Effects of ozone preconditioning on recovery of rat colon anastomosis after preoperative radiotherapy

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Abstract

Background. Anastomotic leakage is a devastating complication of colorectal surgery. Neoadjuvant radiotherapy for colorectal cancer can affect the mechanical and biochemical parameters of anastomotic healing. It has been reported that ozone increases antioxidant enzyme activity and stimulates adaptive processes to oppose the pathophysiologic conditions mediated by reactive oxygen species (ROS).

Objectives. The objective of this study was to investigate the effect of controlled administration of ozone on the healing of anastomosis and the activation of antioxidant enzymes in the colon after radiotherapy.

Material and methods. Rats (n = 48) were randomly assigned to the following groups: control groups (1 and 2), saline-treated and irradiated (IR) groups (3 and 4) and ozone oxidative preconditioning (OOP) and IR groups (5 and 6). Rats were exposed to whole-body IR (6 Gy) after pretreatment with either saline or ozone. Rats in groups 1, 3 and 5 were euthanized on postoperative day 3, whereas those in groups 2, 4 and 6 were euthanized on postoperative day 7. The anastomoses were performed on day 7 post-IR. The anastomotic segment was resected to measure hydroxyproline (HPO) content, myeloperoxidase (MPO) activity and malondialdehyde (MDA) concentration and for histopathological evaluation.

Results. The mean bursting pressure of the groups that underwent radiotherapy was lower than that of the control groups (p < 0.001). In groups 5 and 6, the tissue HPO concentrations were higher than those in groups 3 and 4. Although mean values for MPO activity in groups 5 and 6 were higher than those in groups 3 and 4, the differences were not significant. Regarding oxidative damage markers, MDA concentrations were significantly lower in group 5 than those in group 3.

Conclusions. In this experimental model, OOP exerted favorable effects on colon anastomotic healing after radiation exposure.

Key words: colon anastomosis, ozone oxidative preconditioning, radiotherapy, anastomotic leakage

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Introduction

Anastomotic leakage after colorectal surgery is a dreaded complication, as it greatly increases morbidity and mortality and has been associated with high local recurrence and diminished survival after colorectal cancer surgery.[1] Preoperative radiotherapy is being successfully used as an adjuvant in rectal cancer therapy, but the ionized beams used in radiotherapy can potentially damage organs by increasing the cellular oxidative stress as a result of molecular ionization, leading to the overproduction of reactive oxygen species (ROS).[2,3] Ozone (O_3) therapy is widely used in medicine for its antioxidant, anti-inflammatory and antimicrobial effects.[4] The therapeutic effect of O_3 particularly targets reactive oxygen products, hydrogen peroxide and lipid oxidation products (LOPs).[5] Thus, it has been hypothesized that O_3 is effective in preventing ischemia–reperfusion damage and has been used as a therapeutic option in ischemia–reperfusion studies.[6,7] The term 'O_3 oxidative preconditioning' (OOP) implies the triggering of an adaptation to oxidative stress through the application of O_3 at repeated non-toxic doses. The claimed efficacy of OOP therapy in preventing ischemia–reperfusion damage in various organs, such as the liver, heart and kidneys, has been demonstrated in experimental studies.[6–8] We aimed to determine whether the antioxidant capacity of the O_3 applied would ameliorate any damage after radiotherapy as well as suppress the anti-inflammatory process at the anastomotic site and to obtain histopathological evidence of this effect.

Material and methods

This experimental study was conducted after receiving approval from the Animal Research Committee in the Bülent Ecevit University (Zonguldak, Turkey). All animals were handled in accordance with the recommendations of the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (Ankara, Turkey).

Animals and groups

Forty-eight female Wistar albino rats aged 10–12 weeks and weighing 200–250 g were fed with standard rat feed and fresh potable water ad libitum throughout the study period. They were kept under constant environmental conditions at an average temperature of 21 ±1°C and humidity of 50–60% in transparent plastic cages lined with wood shavings. The rats were randomized in a blinded manner to 6 groups comprising 8 rats each.

In control groups (groups 1 and 2), rats were administered 0.5 mL of intraperitoneal saline for 5 consecutive days. After the administration of the final dose, left colon anastomosis was performed on the rats. On days 3 and 7 following the anastomosis procedure, the rats in group 1 and 2 were euthanized.

In saline-treated and irradiated (IR) groups (groups 3 and 4), rats were administered 0.5 mL of intraperitoneal saline for 5 consecutive days. A single-dose pelvic IR with 500 cGy was performed 1 h after the final dose was administered. On day 7 post-IR, left colon anastomosis was performed. After the procedure, the rats were euthanized on day 3 (group 3) and day 7 (group 4).

OOP and IR groups (groups 5 and 6)

An intraperitoneal injection containing a mixture of 0.7 mg/kg of O_2/O_3 gas was administered each day for 5 consecutive days. A single-dose pelvic IR with 500 cGy was performed 1 h after the final dose was administered. On day 7 post-IR, left colon anastomosis was performed. The rats were euthanized on day 3 (group 5) and day 7 (group 6) after the procedure. The presence/absence of any complications related to the wounded area in rats and any intra-abdominal abscess, anastomotic leakage or stenosis was also noted. Evaluations of the bursting (blast) pressure and hydroxyproline (HPO) concentration during the anastomosis as well as those of the histopathological and biochemical aspects were performed. During the biochemical examination, malondialdehyde (MDA), myeloperoxidase (MPO) and superoxide dismutase (SOD) concentrations were examined as the oxidative markers in the tissue.

Surgical procedure

No bowel preparation was preoperatively applied to the rats. To induce dissociative anesthesia, 80 mg/kg of ketamine HCl (Ketalar® vial; Eczacıbaşı Pharmaceutical Industry and Trade Inc., Lüleburgaz, Turkey) and 10 mg/kg of xylazine HCl (Rompun® 2%; Bayer, Leverkusen, Germany) were intramuscularly injected into each rat. The laparotomy procedure was performed through a 4-centimeter standard mid-line incision. Left colon is transected with a scalpel to perform an anastomosis. Vascular supply of colonic edges is preserved. Colonic contents were cleaned up 2 cm proximally and distally using the stripping method. To minimize the effect of the suturing material on anastomotic healing, 6/0 non-absorbable monofilament round-needle polypropylene sutures were used. An edge-to-edge anastomosis procedure was performed, with approx. 6–8 pieces of primary interrupted sutures.

O_3 production

Ozone was generated by an O_3 generator (Bozon N; Econica, Odessa, Ukraine), allowing control of the gas flow rate and O_3 concentration in real time, using a built-in ultraviolet spectrometer; it was immediately administered at a dose of 1.2 mg/kg daily via an intraperitoneal route. The volume of the injected mixture was approx. 1 mL.

The OOP was performed with 5 applications (once daily) of the O_3/O_2 mixture. The O_3 flow rate was kept constant at 3 L/min, representing a concentration of 60 mg/mL and a gas mixture of 97% O_2 + 3% O_3. Tygon polymer tubes and single-use silicon-treated polypropylene syringes (O_3 resistant) were used throughout the experiment to ensure containment of O_3 and consistency of concentration.[9]

Euthanasia

Groups 1, 3 and 5 were euthanized on postoperative day 3, while groups 2, 4 and 6 were euthanized on postoperative day 7 using the intra-cardiac blood collection method. After the bursting (blast) pressure was measured, the colon segment was resected, including 2 cm of the proximal and 2 cm of the distal part of the anastomotic line. Then, it was divided into 2 equal parts vertically passing through the middle of the anastomosis. For the histopathological examination, a section of tissue, including the anastomosis, was placed in 10% formaldehyde solution. Another section was frozen at −80°C for measuring HPO, MPO, SOD, and MDA concentrations.

Measurement of the bursting (blast) pressure

To measure the bursting pressure, an infusion pump (Infusion pump Hospira Plum A+; Abbott, Irving, USA), a pressure transducer (Transpac IV, Abbott) and a monitor (Petas KMA 460-R; Petas, Ankara, Turkey) were provided. The proximal end of the anastomosis was transected, and the fecal content within (if any) was removed. A 6-Fr catheter that was connected to the monitor along with the pressure transducer was placed within the anastomosis. To avoid any air or fluid leakage, the catheter was fixed with a 2/0 silk suture, and a closed system was established. Fluid was transferred from the catheter placed in the proximal part of the colon by means of a perfusion pump at a speed of 50 mL/h, and the pressure was tracked on the monitor. Fluid leakage or a sudden decline in pressure in the course of the blast at the anastomosis site was determined, and the monitor indicator at that moment was recorded as the bursting (blast) pressure of anastomosis.

Radiation procedure

A single pelvic IR with 500 cGy was applied to the rats while they were in the prone position by a masked investigator. A computed tomography (CT) simulation of a rat anatomy was performed in 1-millimeter slices, and a dose calculation was performed with the help of Eclipse Treatment Planning System v. 8.9 (Varian Medical Systems, Palo Alto, USA). The animals were brought back to their cages in the post-IR period. The animals in the group 1 and 2. were anesthetized but not exposed to radiation.

Biochemical examinations

Biochemical analyses were performed in the Research Laboratory of Nutrition and Dietetics in Muğla Health College, Muğla Sıtkı Koçman University, Turkey. Hydroxyproline concentrations in the tissue were measured according to a modification of the method of Jamall et al.[10] After being weighed, the samples were hydrolyzed with 6 N HCl in an autoclave (Nüve OT4060; Nüve Sanayi Malzemeleri, Izmir, Turkey) at 121°C for 15 min. Twenty-five microliter of hydrolysate were taken and lyophilized and then were dissolved in 1 mL 50% (v/v) isopropyl alcohol. Chloramine-T was added to these samples 10 min later. Then, 1 mL of Ehrlich solution was added, and the samples were incubated at 50°C for 90 min.

Under the same circumstances, 0.4-, 0.8-, 1.2-, and 1.6-microgram L-HPO standards were studied. The color change that occurred during the reaction was spectrophotometrically measured at a 560-nanometer wavelength (PG Instruments T80+; PG Instruments Limited, Leicestershire, UK). Hydroxyproline levels were calculated from a standard curve prepared from L-HPO and expressed as microgram per gram tissue (μg/g tissue).[10,11]

Tissue MDA concentrations was measured in a 96-well microliter plate using an enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech Co., LTD., Newark, USA) according to the manufacturer's guidelines. Tissues were homogenized (Pro-Scientific 200; Pro-Scientific, Oxford, USA) in phosphate-buffered saline (PBS). The homogenates were centrifuged for 5 min at 5,000 × g (Eppendorf 5804R; Eppendorf, Hamburg, Germany). The supernatants were used for the determination of MDA levels. Optical density of each well was measured with a microplate plate reader at 450 nm absorbance (Biorad Model 680 microplate reader; Biorad, Hercules, USA). The sensitivity of the assay was 7.81 pmol/mL and the linear range of the standard was 31.25–2,000 pmol/mL. The intra-assay and inter-assay coefficients of variation were <8% and <10%, respectively. The concentrations of MDA were represented as nanomoles per milligram protein (nmol/mg protein).

Tissue MPO concentrations were measured in a 96-well microliter plate, using an ELISA kit (USCN Life Science, Wuhan, China) according to the manufacturer's guidelines. Tissues were homogenized (Pro-Scientific 200; Pro-Scientific Oxford, USA) in ice-cold PBS. The homogenates were centrifuged for 5 min at 5,000 × g (Eppendorf 5804R). The supernatants were used to determine MPO concentrations. Optical density of each well was measured with a microplate plate reader at 450 nm absorbance (Biorad Model 680 microplate reader). The concentrations of MPO were represented as nanogram per milligram protein (ng/mg protein).

Tissue SOD activity was measured using the spectrophotometric method developed by Sun et al.[12] Tissues were homogenized (Pro-Scientific 200) in PBS were centrifuged for 5 min at 5,000 × g (Eppendorf 5804R). Supernatant were

mixed to equal volume chloroform/ethanol (3:5 ratio) and centrifuged (Eppendorf 5804R) at 5000 × g for 2 h at 4°C. Then, SOD activity and protein levels were measured in supernatants. One unit of SOD was defined as the amount of enzyme causing 50% inhibition of the reduction rate of nitroblue tetrazolium. Superoxide dismutase activity was given as units (U)/mg protein.

Total protein content of tissues was measured using the method of Lowry et al.[13] with BSA as a standard.

Histopathological evaluation

From the tissue samples in 10% formaldehyde solution, paraffin-embedded blocks were prepared and sections of 4–5-micron thickness were stained with hematoxylin and eosin (H&E). The sections were then evaluated under light microscopy (Leica DMLS; Leica Camera AG, Wetzlar, Germany) by a single pathologist in a blinded manner. Mucosal wound recovery was scored according to the scale proposed by Houdart et al.[14] Granulocyte infiltration, mononuclear cell infiltration, fibroblastic proliferation, focal necrosis, and exudate formation pertaining to the anastomotic wound recovery were evaluated and scored according to the modified parameters[14,15] as: 0 – none, 1 – mild, 2 – moderate, and 3 – severe.

Statistical analysis

Statistical analyses were performed using SPSS v. 13.0 (SPSS, Inc., Chicago, USA) statistical software. For continuous variables with the normal distribution, the Shapiro–Wilk test was used, whereas in 3 or more group comparisons of the variables showing non-normal distribution, the Kruskal–Wallis test was used. For group comparisons, the Mann–Whitney U test was used. P-values ≤0.05 were considered statistically significant.

Results

When the groups were evaluated in terms of the bursting pressure, the bursting pressure of the groups that underwent radiotherapy (groups 3–6) was lower than that of the control groups (groups 1 and 2) (p < 0.001). The bursting pressures in the group that received OOP prior to IR were significantly higher than those in the groups treated with saline prior to IR (p < 0.001). The distribution of the bursting pressure values of anastomosis according to the groups is shown in Fig. 1 and Table 1.

The tissue HPO concentrations in the groups that were treated with saline prior to irradiation were lower than those in the control groups (p < 0.05). Conversely, the HPO values of groups 5 and 6 were statistically higher than those of groups 3 and 4 (Fig. 2).

The MDA concentrations of the groups that underwent radiotherapy (groups 3–6) were higher than those

Table 1. Distribution of the bursting (blast) pressure and standard deviation (SD). Values of anastomosis by group

Groups	Average bursting (blast) pressure (mm Hg) ±SD	Median	Minimum	Maximum
Group 1	145.38 ±12.716	144.5	123	162
Group 2	149.63 ±37.489	157.5	90	192
Group 3	50.75 ±18.722	52.5	15	75
Group 4	74.13 ±25.737	84.0	40	104
Group 5	86.00 ±19.479	82.5	60	118
Group 6	140.88 ±31.832	142.5	90	185

of the control groups (groups 1 and 2); however, the difference was statistically significant only in the comparison between group 3 and the control groups (p = 0.014). Similarly, although a decrease in the MDA concentrations of groups 5 and 6 was maintained, a statistically significant decline was observed in the MDA concentrations of group 5 (p = 0.014) in comparison with those of groups 3 and 4 (Fig. 3).

The MPO concentrations in the groups that underwent radiotherapy (groups 3–6) were lower than those in the control groups (groups 1 and 2). The increase in the MPO concentrations in the group that underwent
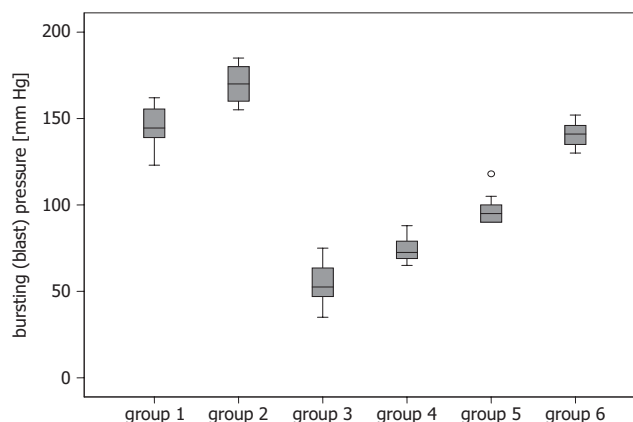

Fig. 1. Distribution of the bursting (blast) pressure values of anastomosis by group
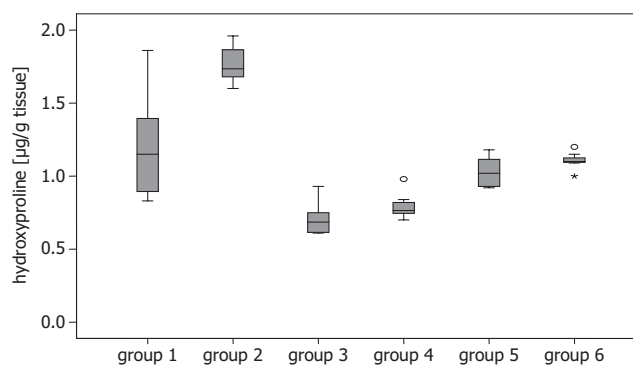

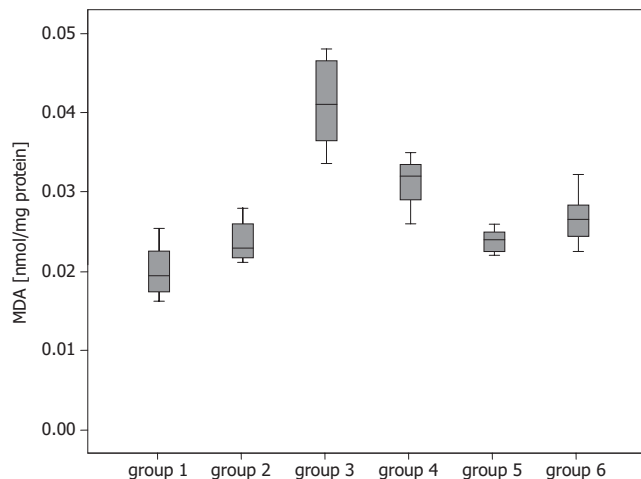Fig. 2. Hydroxyproline (HPO) concentrations in the anastomotic tissue by group [μg/g tissue]

Fig. 3. Malondialdehyde (MDA) levels in the anastomotic tissue by group [nmol/mg protein]
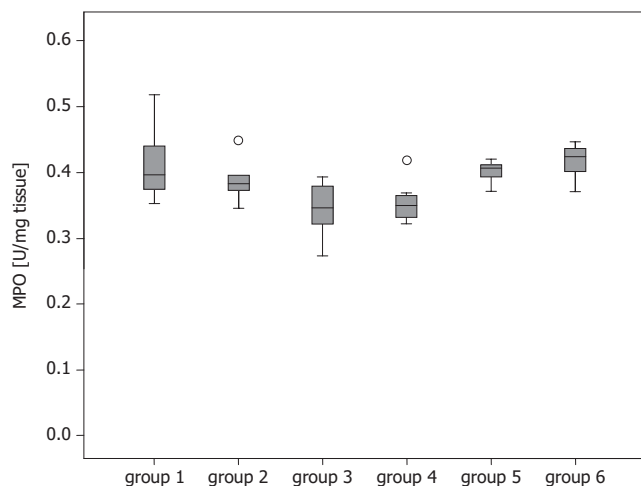

Fig. 4. Myeloperoxidase (MPO) concentrations in the anastomotic tissue by group [ng/mg protein]

Fig. 5. Superoxide dismutase (SOD) activities in the anastomotic tissue by group [U/mg protein]

OOP (groups 5 and 6) compared with those in the groups treated with saline was only clinically significant. There were no statistically significant differences between the groups (Fig. 4).

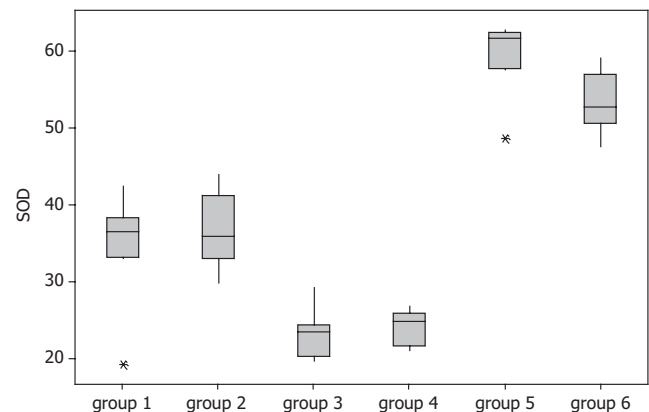The tissue SOD concentrations of groups 3 and 4 were lower than those of groups 1 and 2 ($p < 0.05$). Conversely, the SOD concentrations of groups 5 and 6 were statistically higher than those of groups 3 and 4 ($p < 0.05$) (Fig. 5). Table 2 shows the HPO, MDA, MPO, and SOD concentrations in the anastomotic tissues in control and saline- or O_3-treated irradiated groups euthanized on day 3 or day 7 after IR.

Upon examining the anastomotic wound healing, granulation tissue development and histological changes corresponding to the local inflammatory response of the study groups (scored from 0 to 4), we observed that the inflammatory cell infiltration in all groups was more intense than in the control groups. The fibroblastic proliferation between the groups was similar; however, it was not statistically significant (Table 3).

Discussion

Anastomotic leakage is one of the major and devastating complication of colorectal surgery. The occurrence of postoperative anastomotic leakage can vary between 10% and 20%.[16] Ischemia is regarded as one of the causes of anastomotic leakage. Wound recovery is negatively affected by hypoxia.[17]

Cronin et al. reported that during the measurements of bursting pressure of the anastomosis, the force to be

Table 2. HPO [μg/g], MDA [nmol/g], MPO [nmol/mg], and SOD [U/g] concentrations in the anastomotic tissue by group in control, and saline- or ozone-treated irradiated groups euthanized on day 3 or day 7 after IR. Each group consisted of 6 rats

Oxidative stress markers	Control		IR			
	day 3	day 7	day 3		day 7	
	group 1	group 2	saline-treated group 3	ozone-treated group 5	saline-treated group 4	ozone-treated group 6
HPO [μg/g]	1.071 ±0.241	0.912 ±0.100	0.786 ±0.129	0.942 ±0.165	0.896 ±0.158	1.025 ±0.098
MDA [nmol/mg]	0.021 ±0.111	0.296 ±0.092	0.396 ±0.093	0.442 ±0.076	0.422 ±0.135	0.496 ±0.090
MPO [nmol/mg]	0.357 ±0.111	0.296 ±0.092	0.396 ±0.093	0.442 ±0.076	0.422 ±0.135	0.496 ±0.090
SOD [U/g]	34.66 ±6.86	36.86 ±4.83	23.22 ±3.07	59.36 ±4.75	24.16 ±2.18	53.24 ±3.84

HPO – hydroxyproline; MDA – malondialdehyde; MPO – myeloperoxidase; SOD –superoxide dismutase; IR – irradiation.

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Table 3. Histological changes in the anastomotic tissue by group in control, and saline- or ozone-treated irradiated groups euthanized on day 3 or day 7 after irradiation (IR). Each group consisted of 6 rats

	Score	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Granulocyte infiltration	0	0	0	1 (12.5%)	2 (25.0%)	4 (50.0%)	3 (37.5%)
	1	3 (37.5%)	4 (50.0%)	4 (50.0%)	5 (62.5%)	4 (50.0%)	5 (62.5%)
	2	1 (12.5%)	3 (37.5%)	2 (25.0%)	1 (12.5%)	0	0
	3	4 (50.0%)	1 (12.5%)	1 (12.5%)	0	0	0
	Score	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Mononuclear cell infiltration	0	0	0	2 (25.0%)	1 (12.5%)	0	0
	1	6 (75.0%)	2 (25.0%)	4 (50.0%)	5 (62.5%)	7 (87.5%)	7 (87.5%)
	2	2 (25.0%)	3 (37.5%)	1 (12.5%)	2 (25.0%)	1 (12.5%)	1 (12.5%)
	3	0	3 (37.5%)	1 (12.5%)	0	0	0
	Score	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Fibroblastic proliferation	0	0	0	2 (25.0%)	1 (12.5%)	0	0
	1	7 (87.5%)	1 (12.5%)	4 (50.0%)	4 (50.0%)	6 (75.0%)	4 (50.0%)
	2	1 (12.5%)	3 (37.5%)	2 (25.0%)	3 (37.5%)	2 (25.0%)	3 (37.5%)
	3	0	4 (50.0%)	0	0	0	1 (12.5%)
	Score	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Focal necrosis	0	8 (100%)	6 (75.0%)	7 (87.5%)	8 (100%)	7 (87.5%)	8 (100%)
	1	0	2 (25.0%)	0	0	1 (12.5%)	0
	2	0	0	1 (12.5%)	0	0	0
	3	0	0	0	0	0	0
	Score	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Exudate formation	0	1 (12.5%)	3 (37.5%)	7 (87.5%)	5 (62.5%)	6 (75.0%)	5 (62.5%)
	1	3 (37.5%)	4 (50.0%)	1 (12.5%)	3 (37.5%)	0	2 (25.0%)
	2	1 (12.5%)	0	0	0	2 (25.0%)	1 (12.5%)
	3	3 (37.5%)	1 (12.5%)	0	0	0	0

exerted postoperative day 3 onwards tended to increase until it reached its maximum pressure on days 7–10.[18]

It was shown that there was an increase in the re-infusion of the blood mixed with O_3 and in the nitric oxide (NO) levels, which caused vasodilatation in the ischemic areas, and that oxidative stress was minimized along with decreased hypoxia, SOD activation and decreased glutathione levels.[19,20] It has currently been reported that antioxidant enzymes, NO pathways and other cellular activities could be regulated by low doses of O_3.[21] Controlled administration of O_3 can minimize the damage induced by ROS by maintaining the adaptation to OOP or stress.[20,22] It was reported that O_3 would enhance healing in ischemic and ulcerous wounds by promoting expression, secretion and activation of growth factors from the activated thrombocytes.[23]

Stevens et al. compared the cases of anterior resection performed after radiotherapy with 5,000 rad and a group that did not undergo radiotherapy.[24] They found a significantly higher anastomotic leakage in the radiotherapy group than in the control group; this was consistent with the findings of some other experimental studies reporting negative effect of radiation on anastomotic healing depending on the applied dose and duration.[25–27]

We performed a colon anastomosis on rats on day 7 following the administration of 6 Gy total body IR. During the measurements performed on postoperative days 3 and 7, it was observed that the bursting pressure was lower in the radiotherapy groups than in the control groups (p < 0.001). It was also determined that OOP caused an increase in the bursting pressure, which was significantly higher than that in the saline-treated and IR groups (p < 0.001). It was observed that the tissue HPO concentration in the saline-treated and IR groups (groups 3 and 4) was low compared with that in the control groups (p < 0.05). The HPO concentrations of groups 5 and 6 were significantly higher than those of groups 3 and 4. Along with these findings, we ascertained that a single, low-dose irradiation performed on preoperative day 7 affected the anastomosis recovery in our rat model.

Our findings regarding the low bursting pressure and HPO concentrations in an experimental rodent model suggest that O_3 exposure prior to radiotherapy contributes to a decrease in the incidence of anastomotic leakage after colorectal surgery during the early stage. Although radiotherapy-based damage occurred as the result of oxidative stress, the MDA concentration of group 3 was a significant indicator of oxidative stress among the study groups.

In other groups, the differences in MDA and MPO values were not significant.

Here, OOP caused the bursting pressure and HPO values to increase in rats during the colon anastomosis procedure. The effect of O_3 exposure on the oxidative indicators/markers in the anastomotic tissue was evidenced by a significant decline in MDA concentrations in group 5. In other groups, the differences in MDA and MPO values were not significant.

Our findings reveal that radiation negatively affected recovery after colonic anastomosis surgery and this is concordant with the results of previous reports. Conversely, based on HPO, SOD and MDA measurements and histopathological examinations, we found that OOP had a positive effect on the recovery of anastomoses performed after radiotherapy. These findings in an experimental rodent model suggest that O_3 exposure prior to radiotherapy contributes to a decrease in the incidence of anastomotic leakage after colorectal surgery. However, there is need for further studies to assess the effects and antioxidant capacity of O_3.

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