6mg/kg ozone+AB), Group-2 (0.9mg/kg ozone+AB) and Group-3 (1.1mg/kg ozone+AB) show statistical differences from the median values of Group-4 (only AB), Group-5 (not treated) and Group-6 (placebo). There was no significant difference statistically between the values of Group-1, Group-2 and Group-3 to whose antibiotic treatment ozone therapy was added (Figure 1).

Table 1. Comparison of serum IL-1, IL-10, and TNF-alpha levels of the groups after treatment.

	IL-1 (pg/ml) median (IQR)	**IL-10** (pg/ml) mean ± SD	**TNF-alpha** (pg/ml) median (IQR)
Group 1 (n=10)	11.00 (9.81-12.56)	148.51±36.49	0.52 (0.39-0.54)
Group 2 (n=10)	16.81 (12.94-18.64)	148.46 ± 12.96	0.51 (0.25-0.58)
Group 3 (n=10)	13.28 (11.45-14.61)	155.96 ± 22.96	0.77 (0.69-0.84)
Group 4 (n=10)	30.83 (27.09-32.03)	160.91 ± 28.78	1.50 (1.16-1.60)
Group 5 (n=10)	31.38 (29.29-34.94)	165.94 ± 28	2.03 (1.97-2.39)
Group 6 (n=10)	94.66 (92.47-99.83)	171.6±23.54	5.17 (4.83-5.51)
p-Value	0.001**	0.295*	0.001**

Data are presented as median ± Interquartile Range (IQR) for IL-1, and TNF-alpha levels. Data are presented as mean ± Standard Deviation (SD) for IL-10 levels. *OneWay Anova Test, **Kruskal Wallis Test

Comparison of IL-10 Values

According to the comparison between IL-10 values, it was identified that there was no statistical difference between the groups (Figure 2).

Comparison of TNF-alpha values

There were statistical differences between Group-4 (only AB), Group-5 (not treated) and Group-6 (placebo) with the values obtained from Group-1 (0.6 mg/kg ozone+AB), Group-2 (0.9 mg/kg ozone+AB) and Group-3 (1.1 mg/kg ozone+AB). It was found that the median values of Group 1 and Group 2 were lower than the median values of Group-3, Group-4, Group-5, and Group-6. It was seen that there was a statistical difference between Group-3 (1.1mg/kg ozone+AB) and other groups. The median value of Group-3 was higher than the median values of Group-1 and Group-2 and lower than the median values of Group-4, Group-5 and Group-6 (Figure 3).

Discussion

Ozone is a gas with a high oxidative potential. It shows bactericidal effect by disintegrating membranes by the oxidation of lipoproteins and phospholipids in bacterial cell membranes (13).

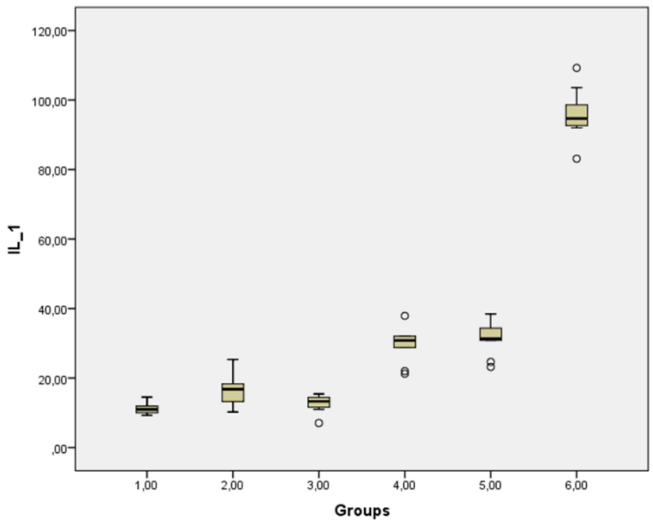

Figure 1. Comparison of serum IL-1 levels of the groups.

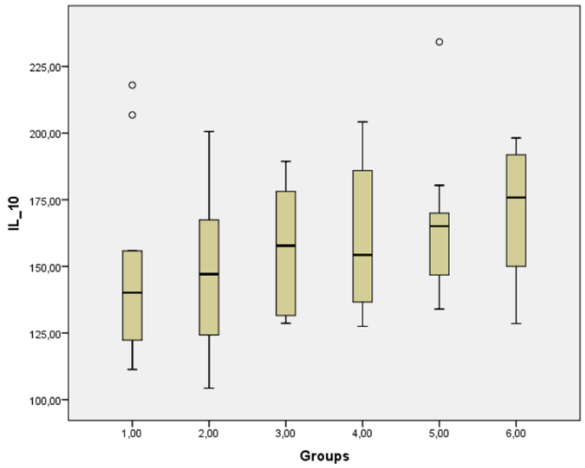

Figure 2. Comparison of serum IL-10 levels of the groups.

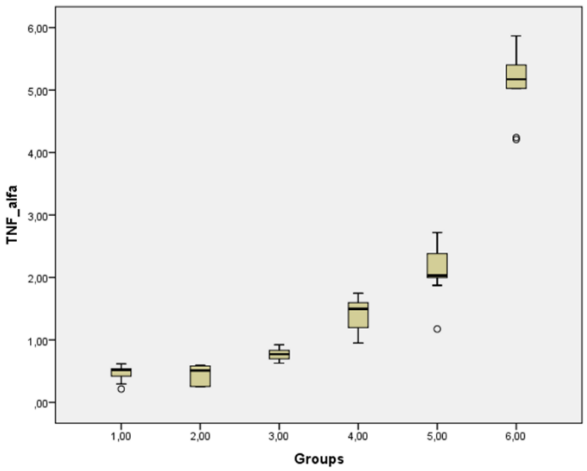

Figure 3. Comparison of serum TNF-alpha levels of the groups.

Use of ozone as a potent antimicrobial agent was subject of various studies (14-19). However, there are still not enough studies regarding the systemic use of ozone. Use of ozone on humans and animals is still being debated because of its side effects. These side effects are especially the effect on the formation of free radicals and its irritant effect on the respiratory system. For these reasons, the use of ozone therapy in clinical practice is quite limited and studies on this subject are mostly experimental. In the studies conducted despite all these limitations, effects of ozone on the immune system were detected and published. Regulation of the phagocytic activities of peritoneal and alveolar macrophages which are the first defense units against bacteria and toxins could be given as an example (20,21). While planning our study, we aimed at bringing a new perspective to the questions about the routine clinical use of ozone regarding the inflammatory process.

Main topics in sepsis treatment generally include source control, supplementary treatment and anti-biotherapy for the factor. In our study, we analyzed and compared different doses of ozone therapy added to the antibiotic treatment for rats in gram (-) sepsis on the levels of cytokine. We conducted our analysis on one anti-inflammatory (IL-10) and two proinflammatory (IL-1, TNF-alpha) cytokine levels.

In the literature, it is mentioned that intraperitoneal ozone injection directly effects peritoneal macrophages and modify its phagocytic activities (22). This is explained by the hypothesis that intraperitoneal ozone injection effects the production and secretion of proinflammatory cytokines in different abdominal organs just like the mechanism where in vitro ozonized blood increase the secretion of TNF-alpha, GM-CSF, IL-2 and IFN-alpha (23-25). Results of our study showed that ozone suppresses the proinflammatory process.

Zullyt B. Zamora et al. (26) analyzed the effect of ozone therapy on TNF-alpha secretion in a study with rats in endotoxic shock and found that TNF-alpha levels are lower in rats receiving ozone therapy. They applied ozone in three different doses (0.2 mg/kg, 0.4 mg/kg, 1.2 mg/kg) and for five days and found that TNF-alpha levels in rats receiving higher doses of ozone were lower. They reported that the effect of ozone applied to septic rats on TNF-alpha levels is caused by the antioxidant system stimulated by the ozone. We also applied three different doses of ozone (0.6 mg/kg, 0.9 mg/kg, 1.1

mg/kg) on rats for five days and found that TNF-alpha levels in groups receiving ozone are lower. However, in our results, TNF-alpha levels of the group receiving higher doses of ozone (1.1 mg/kg) was higher than the groups receiving lower doses. The fact that TNF alpha values are lower in two groups receiving lower doses of ozone may lead us to think that we should use ozone in the lowest doses possible. When the undesired effects of ozone are taken into consideration, it could be said that this comment may encourage the use of lower doses of ozone.

According to the common results of various studies conducted in 1990s, TNF-alpha secretion is a requirement for the secretion of many other inflammatory cytokines (26). We thought that we have to analyze the TNF-alpha parameter while planning our study.

Endotoxemy, sepsis and septic shock are cases related to the formation of reactive oxygen radicals. Formation of reactive oxygen radicals in high numbers causes a considerable increase in oxydative stress with lipid peroxydation in shock. A study shows that 100 mg/kg intraperitoneal lipopolisaccarid (endotoxin) injection to mice caused a marked increase in reactive oxygen radicals and an increase in TNF-alpha and IL-1 secretion from peritoneal leukocytes (27). We studied IL-1 levels as well as TNF-alpha levels as proinflammatory cytokine in our study groups.

Bette et al. (28) applied tazobactam/piperasilin antibiotic regime after creating pneumoperitoneum by ozone/oxygen mixture to wistar rats in sepsis and found an increase in the survival rates and a decrease in the levels of TNF-alpha and IL-1 which are proinflammatory cytokines. In our study, we applied ozone therapy by adding it to a standard antibiotic regime and we saw that ozone increased the activity of antibiotic in the suppression of proinflammatory process.

In another promising study on this subject, Schulz et al. (29) found that mortality rates decreased after intraperitoneal ozone therapy given in repeating doses to rats with polimicrobial peritonitis. In our study, no deaths were recorded for 5 days; however, according to our results we believe we can conclude that mortality in sepsis would decrease.

Rodriguez et al. (30) determined that ozone therapy given to the rats in which they created fecal peritonitis caused an increase in the levels of superoxide dismutase (SOD) and glutadionperoxidase (GPx) and interpreted this as the protective effect. We did not have a chance to analyze the levels of SOD or GPx. More detailed results could be obtained by taking our study one step further and analyzing levels of SOD and GPx.

Torossian et al. (31) obtained results from their study that could be interpreted somewhat differently. Torossian et al. applied ozone therapy to rats induced with peritoneal sepsis for 5 days and determined that survival rate decreased from 50% to 35% and in addition to that there was an increase of TNF-alpha and macrophage inflammatory protein levels in rats receiving ozone therapy. Therefore, authors published that the ozone therapy is proinflammatory by interpreting it. This study claims opposite results to our study and our source studies.

In our study we also examined the levels of anti-inflammatory cytokine, IL-10. IL-10 peak could be observed 24 hours after systemic damages (32,33). IL-10 production is known as a part of the protective mechanism which suppresses the induction of TNF-alpha and IL-1 which are known as proinflammatory cytokines. IL-10 secretion is stimulated by monocytes and macrophages during sepsis (34,35). Sewnath et al. (36) determined that endogen IL-10 plays a role in host's local bacterial defense mechanism and SIRS development during abdominal sepsis. Accordingly, they stated that IL-10 increases the bacterial clearance. We saw in the results we achieved that there is no statistically meaningful difference of IL-10 levels between groups. When the mean values are analyzed directly, we saw that values of the groups receiving ozone therapy are slightly lower than values of other groups, however, this difference was not significant. As stated above, IL-10 peak during sepsis is seen at the end of first 24 hours. Our study was 5 days long. Serums we used were taken from rats at the end of day 5. We think that lack of a significant increase in anti-inflammatory cytokine levels proves that we were able to suppress the proinflammatory process by the treatment we gave.

The fact that we analyzed the presence of E. coli endotoxin in the serums in our study increases the reliability of our study as it shows that we were able to form sepsis in rats.

Many biological markers (such as complement system activation, chemokines, and oxygen radicals) are effective in the diagnosis and follow-up of sepsis. We used only IL-1, IL-10, and TNF-alpha in our study. Therefore, the most significant limitation of our study is that we worked with a limited number of biomarkers.

In conclusion, in our study in which we analyzed the effects of three different doses of ozone added to the antibiotic treatment for rats on cytokine levels in experimental sepsis model, we determined that;

a. IL-1 and TNF-alpha levels in all groups receiving ozone therapy were statistically significantly lower than the groups receiving no therapy and receiving only antibiotic treatment,

b. There was no statistical difference in IL-1 values between three groups receiving ozone therapy,

c. TNF-alpha levels were not statistically different from each other in two groups (0.6 mg/kg and 0.9 mg/kg) receiving lower doses of ozone

therapy, but significantly higher in the group receiving high doses of ozone (1.1 mg/kg),

d. IL-10 levels were not statistically significantly different in all treatment groups.

According to these results, we think that ozone therapy added to classic treatments in the treatment of sepsis which affects all organs and systems and which is a significant cause for mortality in intensive care units could supplement the treatment by decreasing the severity of the systemic inflammatory response.

Ethics Committee Approval: This study was approved by Marmara University Animal Experiments Ethical Committee (approve date: 11.03.2011, approval number: 14.2011).

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