

# Effect of Ozone Therapy (OT) on Healing of Colonic Anastomosis in a Rat Model of Peritonitis

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**Background:** Ozone is a three-oxygen molecule (O<sub>3</sub>). Ozone therapy (OT) is systematically effective when pathological inflammatory and immunologic processes are activated. Among of these conditions are wound healing, macular degeneration related to aging, and conditions that are ischemic or infectious.

**Aims:** The aim of this study was to determine the effects of OT on wound healing of intestinal anastomosis in the presence of peritonitis in a rat model.

**Study Design:** Animal experimentation.

**Methods:** A total of 40 Wistar albino rats were randomized into four groups (n=10) including: sham (S), peritonitis (P), ozone 0 (O0), and ozone 24 (O24). In group S, only cecal dissection was carried out. The S group had only a cecal dissection and intestinal anastomosis performed, but no peritonitis. In all other groups, cecal ligation and puncture (CLP) followed the cecal dissection to induce bacterial peritonitis. 24 h after puncture, a cecal resection and ileocolic anastomosis were performed. In group P, 24 h after CLP, a cecal resection and ileocolic anastomosis were performed and no ozone was administered. In group O0, immediately after the anastomosis, and in group O24, starting 24 hours after the anastomosis, an intraperitoneal 1 mg/kg/day ozone administration was applied for seven days. On the sev-

enth day the animals were sacrificed, the anastomotic bursting pressures (BP) and the hydroxyproline values of the anastomotic tissues were measured, and histopathologic examination of the anastomotic segment was carried out.

**Results:** The highest BP was in group S, with 211±23.13 mmHg. The mean BP of group P was 141±56.25 mmHg, which was significantly lower than in the other two peritonitis groups that received ozone therapy, group O0 and O24, where it was 192±22 and 166±45 mmHg, respectively (p<0.05). The difference in the BP between groups O0 and O24 was not statistically significant (p>0.05). Histopathologic analyses of the anastomotic segments determined there was significantly more oedema and necrosis in the control group rats, and collagen deposition in the anastomotic tissue was significantly higher in the ozone-treated groups on postoperative day 7. Hydroxyproline levels were significantly higher in groups O0 and O24 compared to the peritonitis group (P).

**Conclusion:** Ozone therapy has a beneficial effect on anastomotic healing of the colon in the presence of peritonitis.

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**Key Words:** Anastomosis, colon, ozone, peritonitis, rat

Colonic resection and anastomosis is one of the most common operations in gastrointestinal surgery, both in children and adults. Although these are safe operations, an anastomosis leakage is a frequent problem (1, 2). Age, condition of the patient, colonic flora, inflammation and surgical techniques are the most important factors affecting the outcome of these procedures (3, 4). The presence of peritonitis is a defining factor of anastomosis integrity.

The cecal ligation and puncture (CLP) model was first defined by Hubbard et al. (5) in 1978 to induce peritonitis. Several studies suggest that in the presence of peritonitis, wound healing worsens and anastomotic leakage increases. To avoid

these unwanted effects of peritonitis on wound healing and to increase anastomosis security, various methods and local or systemic agents are being investigated.

Ozone is a three-oxygen molecule (O<sub>3</sub>). Ozone therapy (OT) is systematically effective when pathological inflammatory and immunologic processes are activated. Among these conditions are wound healing, macular degeneration related to aging, and conditions that are ischemic or infectious.

During OT, the gaseous mixture of oxygen and ozone exerts biological actions that may increase certain enzymes and cytokines. Ozone provides its protective effects via autacoids, cytokines, and growth factors. Recurrent OT corrects the dis-



**TABLE 1.** Semiquantitative histopathological parameters used to analyse wound healing in the colon

Score	Necrosis	PMN cells	Mononuclear Cells	Oedema	Mucosal Epithelium	Submucosal/mucosal layer	Granulation Tissue	Neoangiogenesis	Fibroblast	Collagen deposition
0	None	Normal number	Normal number	None	Normal Glandular	Good bridging	None	None	None	None
1	Small patches	Slight increase	Slight increase	Some	Normal cubic	Average bridging	Some	Partial	Partial	Partial
2	Some patches	Marked infiltration	Marked infiltration	Marked	Incomplete cubic	Poor bridging	Marked	Moderate	Moderate	Moderate
3	Massive	Massive infiltration	Massive infiltration	Severe	Absent	No bridging	Severe	Marked	Marked	Marked

PMN: polymorphonuclear lymphocytes

turbed redox equilibrium by affecting the antioxidant system (6-10). Positive effects of OT on caustic oesophagus injuries have also been suggested (11).

The concept of using ozone to accelerate the healing of infected wounds, necrotic or low oxygenated tissue has been explored in orthopaedics, dentistry and skin wounds. The use of these therapeutics in the healing of colonic anastomosis was explored for the first time by Lelyanov et al. (12).

The aim of the present study was to determine the effects of OT on anastomotic healing in a rat peritonitis model.

## MATERIAL AND METHODS

After obtaining instructional animal ethics committee approval, 40 male Wistar albino rats weighing 300-350 g were equally divided into four groups. They were housed in a temperature- ( $21\pm 2^\circ\text{C}$ ) and humidity-controlled environment with a 12-hour light/dark cycle. Each group contained ten rats. The groups included: sham (S), Peritonitis (P) (control), ozone 0 (O0) and ozone 24 (O24). In group S, only cecal dissections were carried out. In the other groups, CLP followed the cecal dissection to induce bacterial peritonitis. We used the modified peritonitis model of Hubbard et al. (5) for this procedure. In group P, 24 h after the previous operation, a cecal resection and ileocolic anastomosis were performed. In group O0, immediately after cecal resection and ileocolic anastomosis, which had been performed 24 hours after the cecal dissection and anastomosis, 1 mg/kg/day ozone was administered intraperitoneally for seven days. In group O24, 24 hours after peritonitis had been induced with the same method, intraperitoneal 1 mg/kg/day ozone was administered for seven days. On the seventh postoperative day the animals were sacrificed the anastomosis bursting pressures (BP) were recorded and histopathologic analyses were performed.

### Bursting pressures

On the seventh day the animals were sacrificed and the anastomotic bursting pressures (BP) were measured. We re-

sected a 10 cm long bowel segment, situating the anastomosis in the middle part, and washed it with saline solution. We then ligated one end with 3/0 silk and connected the other end to a 8 F feeding tube. We connected a Y-shaped adapter at the end of the feeding tube. A sphyngomanometer (Petas, Turkey) and a 50 mL syringe were adapted to either end of the adapter. We submerged the bowel in the saline solution. We then insufflated the colonic segment with air. The bursting pressure was recorded at the time of the first air bubble. In all cases the bursting site was on the anastomosis. An anastomosis segment 1 cm in length was also fixed in formaldehyde solution (13).

### Pathological analysis

Histopathological examination was performed blindly by a pathologist who was not aware of the study groups. After routine procedures, colonic segments were embedded in paraffin blocks. The paraffin blocks were cut into four micron-wide sections and these tissue sections were stained with haematoxylin and eosin. Histopathologic scoring was performed according to a scale previously used by Verhofstad et al. (14) and is shown in Table 1. Fibroblastic activity, neoangiogenesis, and a collagen deposition grading scale were used by Ehrlich and Hunt (15). The scale was used in the modified form where it was converted to a numerical system by Philips et al. (16).

### Hydroxyproline measurement

One gram of the resected tissue containing the anastomotic segment was hydrolysed for five hours at  $121^\circ\text{C}$  in an acidic buffer solution. After the hydrolysis, the samples were centrifuged at 5000 rpm for 20 minutes. The absorbance of the final material was evaluated by the colorimetric method at 560 nm and  $121^\circ\text{C}$  and the tissue hydroxyproline level was calculated as mg/L/g using a commercially available kit (Hydroxyproline Extra [Colorimetric determination], Brand; Far, Italy) (17).

### Statistical analysis

Statistical Package for the Social Sciences (SPSS) software 15.0 version (SPSS, Inc, Chicago, IL, USA) statistical computer programme was used. Descriptive statistical meth-

**TABLE 2.** Bursting pressure and standard deviation for all groups. The difference between the BP results in groups O0 and O24 was not statistically significant ( $p>0.05$ )

	Mean BP	SD
Sham (S)	211	23
Peritonitis (P)	141	25
O0 (ozone 0)	192	22
O24 (ozone 24)	166	45

BP: bursting pressures; SD: standard deviation

**TABLE 3.** Histological scoring of the groups ( $p<0.05$ )

	S	P	O0	O24
Necrosis	3	3	0*	0*
PMNL	1	1	0*	0*
MNL	2	2	2	2
Oedema	3	3	1.5*	1.5*
Mucosa	2	2	2	2
Submucosa	2	2	2	2
Granulation	1	1	1	1
Neoangiogenesis	1	1	1	1
Fibroblast	2	2	2	2
Collagen	1.5	1	3*	3*

PMN: polymorphonuclear lymphocytes; MNL: mononuclear lymphocytes

\*There is statistical difference between the ozone treated groups and others ( $p<0.05$ )

ods were used. ANOVA and *t*-tests were used to compare the group distributions and Kolmogorov-Smirnov and Shapiro-Wilk normality tests were used for the normality analyses. The Kruskal-Wallis test was used for the variance analysis and the Mann-Whitney *U* test was used to compare those groups for which a normal distribution did not exist. Ninety-five percent was accepted as the confidence interval, and a *P* value  $\leq 0.05$  was considered significant for the analysis.

## RESULTS

There was no mortality during the study. All rats showed intra-abdominal sepsis symptoms following CLP, such as anorexia, piloerection, and immobilization following inoculation.

### Body weight analysis

In all groups with CLP, body weight decreased, especially in the first 24 hours after CLP. A body weight loss of 10% was similar between groups II-IV.

### Effects of ozone therapy on colonic anastomosis bursting pressures

The BPs of all groups are shown in Table 2. The highest BP was in group S, with  $211\pm 23$  mmHg. The sham group

only had a cecal dissection and anastomosis performed, but no peritonitis. The mean BP of group P was  $141\pm 25$  mmHg, which was significantly lower than in the other two peritonitis groups that received ozone therapy, group O0 and O24, where it was  $192\pm 22$  and  $166\pm 45$  mmHg, respectively ( $p<0.05$ ). The difference between the BP results in groups O0 and O24 was not statistically significant ( $p>0.05$ ).

### Effects of ozone therapy on histopathologic values

On the seventh postoperative day there were no significant differences between the groups in terms of submucosal/mucosal layer, mucosal epithelium, granulation tissue, and mononuclear cells ( $p>0.05$ ). However, there were significant differences between the groups in terms of necrosis, oedema, and polymorphonuclear cells ( $p<0.05$ ). Necrosis and oedema were more severe and polymorphonuclear cell infiltration was higher in the control group than in the treatment groups. The collagen deposition at the anastomotic line was significantly higher in the ozone groups than in the control group. Compared with the group S and P rats, a marked collagen deposition was observed in the ozone groups after Masson's trichrome staining. The histological scoring of the groups are given in Table 3.

### Hydroxyproline level

The mean hydroxyproline levels were  $439\pm 23.42$  mg/L/g in Group S,  $123\pm 22.32$  mg/L/g in Group P,  $399\pm 29.81$  in Group O0, and  $345\pm 85.45$  in Group O24. Hydroxyproline levels were significantly higher in groups O0 and O24 compared to the peritonitis group (P). There were significant differences in the concentration of hydroxyproline when the O0 and O24 groups and the sham group were compared ( $p<0.05$ ).

## DISCUSSION

One of the most common complications of gastrointestinal operations is anastomotic leaks, which are associated with high morbidity and mortality rates. The anastomotic leak rate is reported at 10% to 20% (16).

Anastomotic strength with maintenance of luminal integrity is a fundamental necessity for a successful outcome of gastrointestinal surgery. Performing a primary anastomosis in a contaminated field is thought to be associated with a failure to heal, anastomotic dehiscence, and peritoneal soilage from gastrointestinal anastomoses. Various agents have been investigated to ameliorate these effects of peritonitis on colonic anastomoses. These agents include hyperbaric oxygen, glucan, erythropoietin, bosentan, growth hormone, and  $H_2$  antagonists like famotidine. All of these agents aim to reduce ischemia (18-23). OT increases tissue oxygenation by shifting the oxyhaemoglobin curve to the right.  $H_2O_2$  formed by the OT causes the production of many growth factors, especially

VEGF (24). We did not evaluate VEGF expression in the current study. This is a further topic of investigation in the future. OT is also an antioxidant agent when used at lower doses by supporting cellular antioxidant systems including glutathione and superoxide dismutase (SOD) (25). In this study, we demonstrated an elevated protective effect of OT on colonic anastomoses in the presence of peritonitis. We evaluated bursting pressures, histopathology, and hydroxyproline levels to measure the anastomotic healing process.

Bursting pressures is reported to be a reliable indicator for wound healing (26). It reveals the mechanical parameters of a colonic anastomosis and reflects growing anastomotic strength (27). The protective effect of OT on wound healing of colonic anastomoses in the presence of bacterial peritonitis is supported by the BP results.

During wound healing, collagen is important in giving strength and integrity to the anastomosis. On the fifth and seventh days following an anastomosis, collagen synthesis is at its peak and the strength of the anastomosis depends on these newly organized collagen fibres (28). Biochemical parameters of anastomotic healing are reflected by the collagen content of perianastomotic tissues. Defining the level of hydroxyproline is a good method of evaluating the amount of tissue collagen. In this study, there was a significant difference in the concentration of hydroxyproline when the O0 and O24 groups and the sham group were compared.

Ozone therapy is supposed to change the levels of matrix metalloproteinases. Therefore, Shurska et al. (29) investigated the effect of ozone therapy on the levels of matrix metalloproteinases but could not prove Ozone therapy may have effects on collagen production and anastomotic healing through influencing the level of matrix metalloproteinases.

Histopathologic examination revealed significantly less necrosis and oedema in anastomotic segments from rats with ozone administration in comparison to the control group. This suggests that ozone therapy may cause a reduction in inflammation. Collagen deposition in the anastomotic segment was also significantly higher than in the control group, which also supports the beneficial effect of ozone therapy by promoting various growth factors, possibly because of an increased collagenase activity.

One of the limitations of the study was the animals in the sham and peritonitis groups received no placebo via the same route by which the solution of ozone was applied in groups O0 and O24. We do not know if the stress caused by this manipulation may have had some effect on the expected response. Having no antibiotic application may be supposed to be a further limitation of the study.

Ozone therapy blocks harmful effects of sepsis-induced injury on collagen synthesis during anastomotic wound healing. This therapy probably exerts its beneficial effects by improving antioxidant mechanisms, increasing tissue oxygenation, and promoting the production of various growth factors. In conclusion, ozone therapy has benefits on anastomotic healing

of the colon in the presence of peritonitis. These effects can be attributed to its anti-inflammatory and antioxidative properties. Better outcomes might have been obtained if OT had been applied immediately after the operation.

**Ethics Committee Approval:** Ethics committee approval was received for this study.

**Peer-review:** Externally peer-reviewed.

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