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Original Article

The Effect of Ozone Treatment to the Inflammatory Response in Lung Contusion

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Abstract

Objectives: The effects of ozone treatment on direct mechanical injury and the inflammation secondary to the injury resulting from blunt thoracic trauma were evaluated with the histopathological results obtained in our study.

Patients and Methods: 24 adult male Wistar albino rats of the same colony with an average weight of 200-250 gr were used in our study. The rats were divided into 3 groups. First group were anaesthetized with ketamine HCl/xylazine. Second group were given contusion with isolated unilateral lung contusion. Third group were given 30 µgr/ml ozone at a dose of 1.1mg/kg intrarectally after the contusion for five days. Four different parameters; atelectasis, parenchymal inflammation, perivascular mononuclear inflammation and bronchial injury, were used in histopathological evaluation and all parameters were scaled.

Results: Cell concentration, perivascular mononuclear inflammation and leukocyte infiltration were significantly higher in the lung tissues of all the rats in the trauma group compared to the anesthesia group. However, when the control, trauma and ozone groups were compared, there was no significant difference in terms of alveolar edema and bronchial injury. Similarly, among subgroubs of trauma and ozone groups, there was not a statistical significance in terms of histopathological data.

Conclusion: When inflammatory stages and cell distribution were compared between the study groups, advanced acute inflammatory changes were found on the fifth day of inflammation in both contusion groups and no significant difference was observed in terms of inflammatory response pattern. Consequently, no positive effect of ozone treatment was shown on the acute and subacute inflammatory response in decreasing the primary and secondary injury caused by the trauma.

Keywords: Ozone, contusion, inflammation

INTRODUCTION

At present, patients presenting to emergency units with blunt thoracic traumas account for a considerable rate of all emergency cases. Cases of blunt thoracic traumas are responsible for 8%-10% of all cases of traumas (1). Pulmonary contusion is the most frequent injury detected in one third of patients presenting with blunt thoracic traumas (1,2).

Pulmonary contusion developing after blunt thoracic traumas predisposes to acute pulmonary damage, acute respiratory failure and pneumonia, which cause mortality in 10-25% of adults with blunt thoracic traumas (3,4).

Pulmonary contusion due to blunt thoracic traumas mainly refers to alveolar congestion and hemorrhage, disruption of the alveolar structure, edema and leukocyte infiltration. Clinical signs of this condition are hypoxemia and increased respiratory workload, the severity and the duration of which depend on hypercapnia. The primary pathophysiological mechanism of the contusion is ventilation/perfusion defect, increased intrapulmonary shunting, increased pulmonary fluid, pulmonary tissue injury at least at the segmental level and decreased compliance (3,5,6). Following blunt thoracic traumas, macrophages and neutrophils are activated. Thus, a progressive deterioration starts (7). Prevention of acute pulmonary damage, acute respiratory failure and pneumonia secondary to thoracic contusion depends on detection and restoration of the inflammatory response developing after pulmonary contusion (1,8).

Medical ozone (O_3) treatment is utilized for a wide variety of conditions due to its antioxidant, anti-inflammatory and antimicrobial effects (9). Unlike treatment with pharmacological drugs, ozone treatment strengthens the body against diseases

through a reaction created by antioxidants and anti-inflammatory methods as strong potentials of the body, not through the drug-receptor relationship (10,11).

The aim of the present study was to investigate the effectiveness of ozone treatment, previously proved to be useful in treatment of many conditions, in pulmonary contusion. To this aim, a unilateral pulmonary contusion model was created and possible histopathological mechanisms underlying ozone treatment were examined.

METHODS

The study protocol was approved by the Ethical Committee of Dokuz Eylül University Medical School (Protocol number: 59/2011). Twenty-four male adult Wistar albino rats weighing 200-250 g were used. The rats were kept at 21-23 °C in the animal experiments laboratory at Dokuz Eylül University in accordance with Guide for the Care and Use of Laboratory Animals. They were exposed to daylight for 12 hours and darkness for 12 hours. The aim of using a rat model was that it was reliable and easy to obtain and had a high rate of reproducibility (12). The rats were fed on standard pellet food and were allowed to eat food and drink water before surgery. They were weighed before the experiments and were anesthetized with ketamine HCl (Ketalar, Pfizer Pharma GMBH, Germany) 40-50 mg/kg combined with xylazine (Alfazyne 2%, Alfasan International, Holland) 7,5 mg/kg through the intraperitoneal route. Spontaneous breathing was maintained during the experiments. Analgesia was achieved by 0.05 mg/kg morphine HCl through the intraperitoneal route (morphine HCl, Galen İlaç San. ve Tic. A.Ş Kadıköy, İstanbul).

Pulmonary contusion was created by using a unilateral pulmonary contusion model, which was a modified version of isolated bilateral pulmonary contusion model described by Raghavendran et al. (3). A cylindrical weight made of aluminum was dropped through a tube made of stainless steel and mounted vertically on a platform. The right lateral pulmonary wall of the rats was positioned under and contacted with the platform on which the weight dropped. The midaxillary line and xiphoid process of the rats were marked to achieve standardization and optimization.

It has been reported that about 2.45 joules of impact energy are necessary to create a pulmonary contusion compatible with life (3). The energy resulting from this mechanism was calculated by using the following formula: E= mgh (E: energy, g: gravity; 9,8 m/s2, h: height; 83,3 cm, and m: weight dropped; 0,3 kg). Since the weight was 300 gr and the height was 83,3 cm, the energy transferred to the chest wall was found to be 2,45 joules.

Ozone treatment protocol:

Ozone 30 µg/ml was administered for five days after the contusion at a dose of 1.1 mg/kg through the intrarectal route. On the fifth day after the trauma, the rats were anesthetized with ketamine/xylazine and all of the blood was sacrificed through the intracardiac route by dissecting the chest wall. Infections, allergic reactions and death were the causes of exclusion from the study.

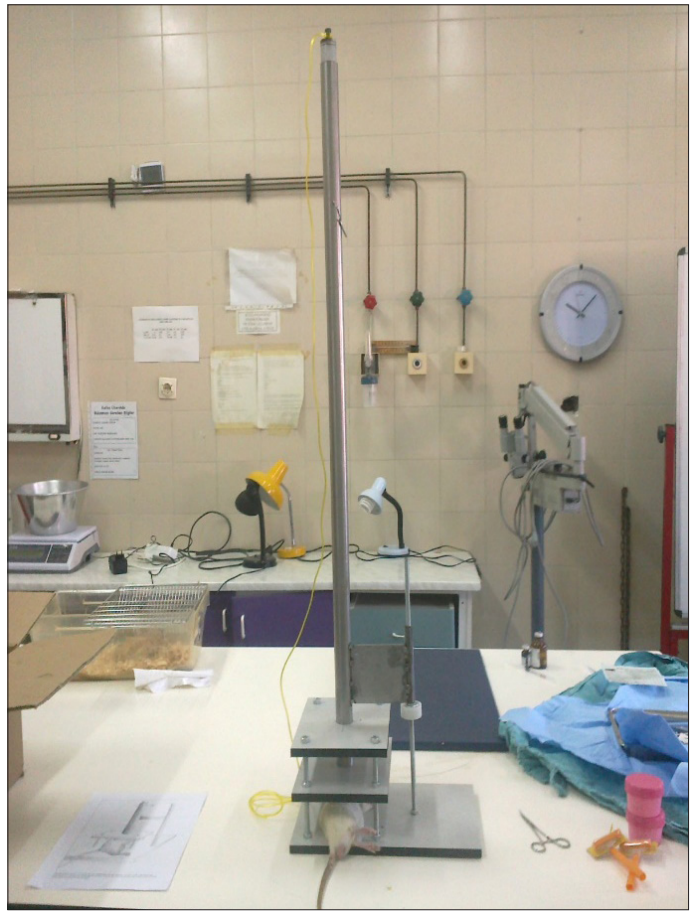

Figure 1. The unilateral contusion model as a modified version of the bilateral contusion model described by Raghavendran et al. (3).

Experimental design:

I. Trauma Group (n=8): The rats in this group were exposed to trauma at an energy of 2.45 joule created by dropping a 300 gr-weight from a height of 83,3 cm under anesthesia in accordance with the trauma model. Five days later, median sternotomy was performed, and the superior, median and inferior lobes of the right lung were removed. This group was not administered any drugs (Figures 2, 3, 6-9).

II. Ozone Group (n=8): After the rats were exposed to trauma under anesthesia, they were administered ozone 30 µg/mL at the dose of 1.1 mg/kg through the intrathecal route for five days. Following treatment, median sternotomy was performed, and the superior, median and inferior lobes of the right lung were removed Figure 4 and Figure 10.

III. Control Group (n:8): The superior, median and inferior lobes of the right lung were removed by means of median sternotomy under anesthesia lasting as in the other groups. Obtained tissues were used to determine base values Figure 5.

Histopathological examination:

For histopathological examination, lung tissues recovered from the sacrificed rats were fixed in 10% buffered formalin. They

were dehydrated with staged alcohol series, cleaned in xylene and embedded in paraffin. They were cut in 5 µm-thickness and stained with hematoxylin and eosin.

Histopathological evaluation was made by using four different parameters. All histopathological examinations of specimens were made by light microscope.

1- Atelectasis, congestion and intra-alveolar hemorrhage were classified into focal, patchy (multiple foci smaller than the magnification of five) and diffuse (continuous throughout the magnification of five). Grade zero corresponded to none, grade one focal, grade two patchy (multiple foci smaller than the magnification of five) grade three diffuse (continuous throughout the magnification of five) Figure 6 and Figure 9.

2- Parenchymal inflammation was considered as mild (single inflammatory cells), moderate (inflammatory cells in groups of two-four) and severe (inflammatory cells in groups of five or more) and the type of inflammation was based on whether there were predominantly neutrophils, mixture of neutrophils and mononuclear cells and predominantly mononuclear cells (Figures 7 and 8). Depending of the intensity of the cells, grade zero corresponded to lack of inflammation, grade one mild inflammation (individual cells), grade two moderate inflammation (groups of five-ten cells), grade three severe inflammation (groups of 10-30 cells), and grade 4 very severe inflammation (groups of more than 30 cells). Based on the type of cells, inflammation was classified into a normal cell distribution, predominance of macrophages, predominance of neutrophils, an equal number of polymorphonuclear leukocyte (PNL) and mononuclear leukocyte (MNL), predominance of MNL, revascularization and fibroblastic stage Table 6. Leukocytic infiltration was utilized to show the severity of inflammation due to contusion. Each type of infiltration had ten subdivisions and leukocytic infiltration was determined under the magnification of x 400. Each subdivision was determined by using the following classification: zero corresponded to lack of extravascular leukocytes, one presence of fewer than ten leukocytes, two presence of 10-45 leukocytes, and three presence of more than 45 leukocytes.

3- Perivascular mononuclear inflammation was classified into mild (individual cells), moderate (groups of cells) and severe (cells forming a cuff around the vessel). Grade zero corresponded to lack of perivascular mononuclear inflammation, grade one mild perivascular mononuclear inflammation (individual cells), grade two moderate perivascular mononuclear inflammation (groups of cells) and grade three severe perivascular mononuclear inflammation (cell deposition in the form of a cuff around the vessel).

4- Bronchial damage was graded based on presence of severe bronchial destruction presenting with focal epithelial damage-focal wall damage and abscess formation. Grade zero corresponded to lack of bronchial damage, grade one focal epithelial damage, grade two focal wall damage and grade three severe damage in the bronchioles accompanied by abscess formation.

Statistical analysis

Statistical Package of Social Sciences 15 (SPSS 15,0,Chicago, IL, USA) was used for the statistical analysis. Data were analyzed with Kruskal-Wallis test to compare the groups. Mann-Whitney U test was utilized for paired comparisons of the groups and all obtained results were expressed in median values (minimum and maximum values). The difference between the groups was accepted as significant when p was <0.05.

RESULTS

A total of 24 rats were used in the study. Macroscopic evaluation made during median sternotomy following sacrifice revealed heterogenous findings in the right lung consistent with contusion.

Histopathological findings:

According to the results of Kruskal-Wallis test performed to compare atelectasis between the groups, the control group was

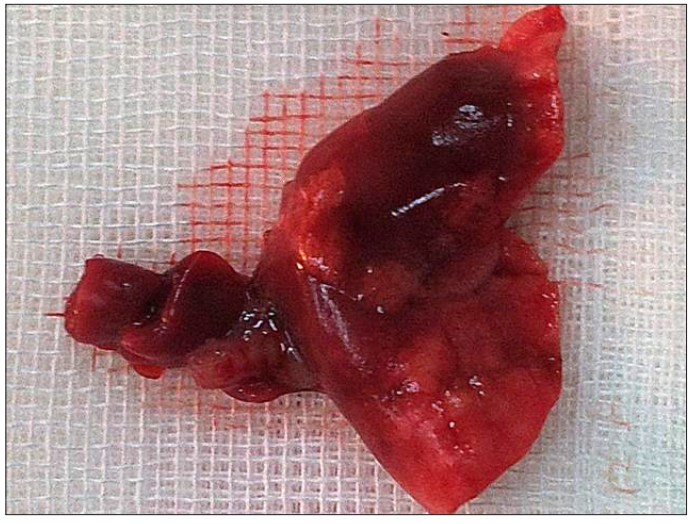

Figure 2. Macroscopy of the Lung Tissue obtained through sacrifice performed after Contusion.

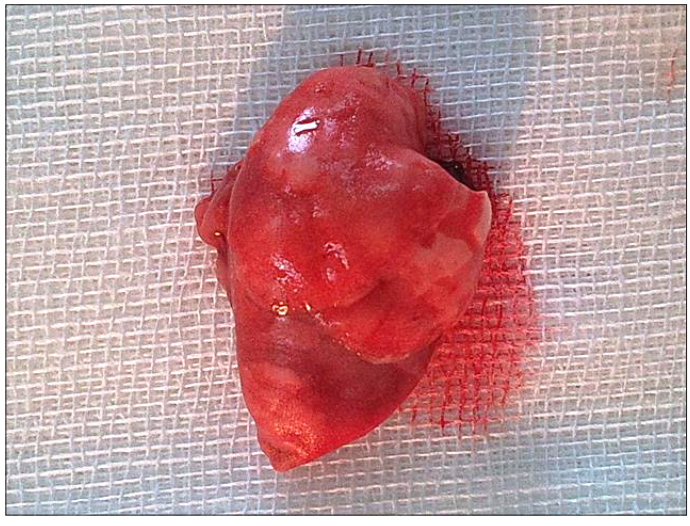

Figure 3. Macroscopy of the Lung Tissue derived through Sacrifice performed Five Days after Contusion.

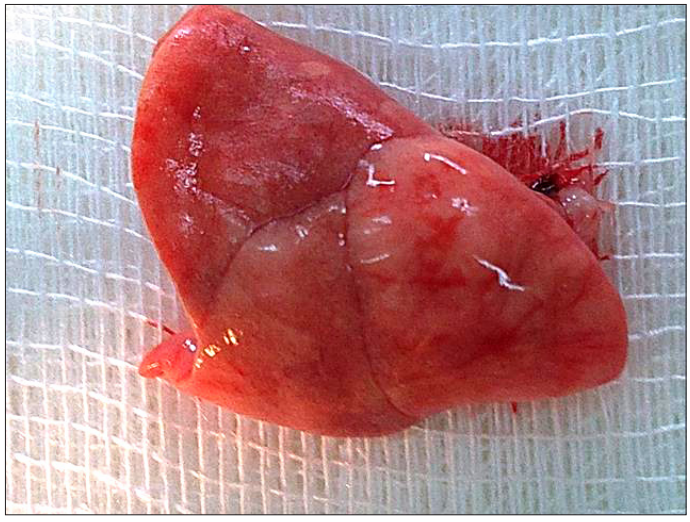

Figure 4. Macroscopy of the Lung Tissue derived through Sacrifice Five Days after Ozone Treatment following Contusion.

found to have significantly low atelectasis (p< 0,05) Tables 1 and 2. The paired comparisons between the control group and the trauma group and the ozone group showed significantly low atelectasis in the control group. However, there was not a significant difference between the trauma and the ozone groups in terms of atelectasis (p>0,05) Tables 3, 4, and 5.

The cellular intensity was significantly lower in the control group than in the other groups (p< 0,05) (Tables 1 and 2). The paired comparisons between the control group, the trauma group, and the ozone group showed a significantly low cellular intensity in the control group (p<0,05). However, the difference between the trauma group and the ozone group was not significant in terms of cellular intensity (p> 0,05) (Tables 3-5).

When all the groups were compared in terms of perivascular mononuclear inflammation, the difference in the control group was significantly lower than in the other groups (p< 0,05) (Tables

Table 1. Median Values (Minimum and Maximum vales) of Histopathological Evaluations in all the Groups

Parameters		Atelectasis	Cellular intensity	Perivascular mononuclear inflammation grading	Bronchial damage grading	Leucocytic infiltration
Anesthesia	N	8	8	8	8	8
	Mean	0,38	0,38	0,25	0,00	0,25
	Std. Deviation	0,518	0,518	0,463	0,000	0,463
	Median	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**
	Minimum	Grade 0	Grade 0	Grade 0	Grade 0	No extravascular leukocytes
	Maximum	Grade 1	Grade 1	Grade 1	Grade 0	fewer than ten leukocytes
Contusion	N	8	8	8	8	8
	Mean	2,38	3,63	2,63	0,25	2,63
	Std. Deviation	0,518	0,518	0,518	0,463	0,518
	Median	**2,00**	**4,00**	**3,00**	**0,00**	**3,00**
	Minimum	Grade 2	Grade 3	Grade 2	Grade 0	presence of 10-45 leukocytes
	Maximum	Grade 3	Grade 4	Grade 3	Grade 1	more then 45 leucocytcs
Contusion + Ozone	N	7	7	7	7	7
	Mean	2,43	3,43	2,43	0,29	2,43
	Std. Deviation	0,535	0,535	0,535	0,488	0,535
	Median	**2,00**	**3,00**	**2,00**	**0,00**	**2,00**
	Minimum	Grade 2	Grade 3	Grade 2	Grade 0	presence of 10-45 leukocytes
	Maximum	Grade 3	Grade 4	Grade 3	Grade 1	more then 45 leucocytcs
Total	N	23	23	23	23	23
	Mean	1,70	2,43	1,74	0,17	1,74
	Std. Deviation	1,105	1,619	1,214	0,388	1,214
	Median	**2,00**	**3,00**	**2,00**	**0,00**	**2,00**
	Minimum	Grade 0	Grade 0	Grade 0	Grade 0	No extravascular leukocytes
	Maximum	Grade 3	Grade 4	Grade 3	Grade 1	more then 45 leucocytcs

Table 2. Analysis of Histopathological Parameters in all the Groups

	Atelectasis	Cellular intensity	Perivascular mononuclear inflammation grading	Bronchial damage grading	Leukocytic infiltration
Kruskal-Wallis	16,470	16,514	16,621	2,502	16,621
df	2	2	2	2	2
p-value	**0,000**	**0,000**	**0,000**	**0,286**	**0,000**

Table 3. Comparison of all Histopathological Parameters between the Control Group and the Trauma Group

	Atelectasis	Cellular intensity	Perivascular mononuclear inflammation grading	Bronchial damage grading	Leucocytic infiltration
Mann-Whitney U	0,000	0,000	0,000	24,000	0,000
Wilcoxon W	36,000	36,000	36,000	60,000	36,000
Z	-3,486	-3,486	-3,520	-1,464	-3,520
p -value	**0,000**	**0,000**	**0,000**	**0,143**	**0,000**

Table 4. Comparison of all Histopathological Parameters between the Control group and the Ozone Group

	Atelectasis	Cellular intensity	Perivascular mononuclear inflammation grading	Bronchial damage grading	Leucocytic infiltration
Mann-Whitney U	0,000	0,000	0,000	20,000	0,000
Wilcoxon W	36,000	36,000	36,000	56,000	36,000
Z	-3,356	-3,356	-3,395	-1,569	-3,395
p-value	**0,001**	**0,001**	**0,001**	**0,117**	**0,001**

Table 5. Comparison of all Histopathological Parameters between the Trauma Group and the Ozone Group

	Atelectasis	Cellular intensity	Perivascular mononuclear inflammation grading	Bronchial damage grading	Leucocytic infiltration
Mann-Whitney U	26,500	22,500	22,500	27,000	22,500
Wilcoxon W	62,500	50,500	50,500	63,000	50,500
Z	-0,204	-0,735	-0,735	-0,151	-0,735
p-value	**0,838**	**0,462**	**0,462**	**0,880**	**0,462**

Table 6. The Distribution of Types of Cells in all the Groups

			Groups			
			Anesthesia	Contusion	Contusion + Ozone	Total
Cell Type	Normal cell distribution	Count	6	0	0	6
		Row %	100,0%	0,0%	0,0%	100,0%
	Macrophage domination	Count	2	0	0	2
		Row %	100,0%	0,0%	0,0%	100,0%
	Revascularization	Count	0	4	4	8
		Row %	0,0%	50,0%	50,0%	100,0%
	The stage in which fibroblasts are seen	Count	0	4	3	7
		Row %	0,0%	57,1%	42,9%	100,0%
	Total	Count	8	8	7	23
		Row %	34,8%	34,8%	30,4%	100,0%

1 and 2). The paired comparisons between the control group and the trauma group and the ozone group showed significantly low perivascular mononuclear inflammation (PMI) in the control group (p<0,05). Perivascular mononuclear inflammation did not significantly differ between the trauma group and the ozone group (p> 0,05) (Tables 3-5).

Bronchial damage did not significantly differ between the groups (p> 0,05) (Tables 1 and 2). The paired comparisons between the groups did not show a significant difference in bronchial damage, either (p> 0,05).

Histopathological examination did not show edema in all three groups. The difference in leukocytic infiltration was significantly lower in the control group than in the other groups (p< 0,05). The comparison of the control group with the trauma group and the ozone group showed significantly low leukocytic infiltration in the control group compared to the others (p<0,05). No significant differences were observed between the trauma group and the ozone group in terms of leukocytic infiltration (p> 0,05) (Tables 3-5).

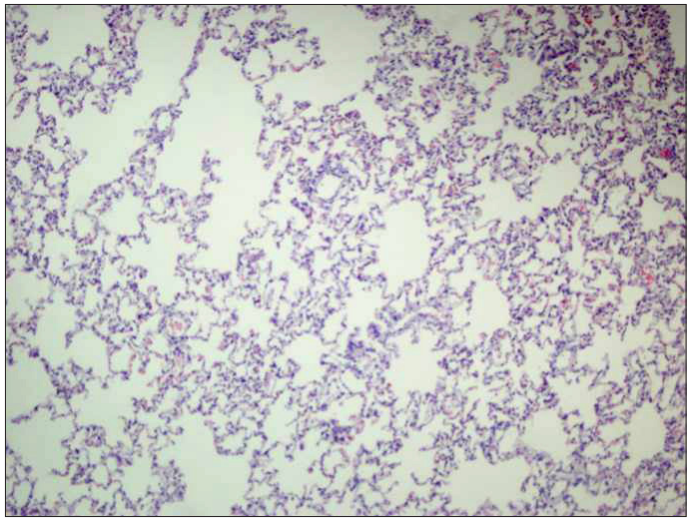

Figure 5. Histopathological View of Normal Rat Lung Tissue stained with H&E under x 100 Magnification

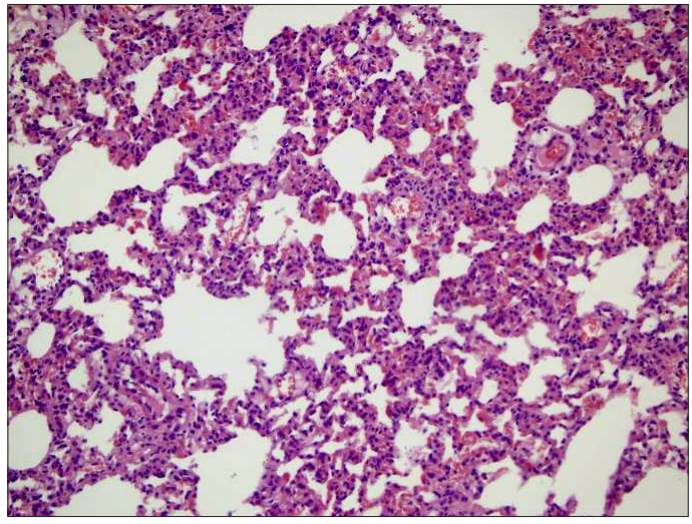

Figure 6. Alveolar Hemorrhage Foci in Rat Lung after Contusion stained with H&E and under x 200 Magnification

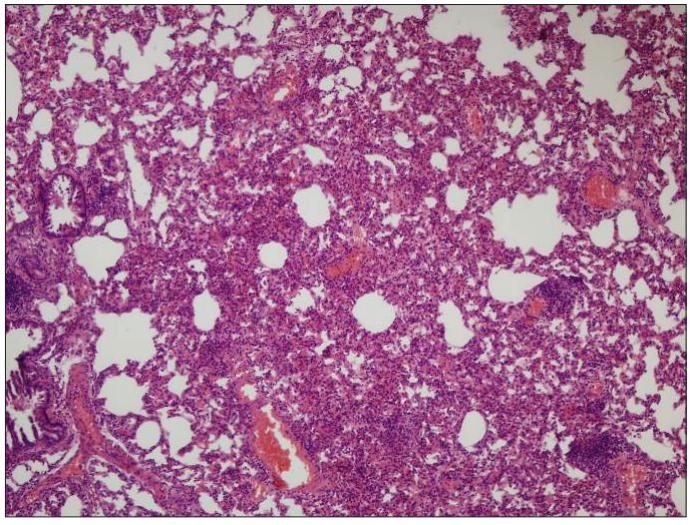

Figure 7. Histopathological View of an Inflammation Focus in Rat Lung stained with H&E x and under x100 Magnification

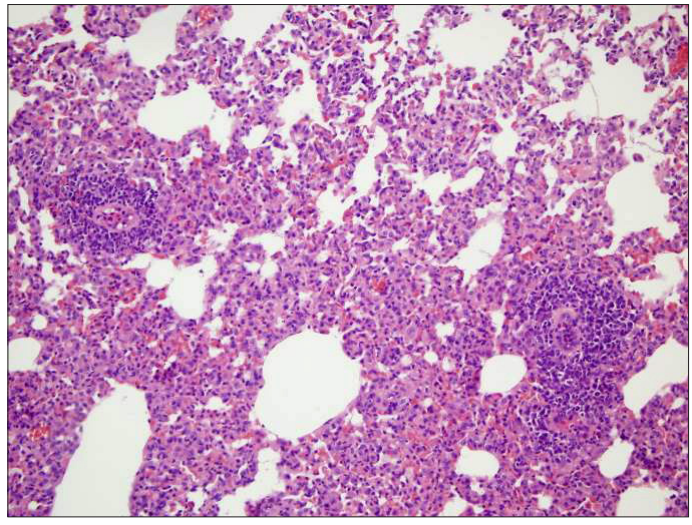

Figure 8. Histopathological View of Lymphoplasmacytic Cell Infiltration around the Vessel in a Rat Lung stained with H&E and under x 200 Magnification

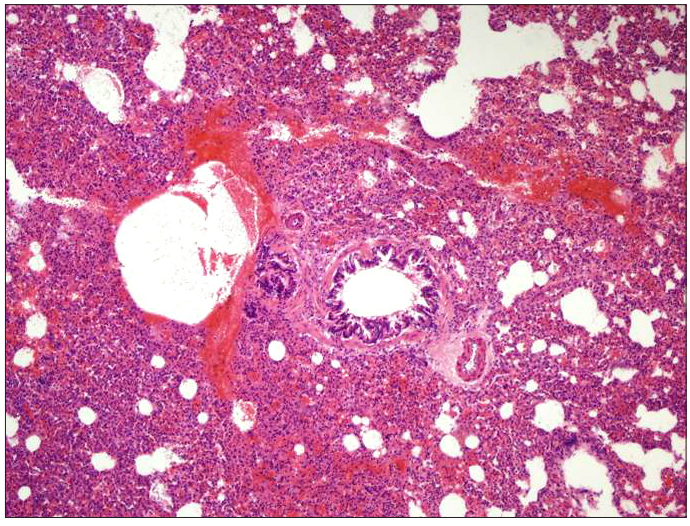

Figure 9. Severe Parenchymal Hemorrhage Foci in a Rat Lung after Contusion stained with H&E and under x 200 Magnification

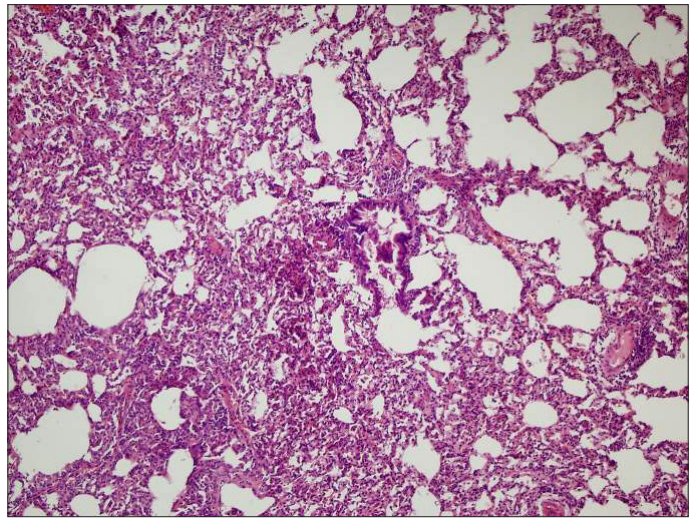

Figure 10. Histopathological View of a Rat Lung Tissue after Ozone Treatment stained with H&E and under x 100 Magnification

DISCUSSION

In the present study, effects of ozone treatment on direct mechanical damage after a blunt thoracic trauma and on inflammation secondary to trauma were evaluated based on the results of histopathological examination. It has been shown in experimental thoracic trauma models that an acute inflammatory response develops after a trauma, which decreases over time and returns to normal around the seventh day of trauma (13). Recent relevant studies have focused on systemic inflammatory processes after trauma, controlling these inflammatory responses and developing procedures supporting the body defense. The aim of the present study was to contribute to studies on systemic inflammatory responses in traumas and histopathological changes in the lung tissue. The results of the study are important in that ozone treatment was utilized to correct pulmonary damage due to contusion created by blunt thoracic trauma and that the results of a control group, a trauma group and an ozone group were compared.

The experimental models available in the literature was primarily based on creation of trauma at a standard impact energy in certain groups (14-16). The model used in the present study to create blunt thoracic trauma is unique and prepared in light of the literature and based on the research facilities available in the study setting (Figure 1). The advantages of this model are that it allows standardization of variability in trauma severity, can be reproducible and is easy to implement, and it is portable and very cost-effective. This model is suitable in terms of the ability to implement a standard trauma.

The most frequent inflammatory changes were increased inflammatory mediators, polymorphonuclear leukocyte infiltration, predominant inflammatory cell type and secondary alveolar edema. It has been shown in the literature that pulmonary injuries are based on inflammation and that the major cells causing tissue injuries are neutrophils (17-19)

Ozone treatment acts by disinfecting, immunomodulating, supplying oxygen to hypoxic tissues through vasodilation, providing a shift to the right in the hemoglobin-oxygen (HbO_2) disassociation curve, quickening tissue repair through release of growth factors and creating an effect on hormone release due to sudden homeostatic change. It is clear that ozone shows its most important antioxidant effect, both local and general by increasing the antioxidant defense capacity (20,21). In a case report by Bernardino Clavo et al., ozone exerted its effect by reversing the oxidative injury in damaged tissue (22).

In an experimental study on rats by Raghavendran et al., histopathological examination revealed predominance of neutrophils in 24 hours of pulmonary contusion and lymphocyte predominance and intra-alveolar edema in 48 hours of the contusion. In their study, parenchymal and perivascular neutrophil and mononuclear cell infiltration started 24 hours after the trauma, but significantly increased 48 hours after the trauma. With increased inflammation, bronchial damage developed at the end of 48 hours (3). In addition, atelectatic changes with surfactant dysfunction were also shown to occur after pulmonary contusion. These changes emerged in the first 24 hours and tended to improve in the 48th hour.

In the present study, respiratory and cardiac performance in the rats exposed to contusion was evaluated subjectively through macroscopy. A striking finding was presence of acute temporary respiratory failure and mild cyanosis, appearing soon after the blunt trauma in all the rats. They improved over seconds and spontaneous regular respiration was maintained.

Gonzalez et al. administered 10 µg/mL medical ozone at a dose of 0.36 mg/kg, 30 µgr/ml medical ozone at a dose of 1.1 µg/kg and 50 µgr/mL medical ozone at a dose of 1.8 mg/kg through the rectal route for five days to three groups of rats to investigate effects of ozone on cisplatin induced nephrotoxicity. They found that 1.1 mg/kg ozone was more effective than other doses and said that rectal insufflation is practical and easy to apply in rats (23). In the present study, the dose and the duration of ozone treatment were arranged in view of Gonzalez et al.'s findings.

Histopathological examination in the current study focused on cellular intensity, cell type, perivascular mononuclear inflammation, alveolar edema, bronchial damage and leukocyte infiltration. The cellular intensity, perivascular mononuclear inflammation and leukocyte infiltration were significantly higher in the pulmonary tissues of rats in the trauma group than in the control group ($p<0,05$).

There was not a significant difference in alveolar edema and bronchial damage between the control group, the trauma group and the ozone group ($p>0,05$). However, other histopathological values were significantly lower in the control group than in the trauma group and the ozone group ($p<0,05$). This suggested that the desired trauma model was created and a marked inflammatory response secondary to the trauma was formed in the present study.

In conclusion, ozone treatment had no positive effects on acute and subacute inflammatory responses in reduction of primary and secondary damage caused by localized trauma in rats. It can be recommended that anti-inflammatory and antioxidant effects of ozone should be investigated in acute and chronic pathologies separately.

Ethics Committee Approval: Dokuz Eylül University Ethics Committee of Animal Experiments. Protocol Number 59/2011.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - AS,FIU; Design - AS,FIU; Supervision - AS; Data Collection and/ or Processing - FIU, AS, EG, DG; Analysis and/or Interpretation - FIU, AS, EG, DG; Literature Search - FIU, AS; Writing - FIU, AS; Critical Reviews - FIU, AS

Conflict of Interest: The authors declare that there are no conflicts of interest.

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