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The Effect of Rectal Ozone Use on Bacterial Translocation and Oxidative Stress in Experimental Colitis Model

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ABSTRACT

Objective: The aim of this study was to examine the effect of rectal ozone on the histopathological healing of the colonic mucosa, tissue oxidative stress, and bacterial translocation in the experimental colitis model.

Materials and Methods: Three groups of rats were randomly formed [Group 1: Sham group, group 2: Control group, group 3: Ozone treatment group]. Microscopic and macroscopic scoring were done histopathologically in all groups. Glutathione-s-transferase (GST), superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) levels were measured in the colon and liver tissue. Blood cultures were taken for the detection of bacterial translocation.

Results: Microscopic damage scores were found as 0.0 (0.0-2.0) in the sham group, 3.0 (3.0-3.0) in the control group, 2.0 (0.0-2.0) in the ozone treatment group (p=0.001). Macroscopic damage scores were found as 0.0 (0.0-1.0) in the sham group, 3.0 (0.0-4.0) in the control group, 0.0 (0.0-1.0) in the ozone treatment group; the scores of ozone treatment and sham groups were found to be statistically different (p=0.004). Compared to the control group, the bacterial translocation in the liver, mesenteric lymph node, portal vein, spleen, and systemic blood was fewer in the ozone treatment group. Statistically significant differences were also observed between the groups' SOD and GST levels in colon tissue and MDA, SOD, and GST levels in liver tissue. Regarding MDA values in the liver tissue of the groups, it was 1.95±0.43 in Group I, 3.63±0.81 in Group II, and 1.19±0.72 U/mg in Group III (p=0.017). When the liver tissue SOD levels of the groups were examined, it was 8.21±0.76 U/mg in Group I, 4.57±0.58 U/mg in Group II, and 8.62±1.13 U/mg in Group III (p=0.029). When GST values in liver tissue belonging to the groups were examined, 0.35±0.16 in Group 1, 0.23±0.03 in Group II, 0.49±0.13 U/mg in Group III (p=0.036).

Conclusion: Rectal ozone application plays a role in increasing the organism's antioxidant activity and has an effective role in increasing the enzyme activities of antioxidant defenses. In addition, rectal ozone application has a positive effect on wound healing at a histopathological level and reduces bacterial translocation in various tissues.

Keywords: Experimental colitis, rectal ozone, oxidative stress

INTRODUCTION

Ulcerative colitis is a recurrent inflammatory disease seen only in the colon part of the digestive system; it is characterized by superficial and continuous ulcers starting from the rectum and ascending towards the proximal colon (1). In ulcerative colitis, pathological changes such as mucosal erosion and ulceration in the colon wall, deterioration of the colon gland structure, edema and inflammatory cell infiltration occur in the submucosa and mucosa (2,3). Although cellular damage has an important place in the

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pathogenesis of ulcerative colitis, the pathogenesis of the event has not been fully explained yet. One of the factors triggering or having etiological potential on ulcerative colitis is oxidative stress (4). Oxidative stress causes variations in oxidative parameters, including myeloperoxidase (MPO), glutathione (GSH) and malondialdehyde (MDA), during the inflammatory response (5). Another factor playing a significant role in the pathogenesis of ulcerative colitis is intestinal microflora (6). The resident microflora is an important building block for the host, with some metabolic functions, trophic effects on the intestinal epithelium, and the barrier function it provides through bacterial antagonism (7). With the presence of robust microflora, the settlement of pathogenic microorganisms is eliminated. This is provided by low luminal pH which is provided by microflora and the consumption of trace molecules necessary for pathogenic microorganisms. Thus, invasion of enteroinvasive microorganisms into intestinal epithelial cells is prevented. Intestinal lesions occur as a result of the inflammatory activity between the microflora and the colonic mucosa. Barrier function of the mucosa is impaired with local mucosal damage and inflammation (8), which causes bacterial translocation that occurs due to the spread of bacteria (alive or dead) in the intestine and the emergence of their toxic products in the liver, mesenteric lymph nodes, spleen and systemic circulation (9). Factors such as changes in normal intestinal flora, problems with the immune system, and disruption of the mucosal barrier have a significant role in the formation of bacterial translocation (10, 11).

Ozone is a molecule containing three atoms and is more unstable than oxygen. Therefore, it is more effective than oxygen in generating a biological response. With its strong oxidant feature, it can react chemically with all oxidizable organic compounds (12). The effect of ozone on metabolism varies depending on its concentration and dose (13). The dose to be selected to obtain a stimulating or suppressive effect varies according to the oxidative load and antioxidant capacity of the tissue to be treated. In applications below the required dose, no response will be obtained, and carbohydrates, enzymes, DNA and RNA may be affected by reactions, since the antioxidant capacity will be exceeded in the use of high doses. In order to apply medical ozone therapy, an ozone-oxygen mixture is obtained from pure oxygen with the help of a generator. The predominantly used systemic treatment dose range is 20-80 µg/ml, and the local treatment dose range is 1-20 µg/ml. Although there are many ozone application methods, the rectal route is among the frequently applied ones.

During rectal administration, ozone reacts with water in the colonic mucosa, secreted antioxidants, mucoproteins and glycocalyx, leading to the release of NO from the endothelium and releasing stem cells, erythrocytes containing high amounts of antioxidant enzymes and 2,3-diphosphoglycerate (2,3-DPG) from bone marrow. Rectal ozone also increases the concentration of oxidative shock proteins and antioxidant enzymes in cells. However, ozone increases oxygen transport and glycolysis capacity in erythrocytes, provides immune activation in leukocytes, and causes the secretion of growth factors from platelets (14, 15).

The present study planned to investigate the effect of ozone, whose antioxidative and anti-inflammatory properties on the healing of the colonic mucosa, tissue oxidative stress parameters, and bacterial translocation were previously reported by the rectal administration in an experimental colitis model created with acetic acid.

MATERIALS AND METHODS

Wistar Albino rats [a total of 24 rats weighting 230-250 grams were used in the 3 groups (n=8)] were acquired from Cumhuriyet University's Animal Center Laboratory (Sivas, Türkiye), and kept in standard conditions: light and dark cycles of 12 hours (turning on the lights at 08:00 a.m.), *ad libitum* food and water were given at the 22±2°C. The experiments were carried out, between 09:00 a.m.-17:00 p.m. The procedures were handled following the National Institute of Health guidelines of "Principles of animal laboratory care". Cumhuriyet University Animal Ethics Committee approved the experimental protocols (Approval date and number: 25.04.2019-65202830-050.04.04-282).

3 groups of rats were formed randomly, which are;

Group I (Sham group) (n=8): Normal rats, nothing has been administered on them.

Group II (Control group) (n=8): For colitis formation, after anesthesia, a 5 cm thin catheter was entered into the rectum in the supine position and 1 mL of 4% acetic acid was administered intrarectally. After 30 seconds it was washed with 1.5 mL of phosphate buffer. In the group with colitis, 4 days i.v. 0.09% saline was applied from the tail vein (Image1).

Group III (Ozone treatment group) (n=8): In the group with colitis, an ozone application at a dose of 20 µg/mL was applied intrarectally for 7 days in the supine position.

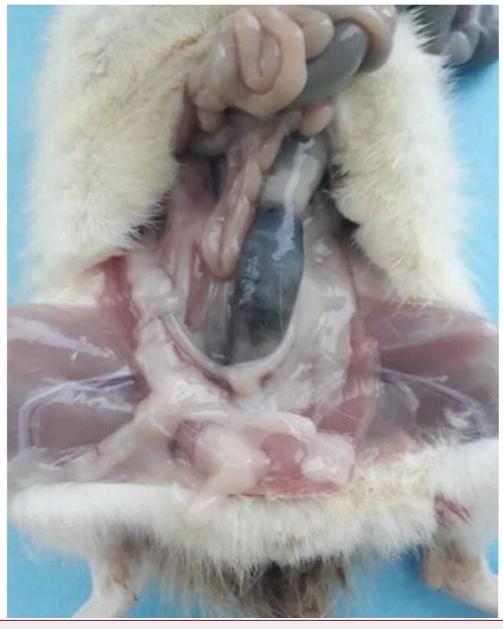

Image 1. Colitis model in rats.

Surgical Procedure

On the 7th day of colitis induction, midline abdominal shaving was carried out in the colitis, sham, and treatment group rats under anesthesia with 8 mg/kg xylazine and intraperitoneal 50 mg/kg ketamine. First site cleaning was performed with an antiseptic solution (10% povidone iodine) than midline laparotomy was performed.

To analyze the blood culture, 4 mL of blood was taken from the vena cava, they were then placed in biochemistry tubes of 2-3 mL. To detect bacterial translocation, 1-2 mL of blood was put into Bact Alert aerobic culture bottles, and attention was paid to sterility. The lymph nodes in the liver, mesentery, and spleen were disintegrated using sterile forceps, homogenized, and transferred into the prepared medium. Then, the colon was resected from the middle of the transverse colon to the rectum.

Rats were anaesthetized by inhalation of lethal doses of ether. Blood samples were placed at -80°C after being centrifuged. Colon resection material was opened longitudinally to measure Glutathione-s-transferase (GST), superoxide dismutase (SOD), GSH, and MDA levels in the colon tissue and to make macroscopic and microscopic evaluation, and the fecal content was cleaned with 0.9% saline. The samples required to measure SOD, MDA, GSH, and GST levels from liver tissue were stored at -80°C for homogenization. The other parts of the tissue were kept in 10% formaldehyde for macroscopic and microscopic evaluations.

Histopathological Evaluation

After keeping the tissue samples in 10% formaldehyde for 24 hours, they were scored macroscopically in the laboratory of the Department of Pathology by the only pathologist who did not know which group they belonged to (Table 1). The classification of the macroscopic changes in the colonic mucosa was made in five different ways, according to the description of Campos et al. (16). Macroscopic morphology scores are given in Table 1.

For microscopic investigation, samples of 2 mm thickness were taken from the column pieces. With the Sakura brand tissue tracking device, dehydration, transparency, and tissue hardening were performed by passing through alcohol, xylene, and paraffin stages in the tissue monitoring process that lasted for 15 hours. Afterwards, the tissues were turned into blocks by embedding paraffin in a Thermo Shandon brand tissue embedding device. After the blocks were cooled in the refrigerator, 3-micron thick sections were taken in the Leica brand microtome device. The slice slides were placed in an oven for 1.5 hours at 65 degrees for deparaffinization. The preparations to be stained with hematoxylin-eosin were taken out of the oven and kept in xylene for 20 minutes. In ethyl alcohol series (100%, 90%, 80%) it was kept for 10 minutes and washed in tap water. After washing, it was left in Hematoxylin paint (Facepath brand) for 2.5 minutes. After washing it in tap water again, it was immersed in acid alcohol solution once and taken back into tap water. It was immersed in ammonia water 3 times and washed again in tap water. After washing, it was left in the eosin solution for 1.5 minutes. The painting process was completed by dipping 4-5 times in alcohol series (80%, 90%, 100%). The preparations whose staining was completed were taken into the oven and left for 10 minutes to become transparent in xylene. Entellan was dropped onto the preparations extracted from xylene and a coverslip was placed on the slide. After the closing process, a microscopic examination was started.

The microscopic changes in the mucosa were evaluated according to the suggestion of Yamamoto et al. The grades between 0 and 3 were given to the microscopic changes in the colonic mucosa as displayed in Table 2 (17).

Bacterial Translocation Detection

The samples placed into a Bact Alert bottle for blood culture were evaluated in the automated culture system. Planted bottles are incubated at 35–37°C, shaken constantly and mon-

Table 1. Classification of macroscopic findings in colonic mucosa.

Degree	Findings
0	Preserved, normal looking mucosa
1	Erythematous areas without edema, congestion, and superficial ulceration
2	Linear superficial ulcerations (7-15 mm), depressed erythematous or darker mucosa appearance, granular ground
3	Ulcerous area (14-45 mm) on uneven ground, normal islets around ulcerated mucosa or edematous mucosa
4	Diffuse irregular and multiple ulcers (>45 mm), thinned bowel wall, granular, irregular base

Table 2. Classification of microscopic findings in colonic mucosa.

Degree	Findings
0	Normal epithelium
1	Single epithelial cell loss, moderate epithelial swelling, single inflammatory cell infiltration in the crypts, mild monocyte-neutrophil infiltration
2	Multiple epithelial cell loss, epithelial flattening, crypt formation and moderate monocyte-neutrophil infiltration
3	Marked epithelial ulceration, crypt abscesses, and marked increase in monocyte and neutrophil levels

itored at 10-minute intervals. The CO_2 produced in the liquid culture bottles was continuously measured according to the colorimetric principle and each cell of the device was monitored by reflectometers. The detection of a positive bottle was reported by audiovisual messages. In this system, eosin-methylene-blue agar plates and blood agar were transferred from the positive bottles and incubated at 37°C for 24 hours. Then, traditional methods were employed to identify the microorganism's growth. Samples lacking a positive signal within 7-day were taken as negative. Tissue samples placed in the brain-heart infusion medium in sterile glass bottles were incubated for 24-48 hours at 37°C. Then passages were made on eosin-methylene-blue agar and blood agar from the liquid medium and incubated at 37°C for 24 hours. Reproducing microorganisms were identified by traditional microbiological methods.

The Collection and Preparation of the Samples for the Analysis

After the sacrification of the experimental animals, liver and intestine tissues were collected. Liver and intestinal tissues extracted from rats were first weighed. Tissues were washed with cold phosphate buffered solution before and during homogenization. They were then centrifuged with external phosphate buffer (pH: 7.4) in ice water at +4°C at 40,000xg for 60 minutes and homogenization was carried out. Supernatants were transferred to eppendorf tubes for MDA, GST, GSH, and protein determination. Supernatants from liver and intestinal tissue samples were put into a chloroform/ethanol mixture of equal volume (3/5 v/v), then vortexed and centrifuged at 6000xg for 10 minutes at +4°C and the upper phase was left to determine. Thus, the extraction for determining SOD activities was completed and the upper phase was used for analysis. All unused samples were kept at -80°C till the analysis.

Biochemical Analysis

MDA analysis in tissue samples were performed as implied by Yoshioka et al. (18). GST and GSH liver tissue protein analysis in cytoplasmic fraction were carried out spectrophotometrically according to the method suggested by Warholm et al. (19). In the study, SOD activity was determined spectrophotometrically as proposed by Sun et al. (20).

Statistical Analyses

The Statistical Package for Social Sciences, ver. 17.0 was used in statistical analysis of the data (SPSS, Inc., Chicago, IL, USA). The Mann Whitney U-test was used as a post-hoc test and the Kruskal Wallis test was employed for analysis of macroscopic scores, microscopic scores, and biochemical parameters between groups. Data are given as mean±standard deviation (SD). Statistical significance was taken at $p<0.05$ level.

RESULTS

The Results of Histopathological Evaluation

In the current study, the scores of macroscopic damages in colon tissue were reported as mean±SD. They were found as 0.13±0.35 in the sham group, 2.88±1.25 in the control group, 1.0±0.53 in the ozone treatment group; the scores of ozone treatment and sham groups were found to be statistically different (p=0.004). The inflammation that occurred significantly in the control group was significantly decreased in the treatment group (p=0.004).

In our study, the microscopic damage score values in the colon tissue were statistically expressed as the mean±SD value. It was found as 0.38±0.74 in the sham group, 3.0±0.0 in the control group, 1.75±0.71 in the ozone treatment group, and it was determined that the values in the groups were statistically differ-

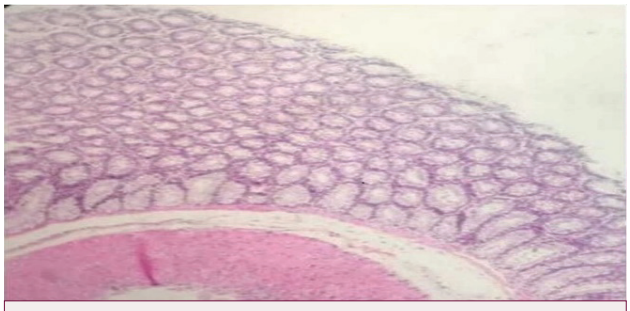

Image 2a. Sham group; normal colonic mucosa.

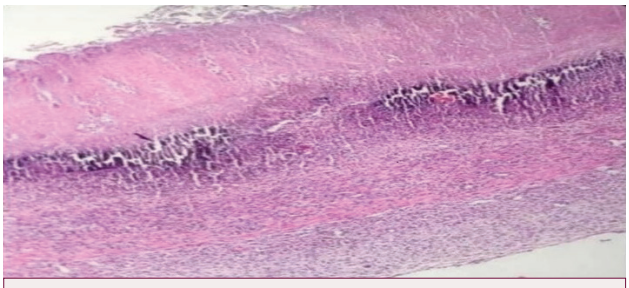

Image 2b. Control group; prominent epithelial ulceration (HEX200).

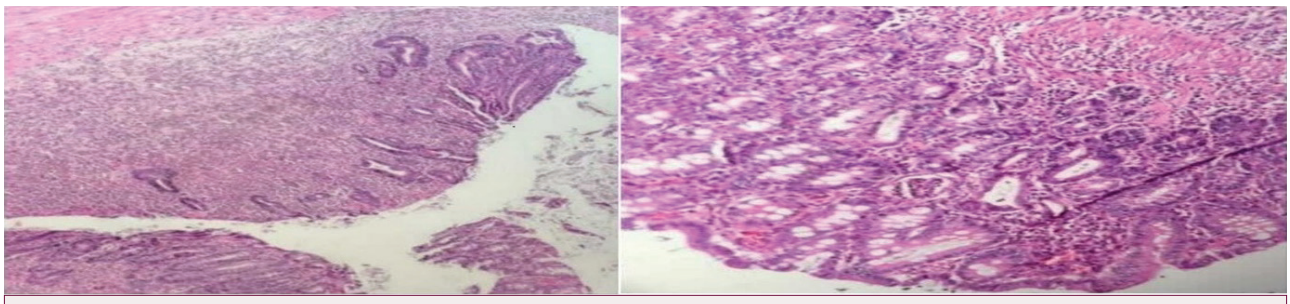

Image 2c -2d. Ozone therapy group; linear superficial ulcerations, loss of single epithelial cells, moderate epithelial swelling (HEX100).

ent from each other (p=0.001). The histopathological images belonging to the groups are shown in Images 2a, 2b, 2c and 2d.

The Results of Bacterial Translocation

No bacterial growth was detected in the sham group. The bacterial translocation frequency was observed to increase significantly in the liver, mesenteric lymph node, spleen, and portal vein blood of the group with acetic acid in which the colitis was formed. The bacterial translocation in the liver, spleen, mesenteric lymph node, systemic blood, and portal vein were observed to decrease in the ozone-treated group compared to control group. The bacterial translocation incidences observed in each group are displayed in Table 3. The translocated bacteria were determined as *Escherichia coli, Enterococcus spp, Proteus spp,* and *Klebsiella pneumoniae*.

Table 3. Bacterial translocation by groups.

Tissues	Sham Group	Control Group	Ozone-treated Group
MLN	0	4	2
Spleen	0	4	2
Liver	0	3	1
Portal Blood	0	4	2
Systemic Blood	0	3	1

Table 4. Biochemical findings.

		Min-Max	Mean±SD	p
Colon tissue MDA	**Group 1**	3.36-9.72	5.91±2.39	0.823
	Group 2	2.39-9.21	6.53±3.64	
	Group 3	3.07-7.73	5.54±1.71	
Liver tissue MDA	**Group 1**	1.60-2.54	1.95±0.43	0.017*
	Group 2	2.73-4.55	3.63±0.81	
	Group 3	0.28-1.85	1.19±0.72	
Colon tissue SOD	**Group 1**	1.50-8.75	5.19±2.63	0.046*
	Group 2	2.72-4.10	3.20±0.63	
	Group 3	6.41-9.02	7.34±1.46	
Liver tissue SOD	**Group 1**	7.38-8.88	8.21±0.76	0.029*
	Group 2	3.75-5.02	4.57±0.58	
	Group 3	7.18-9.56	8.62±1.13	
Colon tissue GSH	**Group 1**	362.07-545.73	453.11±84.64	0.779
	Group 2	344.55-525.76	430.61±89.89	
	Group 3	381.28-643.58	474.77±115.89	
Liver tissue GSH	**Group 1**	362.07-553.71	454.77±89.37	0.334
	Group 2	316.34-488.28	405.63±87.54	
	Group 3	403.36-634.62	500.47±99.13	
Colon tissue GST	**Group 1**	0.34-0.88	0.53±0.23	0.049*
	Group 2	0.19-0.29	0.22±0.06	
	Group 3	0.31-0.81	0.61±0.27	
Liver tissue GST	**Group 1**	0.19-0.54	0.35±0.16	0.036*
	Group 2	0.20-0.26	0.23±0.03	
	Group 3	0.31-0.67	0.49±0.13	

* indicates p<0.05, values shown as U/mg

Two ratio tests, which tested the number of positive tests, were used to compare bacterial translocation in all animals (8 rat-groups; $p<0.05$).

When the SOD levels in the colon tissue of the groups were examined, it was 5.19±2.63 U/mg in group I, 3.20±0.63 U/mg in group II, and 7.34±1.46 U/mg in group III. A statistically significant difference was observed between the colon tissue SOD levels of the groups (p=0.046). When the liver tissue SOD levels of the groups were examined, it was 8.21±0.76 U/mg in group I, 4.57±0.58 U/mg in group II, and 8.62±1.13 U/mg in group III. A statistically significant difference was also observed between the SOD levels in the liver tissue of the groups (p=0.029; Table 4). Colon and liver tissues SOD levels belonging to the groups were shown in Figure 1.

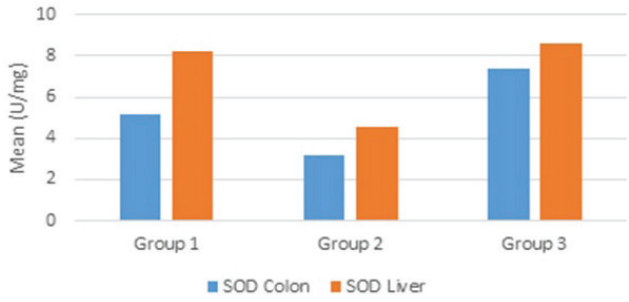

Figure 1. SOD values in colon and liver tissue.

When the values of the MDA belonging to the colon tissue of the groups were examined, it was 5.91±2.39 in group I, 6.53±3.64 in group II, and 5.54±1.71 U/mg in group III. The difference between MDA values in the colon tissues was statistically insignificant (p=0.823). Regarding MDA values in the liver tissue of the groups, it was 1.95±0.43 in group I, 3.63±0.81 in group II, and 1.19±0.72 U/mg in group III. A statistically significant difference was observed between MDA levels in the liver tissue of the groups (p=0.017). Colon and liver tissues MDA values belonging to the groups were shown in Figure 2.

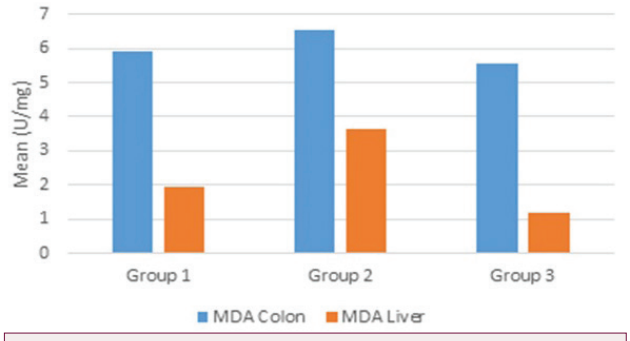

Figure 2. MDA values in colon and liver tissue.

When the GSH values in the colon tissue of the groups were examined, it was 453.11±84.64 in group I, 430.61±89.89 in group II, and 474.77±115.89 U/mg in group III. The difference between GSH values in colon tissue of the groups was insignificant (p=0.779). Regarding GSH values in the liver tissue of the groups, it was 454.77±89.37 in group I, 405.63±87.54 in group II, and 500.47±99.13 U/mg in group III. The considerable differences between GSH values in the liver tissue of the groups was insignificant (p=0.334). Colon and liver tissue GSH values belonging to the groups are shown in Figure 3.

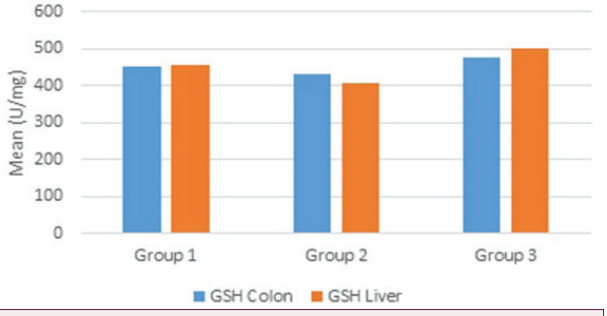

Figure 3. GSH values in colon and liver tissue.

When the GST values in the colon tissue of the groups were examined, it was 0.53±0.23 in group I, 0.22±0.06 in group II, and 0.61±0.27 U/mg in group III. A statistically significant difference was observed between GSH values (p=0.049). When GST values in the liver tissue belonging to the groups are examined, 0.35±0.16 in group 1, 0.23±0.03 in group II, 0.49±0.13 U/mg in group III. A statistically considerable difference was observed between the GST values in the liver tissue of the groups (p=0.036). GST values in colon and liver tissue belonging to the groups are shown in Figure 4.

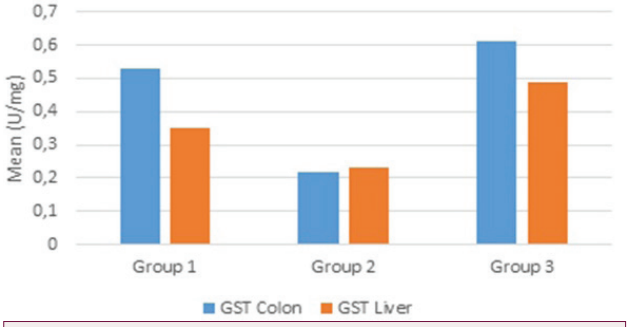

Figure 4. GST values in colon and liver tissue.

DISCUSSION

According to the results of our study, rectal ozone application caused a significant improvement in the colonic mucosa of rats with ulcerative colitis. The decrease in MDA levels in tissues and enzyme changes related to other oxidative reactions indicate that ozone therapy protects the colonic mucosa from oxidative stress. In addition to oxidative stress-related enzyme changes, a significant improvement was observed in the colonic mucosa of the control group with ozone treatment in the histopathological classification of microscopic and macroscopic tissue samples.

Lipid peroxidation occurs due to the free oxygen radicals is one of the most significant factors that causes oxidative damage in the cell membrane containing unsaturated fatty acids. The decrease in MDA in the tissue indicates that ozone therapy protects the colon tissue from oxidative stress. In the studies of Delgado-Roche et al. (21) when the effect of rectal ozone therapy on cellular redox was examined in multiple sclerosis patients, 20 µg/mL ozone was administered by rectal insufflation three times a week for one month. Immunoenzymatic and spectrophotometric tests were used to address the effect of ozone therapy on the biomarkers of inflammation and oxidative stress. Medical ozone was observed to improve the activity of antioxidant enzymes significantly and to increase the cellular reduced glutathione level. Accordingly, a significant decrease was observed in oxidative damage on lipids and proteins in the patients treated with rectal ozone.

In an animal study of Aslaner et al., medical ozone treatment was studied in the acute distal colitis; they have found its possible effect is by means of decreasing inflammation, edema, and affecting the proliferation and the vascularization to treat acute distal colitis (22). We think that the underlying mechanism of medical ozone treatment's anti-inflammatory effect can be a decrease in oxidative stress. In our study, GST, SOD, and GSH levels from colon and liver tissue increased in the rectal ozone group when compared to the control group. Regarding the ozone treatment group, the decrease in the MDA level from colon and liver tissue showed that ozone therapy plays an active role in protecting against oxidative stress. Eliakim et al. (23) studied the effect of an ozonated water enema on inflamed and normal mouse colon mucosa. The ozonated water (20 µg/mL) was prepared with an ozone generator and given intrarectally every day (0.5 mL). The rats were sacrificed on 1, 3 and 7 days after the administration of the rectal ozonated water, and their colons were resected. Damage was evaluated macroscopically and microscopically. Tissue myeloperoxidase and nitric oxide synthase activity was examined, and they found that ozone therapy did not cause macroscopic damage, but induced microscopic colitis accompanied by an increase in nitric oxide activity and myeloperoxidase as well as prostaglandin E2 formation. The groups in our study were evaluated histopathologically and while significant epithelial ulcerations were observed in the control group, linear superficial ulcerations, single epithelial cell loss and moderate epithelial swelling were observed in the ozone treatment group. Macroscopic and microscopic damage scoring results were also found to be better in the ozone treatment group. The study of Guanche et al. (24) was planned with a random distribution in four groups of 5 animals in each group. Group 1 was marked as the control group and received only oxygen; the remaining three groups (group 2, 3 and 4) were treated with a mixture of ozone/oxygen (O_3/O_2) by rectal insufflation with 600, 1400 and 2600 µg dosages in the volumes of 30, 40 and 50 mL, respectively. The study showed that O_3 increased pro-oxidant biomarkers and caused a high activation of the major enzymes of the antioxidant system. In the study, the increase of the antioxidant activity which went up 42 units, caused by the activity of superoxide dismutase, catalase, and glutathione peroxidase enzymes, was significant. The animal studies of León et al., in which carbon tetrachloride (CCl_4) was used as an oxidative threat, demonstrated that an adaptation to oxidative stress can be induced by repeated ozone administration at non-toxic doses, which allows animals to preserve hepatocellular integrity after CCl_4 poisoning (25).

The lack of antibiotic application was a limitation of the study. In conclusion, rectal ozone therapy in the experimental colitis model reduced colon and liver MDA levels significantly compared to the control group; it also improved the histopathological changes in the tissue, microscopic and macroscopic damage scoring results. In addition, the effect of ozone therapy is concluded to be associated with an increase in SOD, GSH, and GST levels in colon and liver tissue.

CONCLUSION

The findings of this study showed that, rectal ozone application plays a role in increasing the antioxidant activity of the organism and the enzyme activities of antioxidant defense systems. Moreover, rectal ozone applications have a positive effect on healing wounds at histopathological level and reduces bacterial translocation in various tissues.

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Ethics Committee Approval: Cumhuriyet University Animal Ethics Committee approved the experimental protocols (Approval date and number: 25.04.2019-65202830-050.04.04-282).

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Author Contributions: Conception/Design of Study - M.E.B., O.A., A.T.; Data Acquisition - M.E.B., O.A., T.K.; Data Analysis/Interpretation - O.A., A.T., T.K., S.G., Y.S.; Drafting Manuscript - M.E.B., O.A., S.G.; Critical Revision of Manuscript - T.K., S.G., Y.S.; Final Approval and Accountability - M.E.B., O.A., A.T.

Conflict of Interest: The authors have no conflict of interest to declare.

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