Protective effects of rectal ozone administration on colon anastomoses following radiotherapy

Tonguç Utku Yilmaz[1,A,D], Eda Yirmibeşoğlu Erkal[2,C], Saffet Çinar[3,D,E], Ceyla Eraldemir[4,B], Çiğdem Vural[5,E], Ezgi Uçar Taş[3,B], Nihat Zafer Utkan[3,F]

[1] Organ Transplantation Unit, Atakent Hospital, Acıbadem Mehmet Ali Aydınlar University, Turkey
[2] Department of Radiation Oncology, Faculty of Medicine, University of Kocaeli, Turkey
[3] Department of General Surgery, Faculty of Medicine, University of Kocaeli, Turkey
[4] Department of Biochemistry, Faculty of Medicine, University of Kocaeli, Turkey
[5] Department of Pathology, Faculty of Medicine, University of Kocaeli, Turkey

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2022;31(12):1355–1364

Address for correspondence
Tonguç Utku Yilmaz
E-mail: drutkuyilmaz@yahoo.com

Funding sources
None declared

Conflict of interest
None declared

Received on December 27, 2021
Reviewed on March 15, 2022
Accepted on July 14, 2022

Published online on August 24, 2022

Abstract

Background. Anastomotic leakage (AL) following rectal surgery is associated with increased mortality and morbidity. Neoadjuvant radiotherapy disrupts the wound healing process in rectal surgery.

Objectives. To evaluate the effects of intra-rectal ozone application on rectal anastomoses after radiotherapy.

Materials and methods. This study was performed on animals. Thirty-two male Wistar rats were randomly divided into 4 groups: control group, ozone group, radiotherapy group, and radiotherapy/ozone group. Ozone was administered intrarectally in the ozone group and water was administered intrarectally in rthe control group for 5 days. The radiotherapy group received 20 Gy of pelvic radiotherapy. The radiotherapy/ozone group received 20 Gy of pelvic radiotherapy after the administration of ozone. Afterward, colon resection followed by an anastomosis were performed under general anesthesia in all groups. Anastomotic segments were resected to evaluate tissue hydroxyproline (HYP) and myeloperoxidase (MPO) levels, perform a histological evaluation, and measure bursting pressure.

Results. There were no statistically significant differences between groups regarding tissue MPO levels ($p = 0.55$). Tissue HYP levels were significantly decreased in the radiotherapy group ($p = 0.04$). Bursting pressure was found to be significantly lower in the radiotherapy group ($p < 0.05$). No significant differences were found between adhesion scores in the control and ozone groups. Exudate formation was significantly lower in the radiotherapy group ($p < 0.05$). The lowest macrophage scores were found in the radiotherapy group ($p < 0.05$). Fibroblast scores were the highest in the control group and the lowest in the radiotherapy group ($p < 0.05$).

Conclusions. Intra-rectal ozone application significantly improved the anastomotic healing process after radiation exposure.

Key words: colon anastomosis, ozone, radiotherapy, anastomotic leakage, rectal administration

Cite as
Yilmaz TU, Erkal EY, Çinar S, et al. Protective effects of rectal ozone administration on colon anastomoses following radiotherapy. *Adv Clin Exp Med*. 2022;31(12):1355–1364. doi:10.17219/acem/152121

DOI
10.17219/acem/152121

Background

Anastomoses are vital steps in colorectal cancer surgery since anastomotic failures can lead to drawbacks such as increased morbidity, increased mortality and potentially worse oncological outcomes.[1] Anastomotic leakage (AL) after colorectal surgery has been reported to occur at a rate of 3–12% in prospective studies.[2,3] Preoperative radiotherapy is a risk factor for AL.[4] Poor blood supply and decreased oxygen delivery lead to hypoxia and have been blamed for the increased risk of AL.[5] After radiotherapy, ischemia leads to oxidative stress and increased production of free oxygen radicals that are the main destructive factor.[6] The overexpression of factors such as tissue growth factor β, vascular endothelial growth factor, tumor necrosis factor, and pro-inflammatory cytokines after radiation lead to uncontrolled matrix accumulation.[7] The uncontrolled matrix accumulation results in fibrosis, AL or a stricture.[7] In order to decrease the deleterious effects of radiotherapy, several agents such as glutamine, steroid, sucralfate, and N-acetylcysteine have been used.[8,9] The aim of using these agents is to improve wound healing by decreasing inflammation and hypoxia. Successful anastomotic healing includes not only histological healing but also the ability of the previously injured bowel to withstand tensile forces. If sufficient tissue strength cannot be restored, the anastomosis may burst upon a challenge with intraluminal pressure.

Ozone, chemically known as O_3, is made up of 3 oxygen atoms and has several functions such as being antimicrobial, antioxidative, regulating the immune response, and causing epigenetic modifications.[10] Oxidative preconditioning by ozone shows its protective effects against free radicals.[11] Ozone promotes macrophage activity and accelerates epithelialization in the colon.[11] The increased epithelialization after ozone administration has been shown to be beneficial in trophic ulcers, burns, gingivitis, and furunculosis.[6] The main mechanisms of ozone in wound healing are attributed to its local antioxidant properties and ability to promote tissue repair. The ozone has been administered via different routes such as intravenous, intra-arterial, subcutaneous, intramuscular, intra-articular, and via an enema.[12] An ozone enema induces a significant increase in mucosal prostaglandin E_2 production during the early period and increases nitric oxide synthase activity during the later period. However, the amount and concentration of ozone in the enema are very important.

Objectives

This study aimed to show the protective effects of rectal ozone administration on colon anastomoses following radiotherapy. This study is the first experimental study evaluating rectal O_3 administration. The relationship between ozone therapy and radiotherapy is a new area for future clinical and experimental studies.

Materials and methods

The experiment was approved by the Animal Ethics Committee at The University of Kocaeli, Turkey (approval No. 10/2-2015). The National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals were followed in handling of all animals.

Ozone

An ozone generator (Bozon N; Econica, Odessa, Ukraine) was used for producing ozone at a dose of 20 μg/mL. The injected mixture had a total volume of 2.5 mL. Five ozone applications performed once daily were used to achieve oxidative preconditioning. The flow rate of O_3 was maintained at 2 L/min. The gas mixture was composed of 96% O_2 and 4% O_3. Intra-rectal ozone applications were performed using 16 gauge silicone catheters that were inserted in the anus under sedation (using ether). The catheters were kept closed for 5 min after injection and then removed.

Animals and experiment

Thirty-two male Wistar rats weighing 300–450 g were housed individually in cages and given free access to regular rat meal and water both before and after the experiment. The animal rooms had no windows and were controlled for temperature (23 ±2°C) and light (12 h day and 12 h night). The rats were divided into 4 groups: control group, ozone group, radiotherapy group, and radiotherapy/ozone group. They were anesthetized with 100 mg/kg ketamine (Ketalar; Parke Davis Co. Inc., New Jersey, USA) and 5 mg/kg xylazine (Rompun; Bayer AG, Leverkusen, Germany) administrated intraperitoneally. The laparotomy procedure was performed through a standard 4-cm midline incision. A 1-cm resection of the left colon was performed from 2 to 3 cm above the peritoneal reflection. The continuity of the bowel was restored with an end-to-end anastomosis using 8–10 interrupted 6-0 monofilament Prolene sutures (Ethicon Ltd., Edinburgh, UK). Running sutures were used for abdominal and skin closure of the rats. The rats were euthanized with intracardiac blood collection.

In the control group, the intra-rectal water administration was performed for 5 days under ether sedation. Afterward, colon resection and anastomosis were performed. Five days after the colon resection and anastomosis, the rats were anesthetized for in vivo analytic procedures.

In the ozone group, the intra-rectal ozone administration was performed for 5 days under ether sedation. Five days after the last administration of ozone, the animals were anesthetized for colon resection and anastomosis. Five days after colon resection and anastomosis, the rats were anesthetized for in vivo analytic procedures. For in vitro analytic procedures, the animals were euthanized.

In the radiotherapy group, the animals underwent ether sedations for 5 days. Five days after the last sedation, the rats underwent radiotherapy. While under anesthesia, each rat was placed in prone position on a styrofoam board. For radiotherapy planning, a volumetric computed tomography (CT) scan was obtained using a CT scanner (SOMATOM Definition AS; Siemens, Munich, Germany). The rectum was contoured in its entirety to define the critical target volume (CTV) with uniform margins of 3 mm added in each direction in order to define the planning target volume (PTV). An isocentric technique was used to deliver a dose of 20 Gy using opposed anterior and posterior portals on a linear accelerator (Oncor; Siemens) with 6 Mv photons. Five days after radiotherapy, the animals were anesthetized for colon resection and anastomosis. Five days after colon resection and anastomosis, the rats were anesthetized for in vivo analytic procedures. For in vitro analytic methods, the animals were euthanized.

In the radiotherapy/ozone group, the intra-rectal ozone administration was performed for 5 days under ether sedation. Five days after the last administration of ozone, the animals underwent radiotherapy treatments as described above. Five days after radiotherapy, the animals were anesthetized for colon resection and anastomosis. Five days after colon resection and anastomosis, the rats were anesthetized for in vivo analytic procedures. For in vitro analytic methods, the animals were euthanized.

Analytic procedures

The existence of infection, dehiscence and intra-abdominal adhesions were regarded as complications. For grading intra-abdominal adhesions, the method described by Knightly et al. was used.[13] A grade of 0 indicated no adhesion; grade 1 – a single, thin, easily separable adhesion; grade 2 – less extensive but weak adhesions that withstood traction poorly; and grade 3 – numerous extensive visceral adhesions that involved the adjacent abdominal wall. Polypropylene sutures were used to identify the anastomotic line. Without removing adhesions, the burst pressure (BP) was measured in situ. The BP of each anastomosis was measured with a fluid pump (B. Braun, Frankfurt, Germany) working at 5 mL/min and a pressure transducer (Abbot Monitoring Kit Transpac II; Abbott Ireland Ltd., Sligo, Ireland) to determine the strength of the anastomosis. Colonic segments of at least 2 cm were prepared separately from the anastomosis. The infusion pump was inserted in the proximal part of the bowel segment using a 6-Fr catheter and the distal part was occluded using a 2-0 silk suture to avoid any air or fluid leak. Fluid was transferred from the catheter at a speed of 30 mL/h. The pressure was observed and leakage was viewed using a magnifying lens and identified by a sudden loss of pressure. After measuring the BP, the rats were euthanized. The anastomosis segment of the colon was resected. Half of the segment was fixed in 10% formaldehyde and embedded in paraffin. The other half was frozen for a subsequent determination of collagen content by hydroxyproline (HYP) measurements. Hydroxyproline measurements were performed using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology, Houston, USA) and the results were provided in ng/mg.[14] Myeloperoxidase (MPO) activity was measured by means of an ELISA kit (Hycult Biotech, Uden, the Netherlands) and the results were provided in ng/100 mg.[15]

After staining with hematoxylin and eosin (H&E), the anastomosis was evaluated histologically in a blinded fashion using the histological scoring described by de Roy van Zuidewijn et al.[16] The apposition of the wound edges of the mucosa and the muscularis mucosa were graded as 1 – good, 2 – moderate and 3 – poor to control for the surgical technique. The epithelialization of the wound healing procedure and the re-epithelialization of the mucosa were examined using a 7-point scale ranging from 1 – none to 7 – normal glandular mucosa. The regeneration of the muscularis propria was considered positive or negative (point scale, 1 = +, 2 = –). Other histological aspects such as necrosis, inflammatory exudate, granulation tissue, and the degree of granulocytes, macrophages and fibroblasts in the granulation tissue were evaluated. The histological parameters mentioned above were evaluated on a 4-point scale, as follows: 0 – negative, 1 – low, 2 – moderate, and 3 – high. Adhesion scoring was graded as 0 – weak adhesion, 1 – easily separable adhesion, 2 – moderate adhesion, and 3 – strong adhesion.

Statistical analyses

The statistical analysis was performed using IBM SPSS v. 22.0 software (IBM Corp., Armonk, USA). The Shapiro–Wilk test was used to determine whether the data fit a normal distribution. The Levene's statistic was used to assess the homogeneity of variance. A value of $p > 0.05$ was considered homogeneous (Table 1). One-way analysis of variance (ANOVA) was used to compare data with normal distribution. The Tukey's post hoc test was used for pairwise comparisons of the groups. Continuous variables showing non-normal distribution were compared using the Kruskal–Wallis test. A value of $p < 0.05$ was considered statistically significant for each test. For group comparisons, the Mann–Whitney U test with a Bonferroni correction was used. After the correction, a value of $p < 0.0083$ was considered statistically significant.

Results

All of the rats survived the surgery. As seen in Table 2, Fig. 1 and Fig. 2, the tissue MPO and HYP levels in the radiotherapy group were lower than in the other groups. Myeloperoxidase and HYP values were compared between the 4 groups using a one-way ANOVA test. There was

Table 1. Variance homogeneity of MPO, HYP and burst pressure

Variables	Levene's statistic	df1	df2	p-value
MPO levels	1.830	3	31	0.167*
HYP levels	2.025	3	31	0.135*
Burst pressure	2.187	3	31	0.112*

* p > 0.05 was considered sufficient for assumption of homogeneity of variance. MPO – myeloperoxidase; HYP – hydroxyproline; df – degrees of freedom.

Table 2. One-way analysis of variance (ANOVA) and post hoc Tukey's test of MPO and HYP values by groups

Dependent variable	Group (I)	F	df	Group (J)	Average difference (I–J)	p-value
MPO	control	4.686	31	ozone	68.65	0.621
				radiotherapy	110.24	0.064
				radiotherapy/ozone	–33.23	0.428
	ozone			control	–68.65	0.624
				radiotherapy	41.59	0.107
				radiotherapy/ozone	–101.88	0.996
	radiotherapy			control	–110.24	0.064
				ozone	–41.59	0.103
				radiotherapy/ozone	–143.47	0.153
	radiotherapy/ozone			control	33.23	0.424
				ozone	101.88	0.997
				radiotherapy	143.47	0.158
HYP	control	2.320	31	ozone	20.67	0.472
				radiotherapy	**498.46**	**0.000**
				radiotherapy/ozone	–88.67	0.771
	ozone			control	–20.67	0.479
				radiotherapy	**477.79**	**0.000**
				radiotherapy/ozone	–109.34	0.094
	radiotherapy			control	**–498.46**	**0.000**
				ozone	**–477.79**	**0.000**
				radiotherapy/ozone	**–587.13**	**0.000**
	radiotherapy/ozone			control	88.67	0.776
				ozone	109.34	0.098
				radiotherapy	**587.13**	**0.000**

MPO – myeloperoxidase; HYP – hydroxyproline; I – group designated for comparison; J – other groups compared; df – degrees of freedom. Values in bold show statistically significant differences between the groups. The value of p < 0.05 was considered sufficient for statistical significance.

no significant difference between MPO values between the groups (F (4.686), df = 31, p = 0.554). However, there was a statistically significant difference between HYP values (F (2.320), df = 31, p = 0.042). The Tukey's post hoc test revealed that there was a significant difference between the radiotherapy and other groups in comparison to the binary groups (Table 2). The mean MPO levels ± standard deviation (SD) (ng/mg) in the control, ozone, radiotherapy, and radiotherapy/ozone groups were 551.52 ±104.55, 482.87 ±141.07, 441.28 ±79.24, and 584.75 ±374.77, respectively. The mean HYP levels ±SD (ng/mg) in the control, ozone, radiotherapy, and radiotherapy/ozone groups were 1336.06 ±169.56, 1315.39 ±502.19, 837.60 ±258.20, and 1424.73 ±438.43, respectively.

Burst pressure values were compared between the 4 groups using a one-way ANOVA test. There was a statistically significant difference between BP values in the 4 groups (F (50.442), df = 31, p = 0.000) (Table 3, Fig. 3). The Tukey's post hoc test showed that there was a significant difference between the radiotherapy group and other groups (Table 3). The mean burst pressures ±SD (mm Hg) in the control, ozone, radiotherapy, and radiotherapy/ozone groups were 143.7 ±22.43, 162.5 ±36.47, 79.7 ±13.79, and 123.3 ±31.31, respectively.

The adhesion scores were found to be statistically significant and lower in the control and ozone groups compared to the other 2 groups (p < 0.05) (Table 4, Table 5).

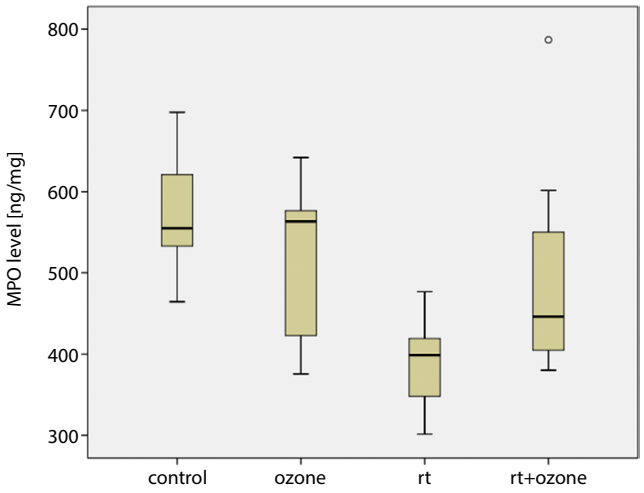

Fig. 1. Tissue myeloperoxidase (MPO) levels

rt – radiotherapy. An outlier was detected in 1 rat in the radiotherapy/ozone group.

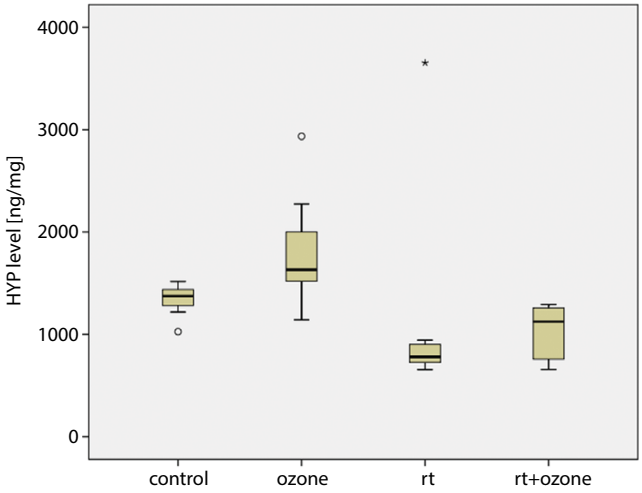

Fig. 2. Tissue hydroxyproline (HYP) levels

rt – radiotherapy. Outliers were detected in 1 rat in each of the control, ozone and radiotherapy groups.

Table 3. One-way analysis of variance (ANOVA) and post hoc Tukey's test of burst pressure values by groups

Dependent variable	Group (I)	F	df	Group (J)	Average difference (I–J)	p-value
Burst pressure	control	50.442	31	ozone	**–18.76**	**0.059**
				radiotherapy	**63.95**	**0.000**
				radiotherapy/ozone	**20.40**	**0.035**
	ozone			control	18.76	0.059
				radiotherapy	**82.71**	**0.004**
				radiotherapy/ozone	**39.16**	**0.007**
	radiotherapy			control	**–63.95**	**0.000**
				ozone	**–82.71**	**0.003**
				radiotherapy/ozone	**–43.55**	**0.001**
	radiotherapy/ozone			control	**–20.40**	**0.035**
				ozone	**–39.16**	**0.000**
				radiotherapy	**43.55**	**0.002**

I – group designated for comparison; J – other groups compared; df – degrees of freedom. Values in bold show statistically significant differences between the groups. The value of $p < 0.05$ was considered sufficient for statistical significance.

Upon histological evaluation of the groups, no tissue necrosis was observed. The scores of inflammatory exudate, macrophages, granulocytes, and fibroblasts in the radiotherapy group were significantly lower than those in the control group (Table 6, Table 7; $p < 0.05$). There were no significant differences among the groups regarding their tissue mucosa and muscularis propria apposition levels (Table 8, Table 9). The wound healing scores, except for the macrophage score of the radiotherapy/ozone and ozone groups were higher than those of the radiotherapy group but not statistically significant (Table 6). The macrophage scores of the radiotherapy/ozone group were significantly higher than those in the radiotherapy group (Table 5; $p < 0.05$). No statistically significant differences were found between the regeneration scores and re-epithelialization of the groups (Table 8, Table 10 and Table 11).

Discussion

Anastomosis is a complex process and one of the pivotal steps in the success of colorectal surgery.[1] Anastomotic wound healing is dynamic and involves multiple variables. Radiotherapy is a widely used treatment modality in the treatment of rectal cancer. Despite its widespread use, radiotherapy has many side effects.[4] Radiation has shown a negative effect on anastomotic healing in experimental and clinical studies.[3,4,17] Reducing the side effects of radiotherapy is one of the areas of interest in modern medicine. Therefore, the effects of ozone therapy in rats receiving radiotherapy are being investigated. Ozone therapy is a trending modality due to its hemostatic effects as well as its accelerating effects on tissue healing.[11,12] A controlled administration of O_3 can reduce the damage

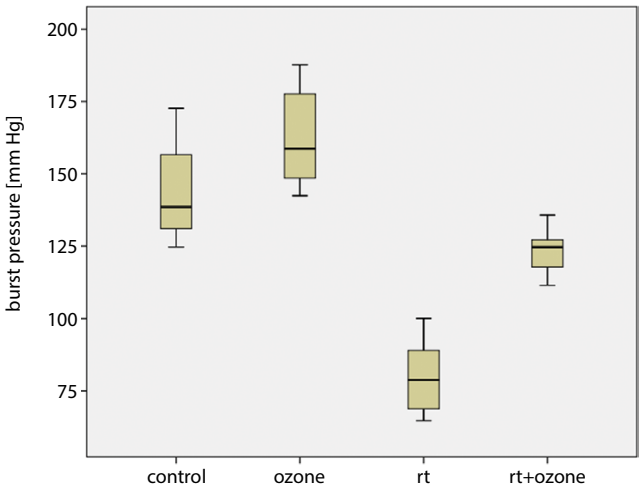

Fig. 3. Tissue burst pressure levels

rt – radiotherapy.

Table 4. Tissue adhesion scores*

Group	Median and SD	Adhesion
Control (n = 8)	median (Q1–Q3)	1 (0.25–0.75)
	SD	0.35
Ozone (n = 8)	median (Q1–Q3)	2.5 (2–3)
	SD	0.54
Radiotherapy (n = 8)	median (Q1–Q3)	3 (3–3)
	SD	0.62
Radiotherapy/ozone (n = 8)	median (Q1–Q3)	3 (3–3)
	SD	0.74
χ^2	overall	24.861
df (total)	overall	31
p-value	overall	0.013

Q1 – 1st quartile; Q3 – 3rd quartile; SD – standard deviation; df – degrees of freedom. * Kruskal–Wallis test was used and Mann– Whitney U test with Bonferroni correction was performed as post hoc test. A value of $p < 0.0083$ was considered sufficient for statistical significance.

Table 5. Adhesion score frequencies of the groups

Variable	Score	Control	Ozone	Radiotherapy	Radiotherapy/ozone
Adhesion	0	2 (25%)	0	0	0
	1	4 (50%)	0	0	0
	2	2 (25%)	4 (50%)	0	0
	3	0	4 (50%)	8 (100%)	8 (100%)

score 0 – weak adhesion; score 1 – easily separable adhesion; score 2 – moderate adhesion; score 3 – strong adhesion.

produced by reactive oxygen species (ROS) by maintaining the adaptation to O_3 oxidative preconditioning or stress. Here, the effects of ozone therapy on colonic anastomosis after radiotherapy were evaluated. For this reason, our study is an O_3 oxidative preconditioning study.

Anastomotic BP is one of the parameters that can show the safety and durability of an anastomosis. It can also provide information about the anastomotic collagen levels. Cronin et al. reported that the anastomotic BP is related to the collagen content.[18] Burst pressure was found to be significantly lower in the radiotherapy group compared to the other groups (Table 3, Fig. 3). This finding points to the negative effects of radiotherapy on anastomoses. The administration of ozone eliminated the negative effects of radiotherapy.

Hydroxyproline is the most important amino acid in collagen formation.[19] Different effects of radiotherapy on HYP levels have been reported in the literature. While some studies have shown that radiotherapy reduces the levels of HYP, others failed to show any relationship between radiotherapy and HYP levels.[20,21] In our study, the HYP level was lower in the radiotherapy group, as expected. Significant differences were found between other groups (Table 2, Fig. 2). The increased collagen synthesis with increased BP indicates that rectal O_3 administration improves wound healing.

Radiotherapy has been shown to play an important role in oxidative stress-related tissue toxicity and inflammation by increasing free oxygen radicals.[22] Under oxidative stress, it has been shown that MPO is secreted from the lysosomes of leukocytes.[23] Increased MPO activity causes tissue damage.[24,25] A reducing effect of ozone application on MPO levels was not detected in our study (Table 2, Fig. 1). Although the main mechanism of O_3 was thought to be related to nitric oxide pathways and antioxidative enzymes, our study did not provide any suggestion about this mechanism.[11] This might be a result of the administration route.

On the 5th day, there was no significant difference between the groups in terms of mucosal re-epithelialization and muscularis propria regeneration. Likewise, there was no significant difference between the groups in terms of their apposition scores (Table 8). These results show that epithelialization and regeneration are neither positively nor negatively affected by radiotherapy and ozone (Table 8). This shows that the dose of radiotherapy was suitable and the concentration of O_3 was not toxic. The most important parameter for the healing of mucosa and muscularis propria layers is the quality of the anastomosis technique.[1] In our study, all anastomoses were performed by a single investigator. No significant differences in adhesion scores were found in the control and ozone groups compared to the radiotherapy and radiotherapy/ozone groups (Table 5). The application of ozone might increase inflammation and accelerate the formation of tissue adhesions.

Table 6. Comparison of histological scores by groups*

Group	Median and SD	Necrosis	Granulation tissue	Inflammatory exudate	Macrophages	Granulocytes	Fibroblasts
Control (n = 8)	median (Q1–Q3)	0 (0–0)	1 (1–1)	0.5 (0–1)	2 (2–2.75)	1 (1–1.75)	2 (2–2.75)
	SD	0	0.51	0.53	0.69	0.64	0.69
Ozone (n = 8)	median (Q1–Q3)	0 (0–0.75)	1 (1–2)	1 (0.25–2)	2 (1.25–2)	2 (2–3)	2 (2–2)
	SD	0.37	0.53	0.75	0.48	0.48	0.37
Radiotherapy (n = 8)	median (Q1–Q3)	0 (0–0)	2 (1–2)	2.5 (2–3)	0.5 (0–1)	2 (2–2.75)	1 (1–1)
	SD	0.35	0.37	0.54	0.53	0.37	0.35
Radiotherapy/ ozone (n = 8)	median (Q1–Q3)	0 (0–0)	1 (1–1.75)	2 (1–2)	2 (1–2)	2 (1.25–2)	2 (1–2)
	SD	0	0.46	0.46	0.70	0.46	0.51
χ^2	overall	2.214	8.402	18.850	16.607	10.044	12.732
df (total)	overall	31	31	31	31	31	31
p-value	overall	0.574	**0.032**a**	**0.000**a,b,e**	**0.000**a,c,d**	**0.000**d**	**0.000**a**

Q1 – 1st quartile; Q3 – 3rd quartile; SD – standard deviation; df – degrees of freedom; * Kruskal–Wallis test was used and Mann–Whitney U test with Bonferroni correction was performed as post hoc test; ** $p < 0.0083$ was considered sufficient for statistical significance. Values in bold show statistically significant differences between groups. [a] – comparison between control group and radiotherapy group; [b] – comparison between control group and ozone group; [c] – comparison between radiotherapy and radiotherapy/ozone group; [d] – comparison between ozone group and radiotherapy group; [e] – comparison between control group and radiotherapy/ozone group.

Table 7. Histological score frequencies of the groups

Variable	Score	Control	Ozone	Radiotherapy	Radiotherapy/ozone
Necrosis	0	8 (100%)	6 (75%)	7 (87.5%)	8 (100%)
	1	0	2 (25%)	1 (12.5%)	0
	2	0	0	0	0
	3	0	0	0	0
Granulation tissue	0	1 (12.5%)	0	0	0
	1	7 (87.5%)	5 (62.5%)	3 (37.5%)	6 (75%)
	2	0	3 (37.5%)	3 (37.5%)	2 (25%)
	3	0	0	0	0
Inflammatory exudate	0	3 (37.5%)	2 (25%)	0	0
	1	5 (62.5%)	3 (37.5%)	0	6 (75%)
	2	0	3 (37.5%)	4 (50%)	2 (25%)
	3	0	0	4 (50%)	0
Macrophages	0	0	0	4 (50%)	0
	1	1 (12.5%)	2 (25%)	4 (50%)	3 (37.5%)
	2	4 (50%)	6 (75%)	0	4 (50%)
	3	3 (37.5%)	0	0	1 (12.5%)
Granulocytes	0	0	0	0	0
	1	6 (75%)	0	1 (12.5%)	2 (25%)
	2	2 (25%)	5 (62.5%)	5 (62.5%)	6 (75%)
	3	0	3 (37.5%)	2 (25%)	0
Fibroblasts	0	0	0	0	0
	1	2 (25%)	1 (12.5%)	7 (87.5%)	3 (37.5%)
	2	4 (50%)	7 (87.5%)	1 (12.5%)	5 (62.5%)
	3	2 (25%)	0	0	0

score 0 – negative; score 1 – low; score 2 – moderate; score 3 – high.

However, as the ozone was administered via the rectal route, there were no significant changes in the adhesion scores of the groups.

It is known that radiotherapy is an important cause of anastomosis leakage due to its effects on reducing tissue blood flow.[4] Decreased blood flow can cause tissue

Table 8. Mucosa and muscularis propria apposition levels. Re-epithelization of mucosa and regeneration of muscularis propria levels*

Group	Median and SD	Mucosa apposition	Muscularis propria apposition	Re-epithelization of mucosa	Regeneration of muscularis propria
Control (n = 8)	median (Q1–Q3)	2 (2–2.75)	2 (2–2)	1 (1–3.75)	1.5 (2–2)
	SD	0.48	0	1.25	0.53
Ozone (n = 8)	median (Q1–Q3)	2 (2–2)	2 (2–2)	1 (1–4.25)	2 (1.25–2)
	SD	0.57	0	1.49	0.48
Radiotherapy (n = 8)	median (Q1–Q3)	2 (2–2)	2 (2–2)	1 (1–1)	2 (1–2)
	SD	0.35	0.35	0	0.51
Radiotherapy/ ozone (n = 8)	median (Q1–Q3)	2 (2–2)	2 (2–2)	1 (1–2.75)	1 (1–2)
	SD	0.35	0	0.91	0.51
χ^2	overall	4.015	3	2.460	1.301
df (total)	overall	31	31	31	31
p-value	overall	0.694	0.457	0.483	0.549

Q1 – 1st quartile; Q3 – 3rd quartile; SD – standard deviation; df – degrees of freedom; * Kruskal–Wallis test was used and Mann–Whitney U test with Bonferroni correction was performed as post hoc test. A value of $p < 0.0083$ was considered sufficient for statistical significance.

Table 9. Mucosal and muscularis propria apposition frequencies of the groups

Variable	Score	Control	Ozone	Radiotherapy	Radiotherapy/ozone
Mucosa apposition	1	0	1 (12.5%)	0	0
	2	5 (62.5%)	6 (75%)	7 (87.5%)	7 (87.5%)
	3	3 (37.5%)	1 (12.5%)	1 (12.5%)	1 (12.5%)
Muscularis propria apposition	1	0	0	0	0
	2	8 (100%)	8 (100%)	7 (87.5%)	8 (100%)
	3	0	0	1 (12.5%)	0

score 1 – good; score 2 – moderate; score 3 – poor.

Table 10. Mucosal re-epithelialization frequencies of the groups

Variable	Score	Control	Ozone	Radiotherapy	Radiotherapy/ozone
Re-epithelization of mucosa	1	5 (62.5%)	5 (62.5%)	8 (100%)	5 (62.5%)
	2	0	1 (12.5%)	0	1 (12.5%)
	3	1 (12.5%)	0	0	2 (25%)
	4	2 (25%)	0	0	0
	5	0	2 (25%)	0	0
	6	0	0	0	0
	7	0	0	0	0

Scores: 1 – none; 2 – little, one-layer, cubic; 3 – large one-layer, cubic; 4 – almost complete, one-layer, cubic; 5 – complete, one-layer, cubic; 6 – one-layer, glandular; 7 – normal glandular mucosa.

Table 11. Muscularis propria regeneration frequencies of the groups

Variable	Score	Control	Ozone	Radiotherapy	Radiotherapy/ozone
Regeneration of muscularis propria	1	4 (50%)	2 (62.5%)	3 (37.5%)	5 (62.5%)
	2	4 (50%)	6 (12.5%)	5 (62.5%)	3 (12.5%)

score 1 – positive; score 2 – negative.

exudate, ischemia and necrosis.[4] The absence of necrosis showed that neither radiotherapy nor O_3 has toxic effects on the colon mucosa. Wound healing is composed of inflammatory exudate, macrophages, granulation tissue, and fibroblast formation. Macrophage and granulocyte cells are a group of cells that take part in the formation of granulation tissue.[15] Fibroblasts are the basic cells of connective tissue, taking part in collagen synthesis during the later stages of wound healing. In our study, all wound healing parameters decreased

in the radiotherapy groups. Although O_3 administration significantly improved wound healing in macrophages, granulocytes and fibroblast levels, granulation tissue formation also improved but was not statistically significant. This is probably due to not enough time for the formation of granulation tissue to occur. However, early wound healing parameters significantly improved with ozone administration when compared to radiotherapy. The negative effects of radiotherapy on wound healing have been shown in previous studies.[15,20] There are studies showing the effect of radiotherapy in preventing the migration of fibroblasts to the wound site.[26] These findings, together with a decrease in exudate formation and the differences in groups receiving ozone, support the hypothesis of our study, namely the positive effects of ozone therapy following radiotherapy (Table 6).

Tissue oxygenation is one of the most important factors for wound healing and, as a result, the prevention of AL. Tissue microvascular patency is the key factor for tissue oxygenation. Surgical devascularization, smoking and diabetes are risk factors for impairing tissue oxygenation. Several diagnostic measurements such as microfabricated oxygen sensors and near infrared spectroscopy have emerged.[27] Hyperbaric oxygen treatments have been shown to be beneficial in preventing AL and increasing transport to tissues.[28] Our study tried to determine if rectal ozone administration can reverse the harmful effects of radiotherapy. Local administration of ozone can improve healing disturbances that occur in the colon mucosa due to radiotherapy.[29] The main difference between our study and previous studies is the application method.[16] The rectal administration method can be translated to human studies and is the main difference between intraperitoneal administration. Besides systemic administration, local treatment can be regarded as an innovative beneficial approach to preventing AL.

The latest theory on the harmful effects of radiation resulting from major changes in the gastrointestinal microflora was put forward by Manichanh et al.[30] After radiotherapy, anaerobes are dominating the microflora of the colon and increasing the risk of leakage.[31] An increase in the amount of oxygen in the colon lumen can decrease the number of anaerobes.[32] Although not proven, the administration of ozone might reverse the harmful effects of radiotherapy on the microflora of the colon. The effect of ozone on the microflora of the colon is a subject of future investigation.

Limitations

The main limitation of our study is the small number of rats (n = 32) used. Another limitation is the absence of different time periods of sampling the rats rather than the 5th day. Also, a more detailed histological evaluation of the colon might have been performed.

Conclusions

As a result of this study, rectal administration of O_3 decreased the negative effects of radiotherapy on colon anastomoses by the O_3 oxidative preconditioning effect. This study is the first experimental study evaluating rectal O_3 administration and might be a subject of future clinical studies. The effect of ozone on tumor cells is a separate issue in the literature that requires more research.

ORCID iDs

Tonguç Utku Yilmaz https://orcid.org/0000-0002-2344-7783
Eda Yirmibeşoğlu Erkal https://orcid.org/0000-0002-6327-7037
Saffet Çinar https://orcid.org/0000-0003-3637-1922
Ceyla Eraldemir https://orcid.org/0000-0001-9410-8554
Çiğdem Vural https://orcid.org/0000-0002-9405-9112
Ezgi Uçar Taş https://orcid.org/0000-0002-2685-6013
Nihat Zafer Utkan https://orcid.org/0000-0002-2133-3336

References

1. Rutegård M, Rutegård J. Anastomotic leakage in rectal cancer surgery: The role of blood perfusion. *World J Gastrointest Surg*. 2015;7(11):289–292. doi:10.4240/wjgs.v7.i11.289
2. Mirnezami A, Mirnezami R, Chandrakumaran K, Sasapu K, Sagar P, Finan P. Increased local recurrence and reduced survival from colorectal cancer following anastomotic leak: Systematic review and meta-analysis. *Ann Surg*. 2011;253(5):890–899. doi:10.1097/SLA.0b013e3182128929
3. Espín E, Ciga MA, Pera M, et al. Oncological outcome following anastomotic leak in rectal surgery. *Br J Surg*. 2015;102(4):416–422. doi:10.1002/bjs.9748
4. Qin Q, Ma T, Deng Y, et al. Impact of preoperative radiotherapy on anastomotic leakage and stenosis after rectal cancer resection: Post hoc analysis of a randomized controlled trial. *Dis Colon Rectum*. 2016;59(10):934–942. doi:10.1097/DCR.0000000000000665
5. Vignali A, Gianotti L, Braga M, Radaelli G, Malvezzi L, Carlo VD. Altered microperfusion at the rectal stump is predictive for rectal anastomotic leak. *Dis Colon Rectum*. 2000;43(1):76–82. doi:10.1007/BF02237248
6. Gültekin FA, Bakkal BH, Sümer D, Köktürk F, Bektaş S. Effects of ozonated olive oil on acute radiation proctitis in rats. *Balkan Med J*. 2013;30(4):369–374. doi:10.5152/balkanmedj.2013.9158
7. Nguyen HQ, To NH, Zadigue P, et al. Ionizing radiation-induced cellular senescence promotes tissue fibrosis after radiotherapy. A review. *Crit Rev Oncol Hematol*. 2018;129:13–26. doi:10.1016/j.critrevonc.2018.06.012
8. Shukla PK, Gangwar R, Manda B, et al. Rapid disruption of intestinal epithelial tight junction and barrier dysfunction by ionizing radiation in mouse colon in vivo: Protection by *N*-acetyl-L-cysteine. *Am J Physiol Gastrointest Liver Physiol*. 2016;310(9):G705–G715. doi:10.1152/ajpgi.00314.2015
9. Vidal-Casariego A, Calleja-Fernández A, Cano-Rodríguez I, Cordido F, Ballesteros-Pomar MD. Effects of oral glutamine during abdominal radiotherapy on chronic radiation enteritis: A randomized controlled trial. *Nutrition*. 2015;31(1):200–204. doi:10.1016/j.nut.2014.08.003
10. Zeng J, Lu J. Mechanisms of action involved in ozone-therapy in skin diseases. *Int Immunopharmacol*. 2018;56:235–241. doi:10.1016/j.intimp.2018.01.040
11. León OS, Menéndez S, Merino N, et al. Ozone oxidative preconditioning: A protection against cellular damage by free radicals. *Mediators Inflamm*. 1998;7(4):289–294. doi:10.1080/09629359890983
12. Eliakim R, Karmeli F, Rachmilewitz D, Cohen P, Zimran A. Ozone enema: A model of microscopic colitis in rats. *Dig Dis Sci*. 2001;46(11):2515–2520. doi:10.1023/A:1012348525208
13. Knightly JJ, Agostino D, Cliffton EE. The effect of fibrinolysin and heparin on the formation of peritoneal adhesions. *Surgery*. 1962;52:250–258. PMID:14457251.
14. Elabscience. *Hydroxyproline (Hyp) Colorimetric Assay Kit (Alkali Hydrolysis Method)*. https://www.elabscience.com/Manual/biochemical_kits/E-BC-K061-S.pdf. Accessed December 20, 2020.

15. Hycult Biotech. *MPO, Rat, ELISA Kit*. https://www.hycultbiotech.com/hk105-01. Accessed December 20, 2020.
16. de Roy van Zuidewijn DB, Schillings PH, Wobbes T, de Boer HH. Histologic evaluation of wound healing in experimental intestinal anastomoses: Effects of antineoplastic agents. *Int J Exp Pathol*. 1992;73(4):465–484. PMID:1390194.
17. Taşdöven İ, Emre AU, Gültekin FA, et al. Effects of ozone preconditioning on recovery of rat colon anastomosis after preoperative radiotherapy. *Adv Clin Exp Med*. 2019;28(12):1683–1689. doi:10.17219/acem/110329
18. Cronin K, Jackson DS, Dunphy JE. Specific activity of hydroxyproline-tritium in the healing colon. *Surg Gynecol Obstet*. 1968;126(5):1061–1065. PMID:5652652.
19. Braskén P, Renvall S, Sandberg M. Fibronectin and collagen gene expression in healing experimental colonic anastomoses. *Br J Surg*. 1991;78(9):1048–1052. doi:10.1002/bjs.1800780908
20. Kerem M, Bedirli A, Karahacioglu E, et al. Effects of soluble fiber on matrix metalloproteinase-2 activity and healing of colon anastomosis in rats given radiotherapy. *Clin Nutr*. 2006;25(4):661–670. doi:10.1016/j.clnu.2006.01.028
21. Terzi C, Sevinç Aİ, Koçdor H, et al. Improvement of colonic healing by preoperative rectal irrigation with short-chain fatty acids in rats given radiotherapy. *Dis Colon Rectum*. 2004;47(12):2184–2194. doi:10.1007/s10350-004-0724-7
22. Kochhar R, Patel F, Sharma SC, et al. Radiation-induced proctosigmoiditis: Prospective, randomized, double-blind controlled trial of oral sulfasalazine plus rectal steroids versus rectal sucralfate. *Dig Dis Sci*. 1991;36(1):103–107. doi:10.1007/BF01300096
23. Ho E, Karimi Galougahi K, Liu CC, Bhindi R, Figtree GA. Biological markers of oxidative stress: Applications to cardiovascular research and practice. *Redox Biol*. 2013;1(1):483–491. doi:10.1016/j.redox.2013.07.006
24. Reiter RJ, Tan DX, Osuna C, Gitto E. Actions of melatonin in the reduction of oxidative stress: A review. *J Biomed Sci*. 2000;7(6):444–458. doi:10.1007/BF02253360
25. Erkol H, Kahramansoy N, Kordon O, Buyukasik O, Serin E, Ulas N. Effects of beta-glucan on hepatic damage caused by obstructive jaundice [in Turkish]. *Ulus Travma Acil Cerrahi Derg*. 2011;17(4):303–307. doi:10.5505/tjtes.2011.88964
26. Seifert WF, Wobbes T, Hoogenhout J, de Man BM, Huyben KMLC, Hendriks T. Intraoperative irradiation delays anastomotic repair in rat colon. *Am J Surg*. 1995;170(3):256–261. doi:10.1016/S0002-9610(05)80010-8
27. Marland J, Gray M, Argyle D, Underwood I, Murray A, Potter M. Post-operative monitoring of intestinal tissue oxygenation using an implantable microfabricated oxygen sensor. *Micromachines (Basel)*. 2021;12(7):810. doi:10.3390/mi12070810
28. Brouwer RJ, Engberts AC, Borger van der Burg BL, van Dongen TT, van Hulst RA, Hoencamp R. Meta-analysis on the effect of hyperbaric oxygen as adjunctive therapy in the outcome of anastomotic healing of experimental colorectal resections in rats. *Diving Hyperb Med*. 2018;48(3):173–185. doi:10.28920/dhm48.3.173-185
29. Reischl S, Wilhelm D, Friess H, Neumann PA. Innovative approaches for induction of gastrointestinal anastomotic healing: An update on experimental and clinical aspects. *Langenbecks Arch Surg*. 2021;406(4):971–980. doi:10.1007/s00423-020-01957-1
30. Manichanh C, Varela E, Martinez C, et al. The gut microbiota predispose to the pathophysiology of acute postradiotherapy diarrhea. *Am J Gastroenterol*. 2008;103(7):1754–1761. doi:10.1111/j.1572-0241.2008.01868.x
31. Brook I, Walker RI, Macvittie TJ. Effect of antimicrobial therapy on bowel flora and bacterial infection in irradiated mice. *Int J Radiat Biol Relat Stud Phys Chem Med*. 1988;53(5):709–716. doi:10.1080/09553008814551081
32. Makanyengo SO, Carroll GM, Goggins BJ, Smith SR, Pockney PG, Keely S. Systematic review on the influence of tissue oxygenation on gut microbiota and anastomotic healing. *J Surg Res*. 2020;249:186–196. doi:10.1016/j.jss.2019.12.022