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The effect of ozonized saline solutions processed under intense electric fields in the treatment of infected necrotizing acute pancreatitis: An experimental mode

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The aim of this study was to study the effect of ozonized saline solutions administered intraperitoneally in rabbits suffering from severe acute pancreatitis with infected necrosis. The acute pancreatitis was induced by infusion of sodium taurocholate into the main pancreatic duct and in the next 24 h, a cecal fistula was created to obtain infection of the pancreatic necrosis with intestinal germs. The rabbits were divided into 3 groups of study and each of them underwent continuous peritoneal lavage 2 times a day for 5 days with simple saline (control group), ozonized saline solution 5 mg/L (group A) and respectively ozonized saline solution 9 mg/L processed under an intense electric field (group B). Serum level of C-reactive protein and total peroxides, bacterial content of the peritoneal liquid and tissue specimens from the pancreas and the peripancreatic area were evaluated. Six days after the induction of pancreatitis all surviving animals were sacrificed. Serum levels of C-reactive protein and total peroxides in rabbits treated with ozonized saline solution were significantly lower as compared to those of rabbits belonging to the control group. Regarding bacterial growth, a significant decrease was obtained in the peritoneal liquid or even no bacterial growth in groups A and B. Histological examination of the tissue showed specific pancreatic changes in all the groups, but less expressed in groups A and B. No significant differences were encountered between rabbits treated with different ozone concentrations. Intraperitoneal ozone therapy is effective in the amelioration of acute pancreatitis by means of laboratory analysis and decreased bacterial growths.

Key words: Severe acute pancreatitis, ozone-therapy, infection of pancreatic necrotic tissues, intense electric fields.

INTRODUCTION

Acute pancreatitis, one of the most frequent gastroenterological diseases, is a leading cause of morbidity and mortality, still having a major economical and emotional impact. Despite later progress in understanding its mechanisms and improving the management strategies, its prognosis did not change significantly as the mortality rates are still high (10 to 50%) (Bhatia, 2002).

Generally, the disease progresses in two phases. The early phase, in the first week, is characterized by a systemic inflammatory response syndrome (SIRS) where the

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late phase is characterized by the presence of complications and it appears only in severe forms of pancreatitis. Infectious complication of the necrotic tissues that occurs in 50% of the cases of severe acute pancreatitis is the leading cause of death, responsible for 70 to 80% of all deaths due to acute pancreatitis (Schmid et al., 1999). Failure in prevention of infection and management of sepsis (Dellinger et al., 2007; Marincaş et al., 2006) shows the need for new therapeutic agents.

Used initially for potable water treatment, ozone is now regarded as one of the most powerful oxidating agents having important antiseptic and antibacterial effects (Bialoszewski et al., 2010).

Proven antibacterial properties *in vitro*, ozone is recommended for application in medicine as an adjunct or alternative treatment to combat various local or systemic infections, especially in those cases where traditional therapy has not given satisfactory results (Bocci, 2007; Dyas et al., 1983; Bialoszewski and Kowalewski, 2003; Lipatov et al., 2002; Silva et al., 2009; Parkhisenko and Glukhov, 2001). Use of ozone in medicine remains controversial because in high concentrations in gaseous form, the compound has a toxic effect on the human body, more pronounced in the respiratory system (Guanche et al., 2010; Bocci et al., 2009). On the other hand, the use of aqueous ozone in different environments, in low concentrations has a beneficial effect on the body both by stimulating host defense mechanisms, and through a direct antimicrobial effect (Oizumi et al., 1998).

Although *in vitro* antibacterial activity of O_3 is obvious even just a few minutes after application (Bocci, 1996), its short life and limited concentration that can be obtained in aqueous solutions significantly decreases its potential action *in vivo*. Applying a high frequency current between certain parameters of intensity, pulse shape, temperature and time of action upon aqueous solutions or upon biological materials generates ozone by corona discharge effect (Kogelschatz and Eliason, 1995).

This study aimed to investigate the anti-inflammatory and antibacterial effects of ozonized saline solution on an experimental model of severe acute pancreatitis infected with intestinal flora. In different concentrations of ozone, maximally ozonized saline solution produced by ozonator will be compared with similar solution further processed in intense electric fields to assess possible differences between their therapeutic effects.

MATERIALS AND METHODS

Surgical interventions in this study were performed at the Center for Experimental Medicine of the University of Medicine and Pharmacy "Iuliu Haţieganu" Cluj-Napoca. The Ethical Commission of “Iuliu Hatieganu” University of Medicine and Pharmacy Cluj-Napoca approved the experimental procedures of the study. A total of 30 Belgian white rabbits were included in the study, weighing 2400 to2950 g housed in standard individual cages, with an average temperature of 22°C.

Induction of acute pancreatitis

In all 30 rabbits, we induced severe acute pancreatitis by injecting sodium taurocholate in the main pancreatic duct, under high pressure (Kudari et al., 2007). The animals received intramuscular acepromazine 1%, 10 mg/kg for sedation and after 10 min; anesthesia was induced by intramuscularly administration of a mixture of xylazine with ketamine (dosages of 10 and 50 mg/kg body weight, respectively). The maintenance during surgery was obtained by intravenous administration of xylazine and ketamine, with a dosage of 5 mg/kg each, oxygen supply through a mask for spontaneous breath.

A median subxiphoidian incision of around 10 to 12 cm was done followed by identification of the main pancreatic duct at approximately 2 cm from the pylorus near the opening in the jejunum and cannulation with a 30 G needle. A total of 2 ml of 5% sodium taurocholate in saline was slowly infused. The intestinal loops were reintroduced in the peritoneal cavity and a double layer laparoraphy was performed with continuous suture using absorbable thread (Mersilene 1). After surgery, the animals were reintroduced in cages with free access to food and water.

Infection of the necrotizing acute pancreatitis

After 24 h, experimental animals were subjected to a new laparotomy under same anesthesia. Induction of intraperitoneal infection was achieved by creating a cecal fistula with a 16 G needle. Viscera were reintroduced inside the peritoneal cavity.

Treatment groups

A system of wash-drainage of the peritoneal cavity consisting of 2 silicone tubes 18 Ch placed near by the pancreas was performed. The 30 rabbits with acute pancreatitis and cecal fistula were randomized into 3 groups and subjected to peritoneal lavage treatment as follows: Control group of 6 rabbits who underwent intraperitoneal lavage with isotonic saline solution (saline); group A of 12 rabbits undergoing intraperitoneral lavage with 5% ozonized saline solution; group B of 12 rabbits who underwent intraperitoneal lavage with ozonized saline processed in intense electric fields.

Peritoneal lavage was initiated 2 h after the creation of the cecal fistula being performed twice a day (every 12 h) for 4 days, 200 ml per session. Duration of each lavage session was 10 min with the solution maintained at a temperature of 36°C.

Ozonized saline solution 5% for group A was obtained in an ozone generator type COM-AD-01-IP (Anser, Germany) with an ozonometer type GM-6000-OEM (Anser, Germany) regulating the inflow of 100% oxygen, a flux of O_2 flow=50 l/h, at a 0.2 atmospheric pressure of oxygen, bubbling time of O_3 in aqueous solution=10 min at 20 to 22°C.

For group B, saline solution (normal saline) was used in which ozone produced by the generator (concentration 5 mg/l) was have bubbled, and was immediately subjected to a corona discharge in an oxygen environment using an alternating power (U = 20 kV, I = 0.1 mA and a discharge gap of 20 mm), thus obtaining a concentration of ozone of about 9 mg/L.

Sample collection (blood, histology, cultures)

Five days after the second laparotomy, all animals were subjected to a new intervention. After onset of anesthesia, blood samples (2 ml) were obtained from each rabbit by venous puncture of one of the limbs. Pancreatic and peripancreatic tissue was taken for histopathological examination and intraperitoneal fluid samples for bacteriological examination. All animals were sacrificed afterwards receiving an overdose of anesthetic agent.

Table 1. Score of histopathological modifications.

Score	0	1	2	3
Edema	Absent	In the interlobular septum	Mild or interacinar	Diffuse
Hemorrhage	Absent	In the interlobular septum	Mild or interglandular	Diffuse
Necrosis	Absent	In 1-2 lobules	In 3-4 lobules	More than 5 lobules
Leukocytes infiltration	Absent	In 1-2 lobules	In 3-4 lobules	More than 5 lobules
Citosteatonecrosis	Absent	Mild	Moderate	Severe
Fibrosis	Absent	In 1-2 lobules	In 3-4 lobules	More than 5 lobules

Hematological and biochemical analysis were performed in the laboratory of medical analysis at the Cluj-Napoca Rehabilitation Center, bacteriological examinations in the Department of Microbiology of University of Veterinary Medicine, Cluj-Napoca.

C-reactive protein and the total amount of peroxides for indirect assessment of oxidative stress was investigated. Identification of the pathogenic agents was performed by inoculating the samples on sheep blood nutrient agar and Mueller Hinton agar and then incubated at 37 ±10°C for 24 h. Following the differentiation between morphology, culture and biochemical preliminaries, colonies were passed on API galleries (API Staph, API 20 Strep, API 20E, API 20 NE). Galleries were incubated at 37 ±10°C for 24 h and then were read by the APIWEB program. Afterwards, they were included under different categories of species according to the codes obtained on reading of the galleries. The tests used to identify each type of microorganism were mannitol fermentation test, citrate plasma clotting test, catalase test and oxidase test.

Histopathological examinations were performed in the Department of Pathology of the University of Veterinary Medicine, Cluj-Napoca. They analyzed macroscopic changes occurring in pancreatic and peripancreatic tissues and in the intraperitoneal fluid.

Tissue specimens were fixed in formalin (10%, pH=7) for 24 h, then embedded in paraffin and cut into 4-µm; slides were stained with hematoxylin and eosin. Microscopic examination described specific histological features found in acute pancreatitis: edema, inflammatory infiltration, fat necrosis, parenchymal necrosis and hemorrhagic vascular lesions and fibrosis. Microscopic images were obtained with an Olympus BX 51 microscope, captured with an Olympus SP 350 digital camera and processed using the "Cell B software" program.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Student t test was used to assess statistical significance between groups; correlations between different variables were studied using Mann-Whitney U test (SPSS software). P values were considered statistical significant at <0.05.

RESULTS

The observation period for each animal included in the study was 6 days. Out of the six rabbits in the control group, two died (33.3% death rate). One of them died 30 h after induction of pancreatitis (6 h after the cecal fistula) and the other on the third day after creation of the cecal fistula. In group A, two rabbits died on the 4 and 5th days (death rate 16.6%) while in group B there has been a single death, on the 4th day (8.33% death rate) from the beginning of the study.

Serum levels of C-reactive protein, analised on the 6th day from the induction of acute pancreatitis, were significantly lower in groups A (0.5083±0.267 vs. 1.140±0.114 mg/dl, p=0.0001) and B (0.550±0.124 vs. 1.140±0.114 mg/dl, p=0.0001) than controls, but without statistical significance between the two groups treated with ozonized solution. The values of total peroxides were significantly lower in groups A (758.08±196.75 mmol/L, p=0.021) and B (627.716±171.95, p=0.001) compared to the control group, without significant differences in antioxidant property of the two ozonized solutions.

Bacteriological examination of intraperitoneal fluid harvested five days after contamination of the pancreatic necrotic tissues showed the presence of infection in all surviving rabbits in the control group. The organisms identified in various combinations include *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Bacterial cultures obtained from two rabbits in group A and three rabbits in group B were negative. In all positive samples obtained from groups A and B, infection was monomicrobial or with two germs, unlike the control group where we found up to four different species in the same sample. Thus, peritoneal contamination was significantly lower in group A (p=0.002) and group B (p=0.001) compared to the control group. No statistical significance was found between the number of bacterial strains in peritoneal fluid from animals in groups A and B (p=0.603), and no absolute antibacterial effect of ozonized saline drips were observed on any of the four bacteria present.

The global effects of the two ozonized solutions tested on necrotic acute pancreatitis infected with intestinal flora were assessed by means of pancreatic histopathological changes. Histopathological criteria followed were edema, hemorrhage, leukocyte infiltration, pancreatic necrosis, peripancreatic fat necrosis and fibrosis (Yilmaz et al., 2009). Each parameter received a grade from 0 to 3 (Table 1), and by adding the score, total histopathological score was obtained. Histopathological changes as edema, hemorrhage and necrosis of pancreatic parenchyma were significantly more pronounced in the control group than the two other groups. Regarding the fibroblast proliferation, there were no major differences between

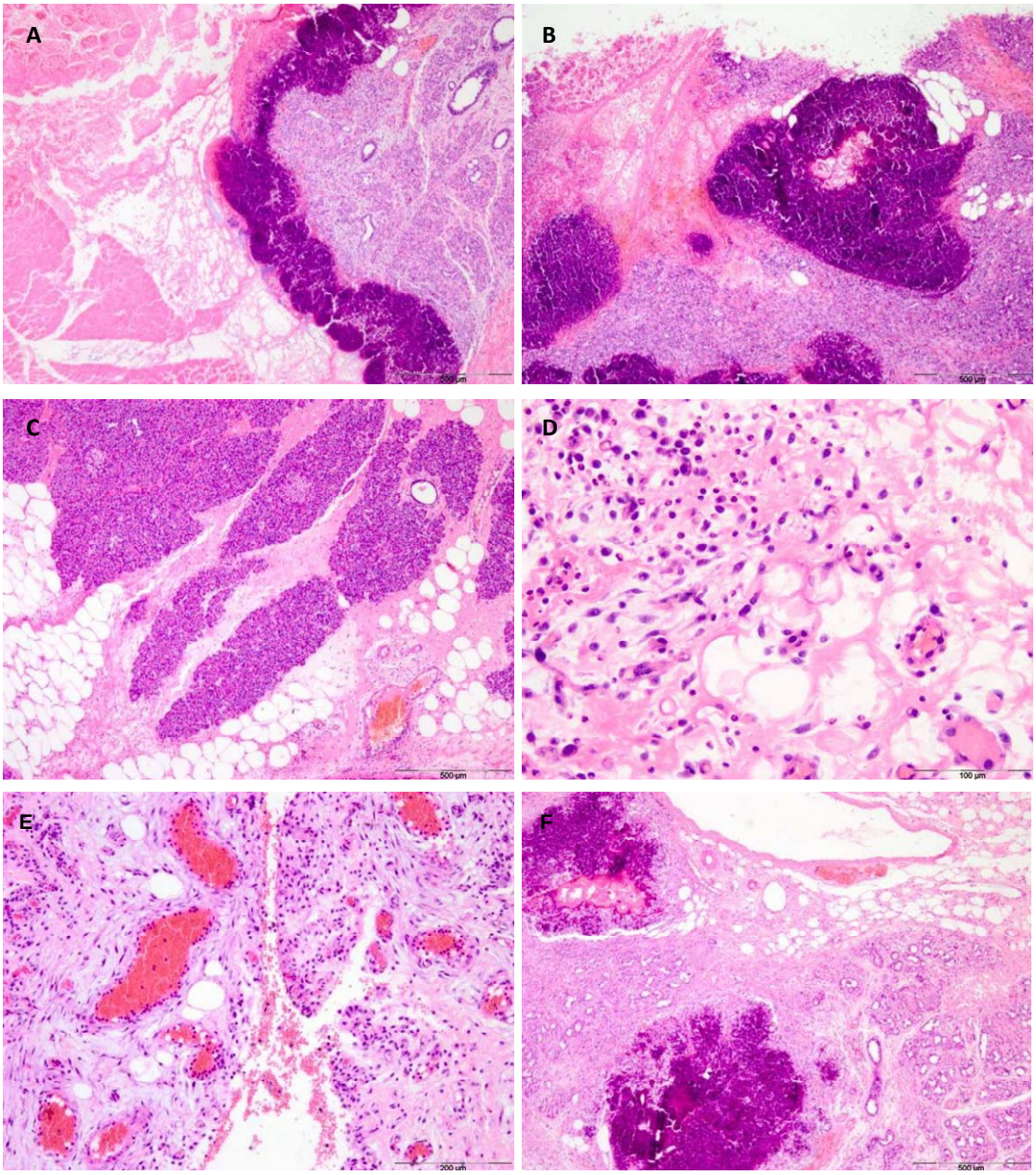

Figure 1. Histopathological aspects of pancreatic and peripancreatic tissues belonging to the rabbits in the control group I, without treatment: (A) diffuse parenchymal necrosis, severe (*), demarcated from normal tissue by a band of neutrophilic and macrophagic leukocyte, HE Bar = 500, (B) necrotico-purulent foci with abscess formation, inflammatory edema, hemorrhage, massive infiltration of neutrophils and macrophages, HE Bar = 500, (C) interlobular, interstitial and intraglandular serofibrinous exudates with parenchymal dissociation, HE Bar = 500, (D) diffuse peripancreatic citosteatonecroză with neutrophilic and macrophagic infiltration, HE Bar = 100, (E) septal fibroblast proliferation at interlobular and interacinar level with parenchymal atrophy, vacuolar degeneration of pancreatic cells, moderate inflammatory infiltrate with neutrophils and rare eosinophils, HE Bar = 100, (F) congestion, edema, leucodiapedesis and perivascular fibroplasia in the peripancreatic tissues (bar, 200 µm).

the groups. Overall, the pancreatic and peripancreatic histological characteristics were statistical significant more altered in the control group than the group A (U=0, p=0.001) and B (U=0, p=0.001) (Table 1, Figures 1A, B, C, D, E, F, 2A, B, 3A, B, C, and D).

DISCUSSION

The most important factor in the evolution of acute severe pancreatitis is infection (Bourgaux et al., 2007). Mortality associated with infection of the pancreatic and

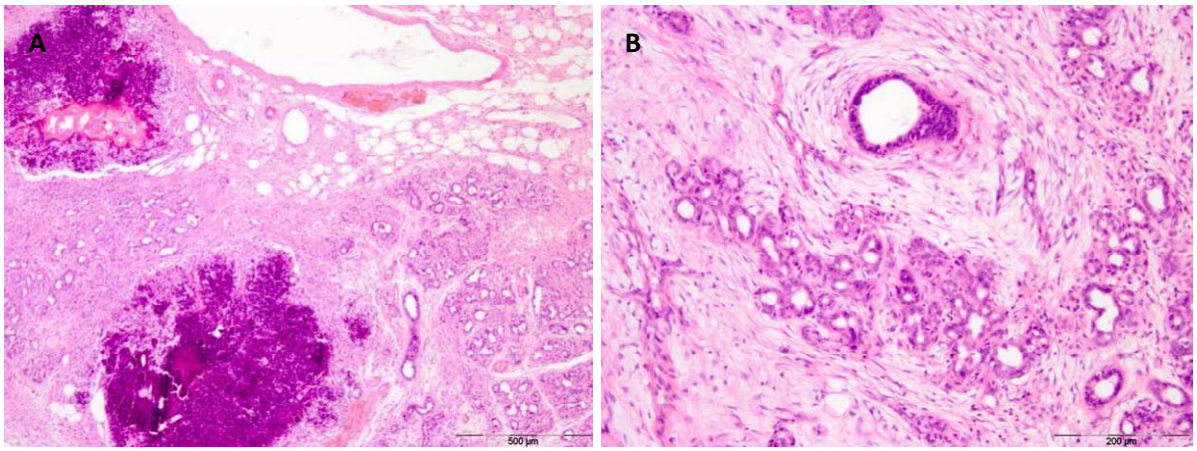

Figure 2. Histopathological aspects of the pancreatic tissues of the rabbits belonging to the study group treated with ozonated saline: (A) focal parenchymal necrosis with the formation of microabceses, edema, hemorrhage, mild neutrophilic inflammatory infiltrate and fibroblast proliferation in adjacent areas, HE Bar = 500, (B) fibroblast proliferation and edema at interlobular interstitial and intraglandular level with ductal swelling, reduced inflammatory infiltrate of neutrophils and macrophages, HE Bar = 200.

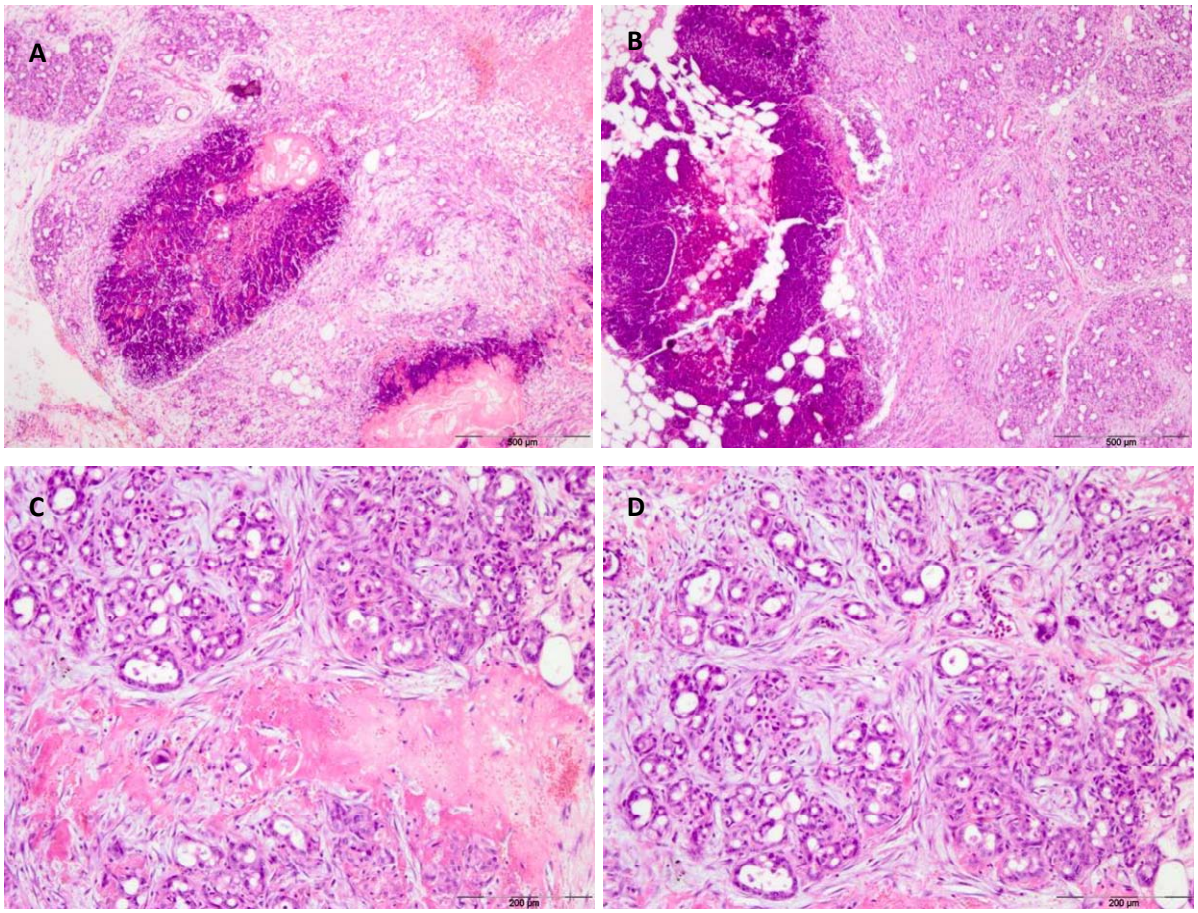

Figure 3. Histopathological aspects of pancreatic tissues of the rabbits belonging to the study group B treated with ozonated saline processed in intense electric fields: (A and B) focal parenchymal necrosis, edema, hemorrhage, moderate neutrophilic inflammatory infiltrate and fibroblast proliferation in adjacent areas, HE Bar = 500, (C) focal parenchymal necrosis, edema, hemorrhage and discrete inflammatory infiltrate, HE Bar = 200, (D) fibroblast proliferation edema at interlobular interstitial and intraglandular level with swelling of the ducts, HE Bar.

peripancreatic necrotic tissues remains high, despite administration of expensive but highly active broad spectrum antibiotics (De Waele et al., 2004; Lilja et al., 2008). Finding new means of treatment to control infection of the necrotic acute pancreatitis is still a challenge today.

Ozone is a known oxidizing agent in aqueous solutions and a reliable antimicrobial agent. In acute pancreatitis with infected necrosis, peritoneal lavage is very important; as it reduces the microbial load as well as the amount of inflammatory mediators arrived in the extravascular space along with the reduction of intra-abdominal pressure (Georgescu et al., 2005; D'Egido, 1991). In this study, we obtained a significant reduction in C-reactive protein levels and total peroxides amount in those animals treated with ozone compared with the animals belonging to the saline control group. C-reactive protein (CRP) is a simple, cheap and reliable test for indirect assessment of severity of acute pancreatitis offering a good prognostic accuracy for pancreatic necrosis and mortality (Cardoso et al., 2013).

These results correlate with the histopathological changes occurring in the pancreas. Potential augmentation of the inflammatory process and oxidative stress caused by a larger amount of free oxygen radicals corresponding to a higher concentration of ozone was not confirmed between groups treated with ozonized saline 5 and 9% as this was not statistical significant. The continuous peritoneal lavage with ozonized solution which reduces the action of free oxygen radicals has a beneficiary effect on microcirculation and activation of the immune system (Bourgaux et al., 2007).

Various studies have investigated the antibacterial effect of ozone in infectious diseases, with the premise that reactive oxygen species generated by the interaction of ozone with pathogenic microorganisms is the most natural and effective antimicrobial agent. In conditions such as abscesses, perianal fistulas, furunculosis, osteomyelitis, vulvovaginitis, and necrotizing fasciitis, ozone therapy had dramatic effects on eradication of the infection and on fastening the healing (Madej et al., 1995). In our study, ozonized saline drips showed a significant antibacterial activity. Administered intraperitoneally, ozone significantly reduced the number of bacterial strains from infected pancreatitis in the groups treated as compared to the control group. Higher concentration of ozone used in animals belonging to group B showed a more pronounced bactericidal effect as compared to 5% ozonized serum used in group A (but not statistical significant).

A research group showed in a study that ozone reduced the severity of pancreatitis and prevented bacterial translocation to the pancreas, liver, cecum, and peritoneum besides improving the survival rates (Uysal et al., 2010). There is only one human study about the efficacy of ozone treatment in acute pancreatitis (Kopchak et al., 2008).

Conclusions

In this study, all the biochemical and histological data indicate that ozone therapy had ameliorative effects on acute pancreatitis. Intraperitoneal continuous lavage with saline drips processed in intense electric fields has real benefits in acute pancreatitis with infected necrosis through significant antimicrobial action, consecutively diminishing tissue injury induced by inflammation mediators. Further research needs to be done regarding different concentrations of ozone related to increased antiinflammatory and antibacterial effect.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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