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Effects of antibiotic and intra-peritoneal ozone administration on proinflammatory cytokine formation, antioxidant levels and abdominal organ functions in the treatment of experimentally generated infectious peritonitis in rabbits

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Abstract: In this study we investigated the effects of antibiotic and intraperitoneal ozone administration on pro-inflammatory cytokine, antioxidant levels and tissue damage in the treatment of experimentally infectious peritonitis. Thirty-three adult male New Zealand White Rabbits were used. The study consisted of four groups including the non-treatment group (G_1), antibiotic group (G_2), ozone group (G_3) and ozone + antibiotic group (G_4). Trimethoprim sulfadimethylprymidine was administered at a dose of 50 mg/kg subcutaneously (*s.c.*) and amoxicillin sodium at a dose of 15 mg/kg intramuscularly (*i.m.*). Medical ozone was administered intraperitoneally (*i.p.*) at a concentration of 30 μg O_3/ml and dose of 80 ml/kg. Once peritonitis was produced, blood samples were taken from the animals before treatment and at regular intervals following treatment. Blood samples were used for haemograms and to measure levels of antioxidant and oxidative enzymes and pro-inflammatory cytokine levels. Tissue samples were examined histopathologically. There was no statistically significant difference between groups with respect to levels of pro-inflammatory cytokines. Antioxidant enzymes were found to be higher in groups G_2 and G_3. The granulocyte and lymphocyte values in group G_3 were determined to increase earlier than in the other groups. The peritonitis scores were similar in G_1 and G_3, which is higher compared to G_2 and G_4 groups. Minimal tissue damage was observed in the group G_2. It was concluded that antibiotic use for preventing peritoneum damage in experimental acute peritonitis was more effective than ozone therapy alone.

Keywords: ozone; tissue damage; septic peritonitis; rabbit

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Ozone is a 3-atom molecule, formed as a result of exposure of oxygen to high-energy electric current and ultraviolet (UV) rays. It has bactericidal, virucidal and fungicidal effects. Therefore, it is used to neutralize both microorganisms and to activate the organism's antioxidant systems. Ozone treatment is the administration of low concentration oxygen and ozone mixture to the patient using various methods (Nogales et al. 2008; Silva et al. 2009). It is used in the correction of many pathological conditions such as peritonitis, pulmonary and pleural diseases and pancreatitis (Li et al. 2007; Nogales et al. 2008).

Infectious peritonitis cases are inflammations leading to intestinal perforation caused by various reasons such as trauma or inflammatory diseases and have a high mortality rate. Endotoxins (lipopolysaccharides) released from the bacterial wall initially alert the macrophages in the spleen and liver. This warning starts the synthesis of pro-inflammatory cytokines such as tumour necrosis factor (TNF-α) and interleukin-1β (IL-1β). Pro-inflammatory cytokines cause inflammatory and immune responses. Next, it reaches other tissues and organs via blood circulation and causes systemic cytokine release. As a result, septic shock develops and multiple organ damage occurs (Schulz et al. 2003; Tracey 2007).

In peritonitis cases, while there is a significant decrease in acitivities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), there is a rise in levels of free radicals such as malondialdehyde (MDA). Following infectious peritonitis, disruptions to the liver and kidneys also occur. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine (Cre) activities in blood serum increase significantly (Rodriguez et al. 2009).

Infectious peritonitis cases are usually treated with fluids and wide spectrum antibiotics. In recent years, it has been reported that intraperitoneal ozone administration shows effect in the treatment of infectious peritonitis by increasing antioxidant enzyme amounts and decreasing levels of free radicals (Li et al. 2007; Madej et al. 2007; Nogales et al. 2008; Rodriguez et al. 2009; Souza et al. 2010; Azuma et al. 2014).

Information is limited regarding the effects of intraperitoneal administration of ozone both on the whole organism and on individual abdominal organs and tissues (Silva et al. 2009; Souza et al. 2010). Among earlier researches, no study was found regarding a combined administration of antibiotic and intraperitoneal ozone in the treatment of infectious peritonitis.

The aim of this study is to investigate the histopathological effects of antibiotic and intraperitoneal ozone administration on pro-inflammatory cytokine production, antioxidant levels and tissue damage in the treatment of experimentally generated infectious peritonitis.

MATERIAL AND METHODS

Animals. This study comprised 33 adult male New Zealand white rabbits with a mean age of 1 year and 3–3.5 kg body weight, reared at Uludag University Applied Research Center for Experimental Medicine. The study was carried out under approval No. 110/2010 dated July 29, 2010 gained from the Istanbul University Experimental Animals Local Ethics Committee.

During the experiment, all rabbits were housed in individual cages at 21 °C room temperature, under 12-hour daylight/12-hour darkness and given water and pellet rabbit food (Eris Yem, Istanbul, Turkey) *ad libitum*. Prior to commencing the study, one week was allowed for the rabbits to adapt to the environment.

Experimental groups. The animals were split into four separate groups (n = 8 per group). One of the rabbits was used as a donor for faecal material (32 + 1 = 33).

Group 1 (Non-treatment group – G_1):

No administration was carried out following faecal contamination.

Group 2 (Antibiotic group – G_2):

Combined antibiotics effective against both Gram (+) and Gram (–) bacteria were administered following faecal contamination. For this purpose, trimethoprim/sulfa methoxazole (Favetrim, Vilsan, Turkey) was administered at a dose of 50 mg/kg *s.c.* and clavulanic acid/amoxicillin sodium (Synulox, Pfizer, Turkey) at a dose of 15 mg/kg *i.m.* q12h for 5 days.

Group 3 (Ozone group – G_3):

Following faecal contamination, ozone was administered intraperitoneally (*i.p.*) at a concentration of 30 μg O_3/ml and dose of 80 ml/kg via the right lower abdomen (Viebahn-Hansler et al. 2012). This administration was repeated every 12 hours for 5 days.

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Group 4 (Ozone + Antibiotic group – G_4):

Following faecal contamination, a combination of antibiotic and intraperitoneal ozone administration was given to all cases. Both administrations were continued at the above doses once every 12 hours for a duration of five days.

Medical ozone, composed of a mixture of 5% ozone and 95% oxygen, was obtained from the ozone generator (Humazona® GmbH, Bruchsal, Germany) and was used immediately.

At the end of the study all rabbits were sacrificed via intravenous (*i.v.*) injection of pentobarbital sodium at a dose of 80 mg/kg. The sacrifications were performed after the last blood samples were taken.

Surgical procedure for the donor rabbit and microbiological analysis. The donor rabbit was starved for approximately four hours before the operation. General anaesthesia was achieved with slow *i.v.* injection of propofol (Propofol 1% Fresenius® 200 mg/20 ml, Sweden, 10 mg/kg) via a catheter inserted into the lateral ear vein.

Following necessary asepsis-antisepsis of the surgical site, median laparotomy was performed to enter the abdominal cavity, the caecum was reached and the required faecal material was collected. The caecum and abdominal cavity was closed using routine surgical procedure.

As a result of the bacteriological examination of the caecum sample, the following were isolated: *Kurthia* spp. (aerobic and Gram +), *Bacillus* spp. (aerobic and Gram +), *Lactobacillus acidophilus* (Gram +, anaerobic), *Bacteroides ureolyticus* (Gram – and anaerobic), *Bacteroides uniformis* (Gram – and anaerobic). Suspensions including 1.5×10^8 CFU/ml (McFarland 0.5) of bacteria were prepared from each isolate and used to produce experimental peritonitis.

Generating peritonitis. In order to produce diffuse peritonitis in all groups, faecal suspension was administered into the median abdomen using a sterile syringe and approximately 0.5 ml for each subject.

Assuming that an immune response would occur in relation to anaesthesia and the surgical procedure, and that this could affect study findings, neither anaesthesia nor any surgical intervention was carried out in the experimental groups during injection of the faecal suspension.

Biochemical analysis. Prior to the study, blood was collected from the jugular vein of all rabbits and the following indicators were analysed: erythrocytes (RBC), haematocrit (HCT), haemoglobin (HGB), total leukocytes (WBC), lymphocytes, granulocytes, AST, ALT, Cre, TNFα and IL-1β, SOD, CAT, GSH-Px and MDA. The time of measurement was determined as 0 hour.

Following induction of peritonitis, haemogram, serum MDA, SOD, CAT, GSH-Px, ALT, AST and Cre measurements were determined in blood samples collected at 12 h, 24 h, 48 h, 72 h and 120 h, while serum TNF α and IL-1β levels were determined in blood samples collected at 6 h, 12 h, 18 h and 24 hours. Separated blood serums were kept at –80 °C pending analysis.

Cytokine levels were assessed using the ELISA (Diagnostic system laboratories, inc. Webster, Texas, USA) method in keeping with the instructions of the producing company.

AST, ALT and Cre levels were measured by spectrophotometric method using an automatic biochemistry analyzer (Tokyo Boeki TMS-1024, Tokyo, Japan).

Plasma thiobarbituric acid reactive substances (TBARS) were determined using the method of Yoshoiko et al. (1979). The assay was based on the reaction of two molecules of thiobarbituric acid with one molecule malondialdehyde. This formed a coloured complex with a maximum absorbance at 532 nm. Plasma Cu-Zn superoxide dismutase SOD activity was determined according to method of Sun et al. (1988) by inhibition of nitroblue tetrazolium (NBT) reduction with xanthine-xanthine oxidase used as a superoxide generator. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. CAT activity was determined by modified method described by Yasmineh et al. (1995). The assay was based on the decomposition of H_2O_2 in buffer by catalase enzyme in the plasma. GSH-Px activity was measured using spectrophotometric kits in accordance with the manufacturer's instructions (Glutatyon Peroxidase Randox (Ransel) cat No: RS506) This method is based on that of Paglia and Valentine (1967).Glutathione Peroxidase (GPX) catalyses the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH the oxidised Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm is measured.

Histopathological analysis. For histopathological analysis, specimens were collected from the ab-

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dominal wall including the peritoneum, intestines, kidney, spleen, lung and heart. The specimens were fixed in 10% formal saline solution. After undergoing necessary laboratory procedures, these were embedded in paraffin blocks. Next, using a rotary microtome, they were cut at a thickness of 3–5 μm, stained with Haematoxylin and Eosin (H&E) and examined under a light microscope. In addition, Masson's Trichrome stain were applied to the peritoneum slides.

Statistical analysis. Differences between groups were determined in terms of all parameters measured in the blood samples collected before and after treatment.

In the statistical evaluation of TNFα, IL-1β, MDA, SOD, CAT, GSH-Px, AST, ALT, Cre lymphocyte and granulocyte data, repeated measures of ANOVA and contrast test methods were used to determine the influences of group and measurement time. In the statistical model, groups G_1, G_2, G_3 and G_4 "between-subject factor" measurement time appeared as "within subject factor". One-way analysis of variance and the Duncan test was used to compare the groups at each measurement time. Repeated measure of ANOVA was used for the purpose of comparing different measurement times for each group.

Kruskal-Wallis and Mann-Whitney U test were used to compare groups in terms of peritonitis scores.

The SPSS 10.0 package programme (SPSS Inc. IL, Chicago, USA) was used for statistical analysis of the all tests carried out. *P* values of ≤ 0.05, < 0.01 and < 0.001 were used for evaluating significance.

RESULTS

The faecal suspension was administered to all groups without any problems. In rabbits in groups G_3 and G_4, there was an insignificant abdominal tautness that occurred during ozone administration and disappeared immediately afterwards. No rabbit death occurred during the experimental period of the study.

Table 1. The effect of group on TNFα and IL-1β levels at different measurement times

Indicators	MT[g] (h)	Groups (G)				SEM	Sig.[e]	Significance of main effects[f]		
		G1	G2	G3	G4			G	MT	G × MT
TNFα	0	24.45^{y}	27.36^{xy}	28.78^{x}	28.56^{xy}	0.91	NS	NS	***	NS
	6	32.84^{x}	34.47^{xyz}	36.65^{x}	39.63^{x}	2.21	NS			
	12	27.73^{xy}	39.37^{x}	36.71^{x}	36.46^{xyz}	2.67	NS			
	18	19.63^{z}	21.18^{z}	25.83^{xy}	19.23^{z}	1.58	NS			
	24	23.72^{xy}	22.93^{yz}	22.73^{y}	24.10^{yz}	1.43	NS			
	Sig.[d]	***	*	*	**					
IL-1β	0	23.11^{z}	17.34^{z}	17.48^{y}	18.20	1.57	NS	NS	***	NS
	6	30.09^{y}	26.48^{xyz}	26.90^{x}	42.11	3.14	NS			
	12	43.37^{x}	55.69^{x}	34.64^{x}	50.98	5.18	NS			
	18	32.36^{xy}	29.37^{y}	35.75^{x}	39.34	3.17	NS			
	24	29.63^{xyz}	28.16^{y}	27.42^{x}	26.56	0.98	NS			
	Sig.[d]	***	**	*	NS					

G = group (G1, G2, G3 or G4); G × MT = interaction effects of group and measuring time; MT = measuring time; NS = not significant ($P > 0.05$)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

[x,y,z]Differences between the means of measurement times carrying various letters in the same column are significant ($P < 0.05$)

[d]Significance level of differences between measurement times for the same group according to repeated measurements of ANOVA statistics

[e]Significance level of differences between groups for the same measurement time according to One-way ANOVA statistics

[f]Significance of main effects according to repeated measurements ANOVA statistics

[g]Measurement time (hour)

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Table 2. The effect of group on MDA, SOD, CAT and GSH-Px levels at different measurement times

Indicators	MT[g] (h)	Groups (G)				SEM	Sig.[e]	Significance of main effects[f]		
		G1	G2	G3	G4			G	MT	G × MT
MDA (Nmol/l)	0	5.15	4.85^{z}	3.80^{z}	5.11^{z}	0.42	NS			
	12	4.47^{c}	$11.00^{b,x}$	$9.93^{b,x}$	$14.87^{a,x}$	0.83	***			
	24	5.81	9.89^{xy}	8.67^{xy}	6.46^{yz}	0.66	NS			
	48	4.30^{b}	$5.88^{ab,yz}$	$7.25^{a,y}$	$4.05^{b,z}$	0.44	*	***	***	***
	72	5.08^{c}	$10.31^{ab,xy}$	$6.47^{bc,xyz}$	$11.31^{a,xy}$	0.81	*			
	120	3.92	4.90^{yz}	5.34^{yz}	3.71	0.45	NS			
	Sig.[d]	NS	**	**	***					
SOD (IU/ml)	0	70.73^{x}	64.27	65.62	62.36	2.51	NS			
	12	72.47^{x}	69.03	67.70	67.15	1.09	NS			
	24	71.17^{x}	69.59	68.62	65.27	1.79	NS	NS	***	**
	48	$48.54^{b,y}$	70.54^{a}	66.28^{a}	53.85^{b}	2.51	**			
	72	$52.07^{c,y}$	63.10^{ab}	66.81^{a}	56.20^{bc}	1.62	**			
	120	$71.90^{ab,x}$	62.47^{b}	69.43^{b}	80.41^{a}	1.94	*			
	Sig.[d]	***	NS	NS	NS					
CAT (IU/ml)	0	9.85^{z}	11.98	8.55^{y}	10.93^{y}	0.83	NS			
	12	$16.70^{b,y}$	114.30^{a}	$90.98^{ab,x}$	$123.75^{a,x}$	14.39	*			
	24	23.59^{x}	139.58	53.62^{x}	44.21^{xy}	18.34	NS	**	***	*
	48	$24.11^{b,xy}$	91.50^{a}	$125.93^{a,x}$	$95.01^{a,x}$	12.83	*			
	72	29.83^{xy}	50.78	95.18^{x}	30.20^{y}	10.21	NS			
	120	$23.83^{b,xy}$	93.53^{a}	$98.10^{a,x}$	111.12^{a}	12.62	*			
	Sig.[d]	**	NS	*	***					
GSH-Px (IU/l)	0	119.59^{a}	21.22^{b}	32.05^{b}	36.89^{b}	13.28	*			
	12	68.53	58.80	48.01	36.63	8.20	NS			
	24	54.54	94.84	101.47	96.76	15.64	NS	NS	**	NS
	48	133.96	124.46	90.64	127.08	17.25	NS			
	72	76.05	136.84	115.39	127.58	19.80	NS			
	120	77.59	90.19	109.87	200.28	16.35	NS			
	Sig.[d]	NS	NS	NS	NS					

G = group (G1, G2, G3 or G4); G × MT = interaction effects of group and measuring time; MT = measuring time; NS = not significant ($P > 0.05$)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

[a,b,c]Differences between the means of groups carrying various letters in the same line are significant ($P < 0.05$)

[x,y,z]Differences between the means of measurement times carrying various letters in the same column are significant ($P < 0.05$)

[d]Significance level of differences between measurement times for the same group according to repeated measurements of ANOVA statistics

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Biochemistry results

The statistical evaluation regarding serum TNF α and IL-1β is presented in Table 1 and the difference between groups was found to be non-significant ($P > 0.05$). However, TNF α increase was seen to emerge sooner in group G_1 (6 h) compared to the other groups.

Statistical evaluation regarding serum MDA, SOD, CAT and GSH-Px activities is shown in Table 2. Serum MDA levels were found to be higher at 12 h and 72 h in group G_4 compared to the other groups. In terms of SOD levels, measurement time in groups G_2, G_3 and G_4 was non-significant, while a significant decrease was observed at 48 h in group G_1. Serum CAT levels were lower at 12 h, 48 h and 120 h in group G1 compared to other groups.

Serum ALT, AST and Cre levels are shown in Table 3. The difference was not statistically significant between groups with regards to serum AST

Table 3. The effect of group on AST, ALT and Cre for groups at different measurement times

Indicators	MT[g] (h)	Groups (G)				SEM	Sig.[e]	Significance of main effects[f]		
		G1	G2	G3	G4			G	MT	G × MT
AST (IU/l)	0	38.13	34.25^{x}	42.25^{x}	43.88	2.72	NS			
	12	38.63	31.38^{x}	36.13^{xy}	39.75	1.81	NS			
	24	52.25^{a}	$35.00^{b,x}$	$41.38^{b,x}$	33.75^{b}	1.88	***	*	**	**
	48	49.88^{a}	$29.63^{b,x}$	$27.63^{b,y}$	30.13^{b}	2.80	**			
	72	43.63^{a}	$27.63^{b,x}$	$22.88^{b,z}$	33.38^{ab}	2.32	**			
	120	48.38^{a}	$21.88^{b,y}$	$18.75^{b,t}$	26.75^{b}	3.76	**			
	Sig. [d]	NS	**	***	NS					
ALT (IU/l)	0	69.75	76.50	86.75^{xy}	101.00	8.35	NS			
	12	84.63	86.63	76.88^{x}	63.63	5.55	NS			
	24	77.00	87.50	79.13^{x}	53.50	5.39	NS	NS	*	*
	48	75.13	77.63	71.88^{x}	56.13	4.99	NS			
	72	71.50	71.25	63.75^{y}	55.50	3.,97	NS			
	120	66.38	61.38	42.75^{z}	48.50	3.69	NS			
	Sig.[d]	NS	NS	***	NS					
Cre (μmol/l)	0	1.01	1.09^{x}	1.09^{x}	1.08	0.04	NS			
	12	0.95^{b}	$0.78^{b,y}$	$0.82^{b,y}$	1.15^{a}	0.05	**			
	24	0.94	1.01^{x}	0.85^{xy}	1.02	0.04	NS	*	*	*
	48	0.90	1.03^{x}	0.79^{y}	0.99	0.03	NS			
	72	0.83^{b}	$1.10^{a,x}$	$0.86^{b,y}$	1.08^{a}	0.06	***			
	120	0.92^{b}	$0.99^{b,x}$	$0.83^{b,y}$	1.46^{a}	0.06	**			
	Sig.[d]	NS	**	*	NS					

G = group (G1, G2, G3 or G4); G × MT = interaction effects of group and measuring time; MT = measuring time; NS = not significant ($P > 0.05$)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

[a,b,c]Differences between the means of groups carrying various letters in the same line are significant ($P < 0.05$)

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levels. Serum AST levels were higher in group G_1 compared to the other groups. Serum Cre levels were determined to be higher at measurement times 12 h and 120 h in group G_4 compared to other groups ($P < 0.01$).

Statistical evaluation regarding granulocyte and lymphocyte levels of cases is shown in Table 4. The granulocyte level increased significantly ($P < 0.001$) at 12 h in all groups and was higher, particularly at measurement times 48 h, 72 h and 120 h in group G_4 compared to the other groups. In terms of lymphocyte levels, a general increase in relation to time and higher levels of lymphocytes were determined in group G_1 compared to the other groups.

No difference was seen in statistical significance between groups with regard to erythrocyte, haemoglobin and haematocrit values.

Pathological results

a) Necropsy findings

In the non-treatment group (G_1) and in the ozone group (G_3), peritoneum of most of the rabbits was haemorrhagic and necrotic. In the intestines, particularly in the caecum, the serosa was congested and haemorrhagic and fibrin formation was seen in some areas (Figure 1A and 1B). The lungs of the

Table 4. The effect of group on lymphocyte and granulocyte for groups at different measurement times

Indicators	MT[g] (h)	Groups (G)				SEM	Sig.[e]	Significance of main effects[f]		
		G1	G2	G3	G4			G	MT	G × MT
Lymphocyte (× 10^3/mm^3)	0	4.01^{z}	5.16^{x}	4.61^{y}	5.06^{x}	0.20	NS			
	12	3.83^{z}	3.33^{yz}	3.38^{zt}	3.08^{y}	0.20	NS			
	24	$4.69^{a,y}$	$3.58^{b,yz}$	$4.74^{a,y}$	$3.43^{b,y}$	0.17	***			
	48	$4.70^{a,xy}$	$3.19^{b,yz}$	$4.79^{a,yz}$	$2.94^{b,y}$	0.28	*	*	***	***
	72	$6.56^{a,x}$	$3.73^{b,yz}$	$3.13^{b,t}$	$3.39^{b,y}$	0.31	***			
	120	$7.38^{a,x}$	$4.23^{b,xyz}$	$6.40^{a,xy}$	4.08^{b}	0.39	***			
	168	7.89^{x}	6.38^{xy}	12.16^{x}	3.80	1.05	NS			
	Sig.[d]	***	**	***	***					
Granulocyte (× 10^3/mm^3)	0	2.04^{z}	2.61^{yz}	1.95^{w}	2.16^{t}	0.10	NS			
	12	$3.74^{b,x}$	$6.43^{a,x}$	$5.85^{a,x}$	$6.74^{a,x}$	0.32	***			
	24	$2.23^{b,z}$	$3.26^{ab,y}$	$4.41^{a,yz}$	$4.61^{a,yz}$	0.28	**			
	48	$2.80^{b,x}$	$3.09^{b,yz}$	$2.90^{b,ztw}$	$4.84^{a,y}$	0.23	***	***	***	***
	72	$2.68^{b,xy}$	$2.55^{b,yz}$	$2.05^{b,tw}$	$3.85^{a,z}$	0.18	***			
	120	$2.65^{b,y}$	$2.28^{b,z}$	$3.20^{b,yt}$	6.15^{a}	0.32	***			
	168	$2.28^{b,yz}$	$2.20^{b,yz}$	$4.30^{a,xy}$	4.23^{a}	0.27	***			
	Sig.[d]	***	***	***	***					

G = group (G1, G2, G3 or G4); G × MT = interaction effects of group and measuring time; MT = measuring time; NS = not significant ($P > 0.05$)

[a,b,c]Differences between the means of groups carrying various letters in the same line are significant ($P < 0.05$)

[k,t,w,x,y,z]Differences between the means of measurement times carrying various letters in the same column are significant ($P < 0.05$)

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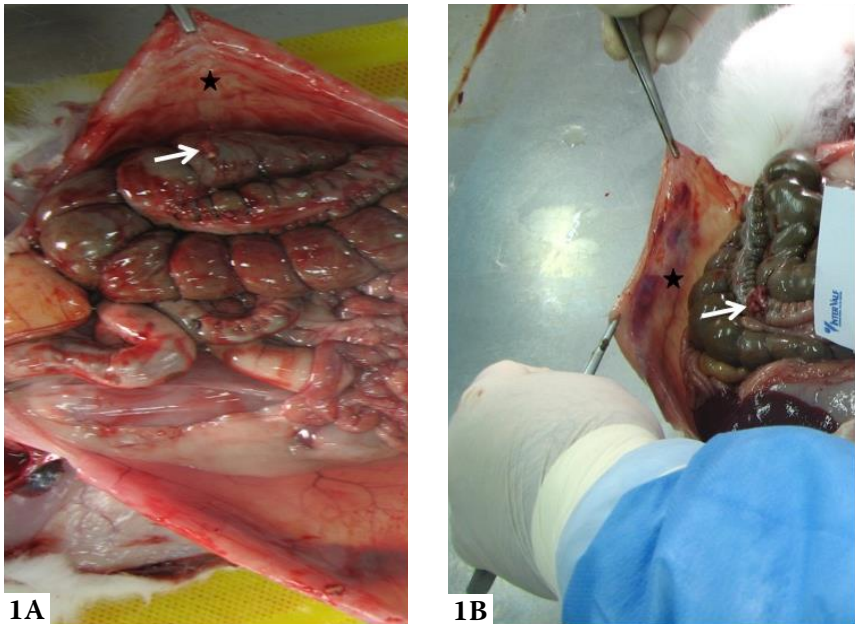

Figure 1. (**A**) Dull appereance on the peritoneum, ecchymotic bleedings on the peritoneum and abdominal wall (star). Bleeding and fibrin formation on the serosa of intestines (white arrow). Untreated Group. (**B**) Haemorrhage and necrosis in the peritoneum (star), fibrin formation on the serosa of intestines (white arrow). Ozone Group. (**C**) Peritoneum and intestines. Antibiotic Group. (**D**) Mild peritonitis. Ozone + Antibiotic Group

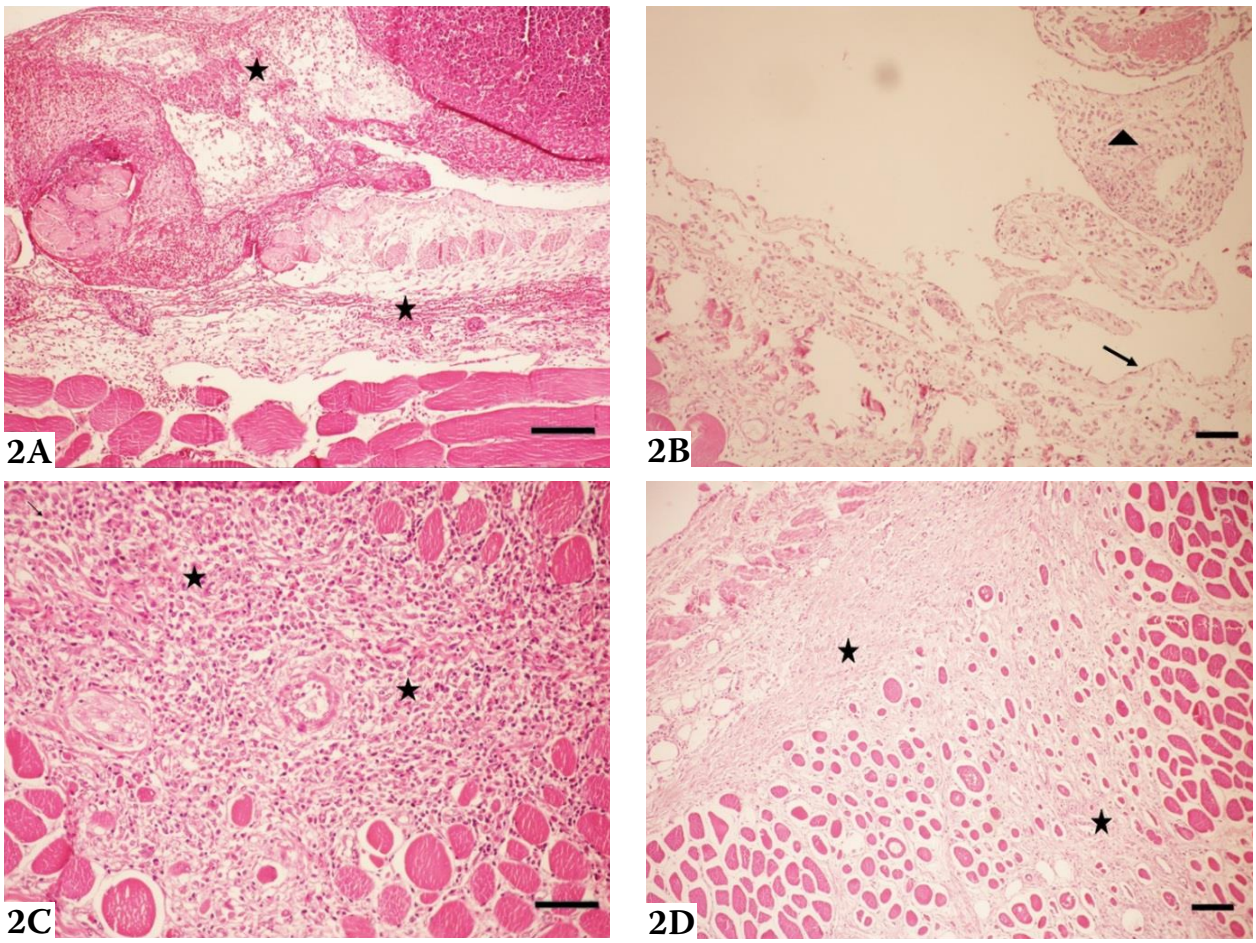

Figure 2. (**A**) Marked fibrin formation and karyorrhectic neutrophil leukocyte infiltration in peritoneal epithelium (star). Non-treatment Group. Bar = 50 µm, H&E. (**B**) Fibrin formation (arrowhead) on peritoneal epithelial surface (arrows). Ozone Group. Bar =200 µm, H&E. (**C**) Severe neutrophil leukocyte infiltration in abdominal wall (stars). Non-treatment Group. Bar = 50 µm, H&E. (**D**) Atrophy in the muscles of abdominal wall and fibrosis. Ozone Group. Bar = 200 µm, H&E

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animals were swollen, oedematous, and haemorrhagic (Figure 1B).

In the antibiotic (G_2) and ozone + antibiotic (G_4) groups, a mildly oedematous and hyperaemic appearance was detected in the lungs. In the livers of some animals mild congestion was observed. There was no significant gross lesion in the peritoneum of the animals in the antibiotic group (Figure 1C). Slight to moderate matte appearance in the peritoneum was seen in most of the animals in the ozone + antibiotic group (Figure 1D). Also, hyperaemia, mild haemorrhage and fibrin were prominent in some of them.

b) Histopathological findings

b1) The non-treatment group (G_1) and the Ozone group (G_3)

Histopathological examination revealed extensive necrosis, haemorrhage, marked fibrin formation and karyorrhectic neutrophil leukocyte infiltrations in the peritoneal epithelium (Figure 2A and 2B). In most of the animals there was severe neutrophil leukocyte infiltration (Figure 2C) between the muscle fibres of the abdominal wall and also atrophy and fibrosis were prominent in some of them (Figure 2D). In the heart muscles, oedema and mononuclear cell infiltration ranging from mild to moderate were observed.

Extensive oedema and collateral hyperaemia were determined in the lungs of the animals in the non-treatment group (G_1) and ozone group (G_3) (Figure 3A). In addition, hyperplasia in the peribronchial lymphoid tissue, extensive fields of emphysema and mild mononuclear cell infiltrations were observed in the interalveolar areas. Fibrin,

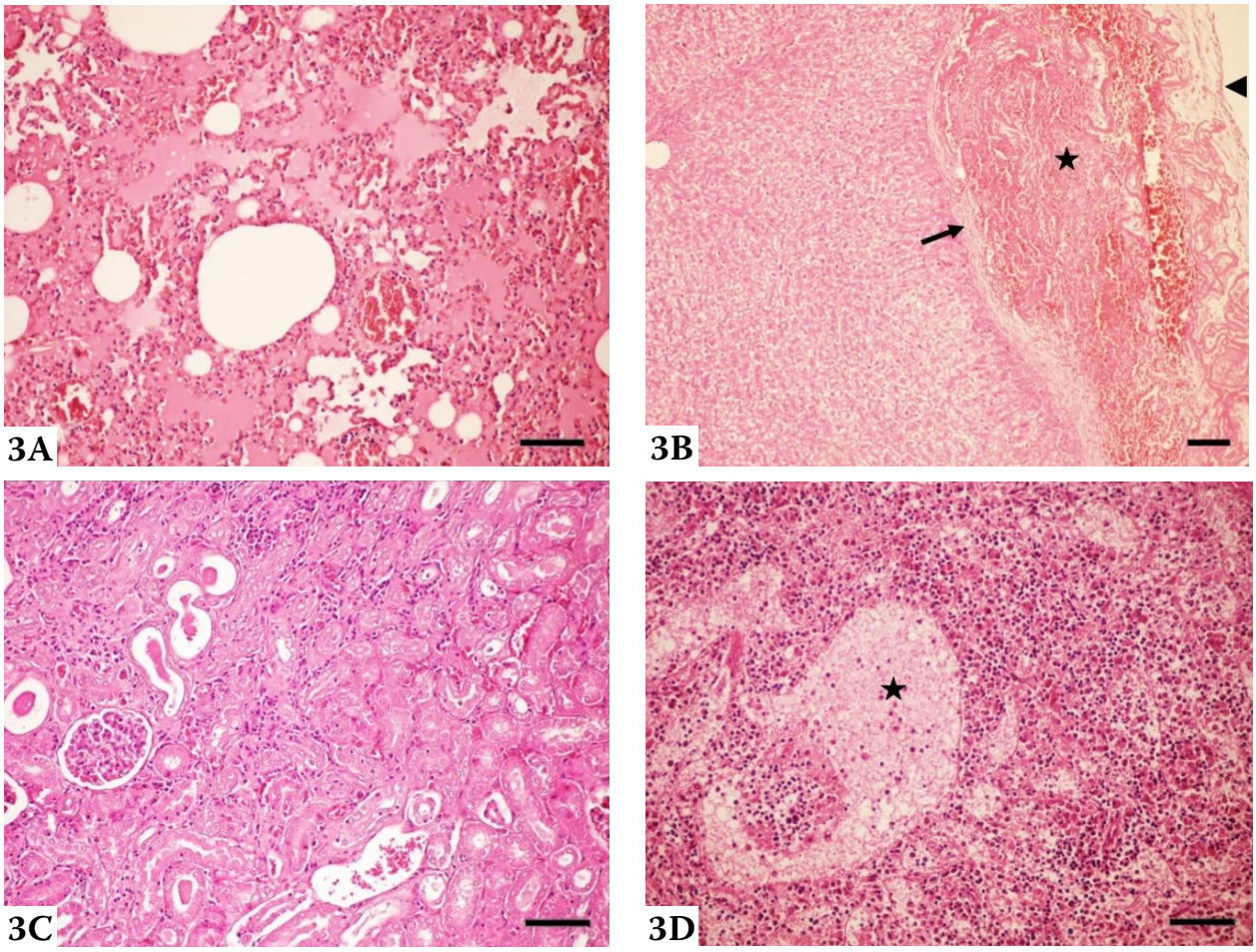

Figure 3. (**A**) Extensive oedema and collateral hyperemia in lungs. Non-treatment Group, Bar = 50 µm, H&E. (**B**) Pseudomembrane formation and haemorrhage (star) on hepatic capsule (arrow). Peritoneal epithelium (arrowhead). Ozone Group. Bar= 200 m µm H&E. (**C**) Hyaline cylinders in the tubular lumens and mononuclear cell infiltration in the intertubular areas in kidney. Non-treatment Group. Bar = 50 µm, H&E. (**D**) Lysis of the white pulp in spleen. Ozone Group Bar = 50 µm, H&E

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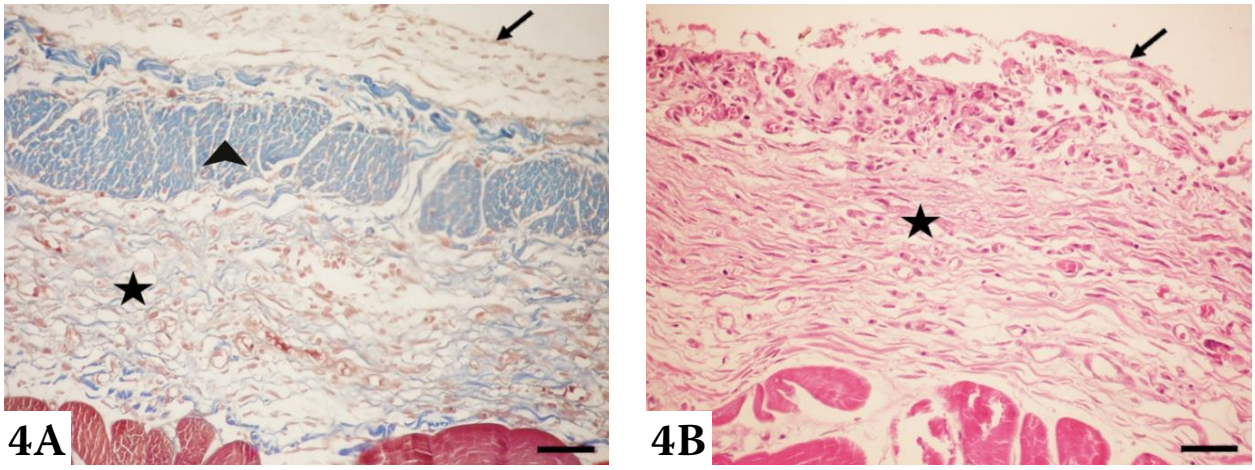

Figure 4. (**A**) Increased collagen fibers in the submesothelial layer (star). Fibrous band (arrowhead). Peritoneal epithelium (arrow). Antibiotic Group. Bar = 30 µm, Masson's Trichrom. (**B**) Erosive changes (arrow) and mild inflammatory cells (star) in the peritoneum. Ozone + Antibiotic Group. Bar = 30 µm, H&E

neutrophilic leukocytes and alveolar macrophages were observed in the alveolar lumens of one animal in the ozone group.

In cross-sections of liver belonging to both groups (G_1 and G_3), extensive parenchyma degeneration and vacuolization were observed. Moderate mononuclear cell infiltration was seen around Kiernan's spaces and in sinusoids. In one animal in the ozone group, pseudomembrane formation in the hepatic capsule and subcapsular bleeding was determined (Figure 3B).

In the kidneys, degeneration in the tubulus epithelium, occasional haemorrhage and hyperaemia in the intertubular area and hyaline cylinders in the tubulus lumens were observed. Proteinous fluid accumulation in the Bowman capsule and proliferation of mesangial cells in the glomeruli were determined. Also in one animal, extensive mononuclear cell infiltration in the intertubular areas and fibrous connective tissue production in some areas were observed (Figure 3C). Lymphoid depletion and fibrin formation of the splenic white pulp and extensive leukocyte infiltration were observed in the red pulp (Figure 3D).

In the intestines, desquamation of intestinal villi, neutrophil leukocyte and mononuclear cell infiltration in the interglandular areas were observed. In addition to these, formation of a pseudomembrane over the villi was determined in one animal.

b2) Antibiotic group (G_2)

In this group, mild peritonitis was determined in three animals only. Slightly erosive areas, mild neutrophil leukocyte infiltration and haemorrhage were observed in the peritoneal epithelium. Also fibrous connective tissue formation was seen in some areas (Figure 4A). Pulmonary oedema was present at a mild level in two animals and a moderate level in two other animals. Severe pulmonary oedema, collateral hyperaemia and interalveolar connective tissue proliferation was observed only in one animal. Mild renal tubule degeneration was observed. Desquamation of intestinal villi was seen in the intestines.

b3) Ozone + Antibiotic Group (G_4)

In this group, in the peritoneal epithelium, the erosive changes seen were mild in three animals, while moderate in one animal (Figure 4B), and oedema, haemorrhage, fibrin and macrophage infiltration in the abdominal wall was also observed. The remaining four animals displayed a normal appearance. Pulmonary emphysema and atelectasia was observed in the animals in general. Mild

Table 5. Descriptive information and statistical comparison regarding peritonitis scores ([A]) in study groups

	G_1	G_2	G_3	G_4	Chi-square/ significance
Mean rank	24.44[b]	7.44[a]	23.25[b]	10.88[a]	21.876
Median	3	0	2.5	0.5	< 0.001
Min/Max	2/3	0/1	2/3	0/2	

[a,b]Differences between the mean ranks carrying various letters are significant ($P < 0.05$)

[A]Scale of peritonitis scores: (0) no peritonitis, (1) mild, (2) moderate, (3) severe peritonitis

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oedema in one animal and mild mononuclear cell infiltration around the bronchi and bronchioli in two animals were observed. Degeneration in the renal tubular epithelium, proteinous fluid collection in the Bowman capsule and mild mononuclear cell infiltration in the interstitial spaces was observed. There was extensive haemorrhage in the large intestine lumen in one animal and desquamation in the intestinal villi of the small intestine.

Peritonitis scores

As a result of the Kruskal Wallis analysis, the effect of the group on peritonitis level score was found to be significant ($P < 0.05$) (Table 5). As a result of the Mann-Whitney U test conducted to compare two groups; the peritonitis scores were found similar in G_1 and G_3, which were higher compared to G_2 and G_4 groups, and while groups G_2 and G_4 had similar peritonitis scores.

DISCUSSION

At present, despite antibiotic treatment and many supporting therapies, sepsis still has a high mortality rate. Therefore, it retains its clinical and financial significance (Staatz et al. 2002; Martin-Barrasa et al. 2015).

The body's first defence system against pathogens is the innate immune system. This system consists of cells, cytokines and mediators (van Westerloo et al. 2005; Martin-Barrasa et al. 2015). LPSs secreted from the bacterial wall activate many intracellular signal pathways such as nuclear Factor KB ($NF\text{-}_KB$) and activates the body's defence mechanism by commencing inflammatory cytokine secretion (Victor et al. 2004; Vaillant et al. 2013; Xing et al. 2015). According to another view (Borovikova et al. 2000; Tracey 2007; Song et al. 2008), regarding body defence, the mechanism activated more rapidly than the peripheral immune system and named "cholinergic anti-inflammatory pathway", has been reported to be more effective in the defence system. Acetylcholine produced by *vagus* nerve stimulation lessens the synthesis of cytokines such as TNFα ve IL-1β from macrophages and causes inflammatory response to decrease.

While the action mechanism of ozone treatment has not yet been fully explained, many different opinions have been reported on the subject. In some studies (Vaillant et al. 2013; Yu et al. 2017), it has been expressed that ozone treatment depresses $NF\text{-}_KB$ activation and lowers TNF alpha and IL-1 Beta levels. Also opinions have intensified which state that the effect of ozone on the body activates various biological processes by triggering the oxidative stress via lipid oxidation products produced as a result of the reaction between unsaturated fatty acids in the cell membrane and hydrogen peroxide produced in liquid medium, and causes an increase in antioxidant enzyme levels (Zamora et al. 2005; Sagai-Bocci 2011; Re et al. 2012; Re et al. 2014; Aslaner et al. 2015; Lee et al. 2017). In contrast, with respect to its cytokine secretion, have expressed that ozone does not have a direct effect on the local immune system (Schulz et al. 2003). In another studies (Sagai-Bocci 2011; Re et al. 2012; Re et al. 2014; Lee et al. 2017), it has been stated that ozone treatment plays a role both in decreasing cell and tissue damage and increasing the effectiveness of antibiotic therapy by breaking down microorganism defence. In the present study, there was no statistically significant difference between groups with respect to TNFα ve IL-1β levels. However, TNFα increase occurred earlier (6 h) in group G_1 compared to the other groups. This result suggests that ozone and antibiotic administrations step in at an earlier stage than bacterial endotoxins and stimulate the "cholinergic anti-inflammatory pathway" before stimulation of the innate immune system. Thus, local cytokine synthesis is supressed and the increase in TNFα level is delayed.

On entering the body, ozone is rapidly converted into reactive oxygen species (ROS). Excessive ROS production then leads to oxidative stress (Lee et al. 2017; Smith et al. 2017). In the study, the highest increase in MDA levels, a free radical, was observed in groups G_2 (12 h), G_3 (12 h) and G_4 (12 h, 72 h). This increase was influenced by the triggering of bactericidal enzymes and free oxygen radical release via stimulation of neutrophils and macrophages by lipopolysaccharides (LPS) produced as a result of the bacterial cell breakdown by both ozone and the antibiotic (Thanomsub et al. 2002; Madej et al. 2007; Shinozuka et al. 2008; Barera et al. 2011). It is also thought that the conversion of ozone into oxidative reactive oxygen species in the organism (Lee et al. 2017; Smith et al. 2017) plays a role in this increase.

Lipopolysaccharides stimulate free radical reactions and markedly increase antioxidant (SOD and

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CAT) activities (Leon et al. 1998; Madej et al. 2007). It has been expressed that, ozone treatment administered in infectious peritonitis cases decreased free oxygen radicals and increased antioxidant enzyme release, therefore providing protection against organ damage (Li et al. 2007; Nogales et al. 2008; Rodriguez et al. 2009; Azuma et al. 2014; Fernandez et al. 2016). In the study, SOD and CAT levels were found to be higher in groups G_2, G_3 and G_4 compared to the non-treatment group. At the same time, the highest MDA value was obtained in the same groups. In contrast to literary sources (Li et al. 2007; Madej et al. 2007; Nogales et al. 2008; Rodriguez et al. 2009; Azuma et al. 2014), this result has demonstrated that the ozone treatment did not show effect by decreasing free radicals, on the contrary, that the increasing free radical level triggered antioxidant production. In agreement with the above information, it has also been demonstrated that both ozone and antibiotics produce the same effect in activating antioxidant systems via their defence mechanisms stimulated by bacterial cell lysis (Thanomsub et al. 2002; Shinozuka et al. 2008).

Faecal contamination causes multiple organ failure including the liver and kidneys and increases serum ALT, AST and Cre levels (Malenstein et al. 2010; Bosmann-Ward 2013). In the present study, while no difference was observed between experimental groups in terms of ALT values, AST values were found to be higher in group G_1. The lowest Creatinine values were seen in group G_3. When these results were evaluated in terms of antioxidant mechanisms, increasing SOD and CAT levels in groups apart from G_1 were observed to be protective against hepatic injury (Gul et al. 2012; Azuma et al. 2014; Fernandez et al. 2016). The marked decrease of creatinine only in group G_3 was evaluated as; in addition to antioxidant mechanisms (Rodriguez et al. 2009), ozone may have increased the oxygen carrying capacity of haemoglobin and raised the amount of oxygen reaching the kidneys (Gornicki-Gutsze 2000; Bocci 2006).

In acute peritonitis cases, neutrophils exhibit a parallel local and systemic increase (Bhan et al. 2016). In this study, leukocyte levels were also examined and the granulocyte and lymphocyte values in group G_3 were determined to increase earlier than in the other groups. This result confirms that ozone has stimulating effects on inflammation production, the innate immune system (granulocyte) and the adaptive immune system (lymphocyte) (Torossian et al. 2004; Bette et al. 2006). Also, antibiotic administration was evaluated as supressing inflammation in the early stages and eliminating factors before the adaptive immune system was activated. In conclusion, compared to ozone administration, antibiotic treatment was seen to be more effective in supressing inflammation.

The systemic inflammation occurring in acute peritonitis accelerates neutrophil migration to the lungs. This increases vascular permeability of the lungs and leads to respiratory distress syndrome (RDS) (Barrera et al. 2011). In the treatment of peritonitis, no literary source was found regarding histopathological changes in the body caused by ozone administration. In the histopathological assessment performed in this study, while in all the animals in groups G_1 and G_3, the lungs had oedematous and bleeding areas macroscopically, mild oedematous and mild hyperaemic appearance was observed in animals in groups G_2 and G_4. This result indicated that antibiotic administration was more effective in supressing systemic inflammation.

The peritonitis scores were found similar in G_1 and G_3, which is higher compared to G_2 and G_4 groups. The ozone alone has not been effective in the treatment of peritonitis. Peritonitis scores were similar in groups G_2 and G_4. This result was interpreted as ozone and antibiotic administrations possessing similar action mechanisms. However, the fact that mild peritonitis was produced only in 3 rabbits in group G_2 showed that antibiotic administration was more effective for treatment. It has been stated that ozone has bactericidal effects on both Gram – and Gram + bacteria and less endotoxin is released from bacteria exposed to ozone (Shinozuka et al. 2008). However, the data obtained from this study showed that the antibacterial efficacy of ozone treatment is not very strong contrary to what is believed. While a combined administration of antibiotics and ozone was expected to increase each other's level of effectiveness and produce more successful results, the results obtained did not support this notion and synergistic effects did not occur.

In conclusion, according to the findings obtained at the end of the study, it was concluded that antibiotic use in the treatment of infectious peritonitis was more effective than ozone therapy alone. At the same time, it is hoped that this study will provide a stepping stone for new research into better understanding the action mechanism of ozone treatment.

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