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Evaluation of the effects of hyperbaric oxygen treatment and enoxaparin on left colon anastomosis. An experimental study

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Abstract. – BACKGROUND: Surgical interventions on left colon lead to high morbidity. The problems in wound healing are the main cause of this morbidity. Hypoxia retards wound healing and hyperbaric oxygen treatment (HBOT) has an anti-hypoxic effect.

MATERIALS AND METHODS: In this experimental study we divided eighty Wistar albino rats into eight groups and numbered between 1 and 8. Normal (non-ischemic) and ischemic left colon anastomosis were performed in the first and second four groups respectively. HBOT and subcutaneous enoxaparin were applied to the groups separately and in combination for four days, except the control groups. (Group-1 and Group-5). We measured anastomotic bursting pressures and performed pathological examinations besides electron microscopic study in one sample from each group after sacrificing the rats on the fourth day.

RESULTS: There were no statistically significant differences in bursting pressures when we compared Group-1 with other non-ischemic groups, and Group-5 with Group-6, but there were statistically significant differences when we compared Group-5 with Group-7 and 8. In pathological examination, there were no statistically significant differences between the groups concerning necrosis, epithelization, granulation tissue formation and collagen deposition. Statistically significant differences were found in the scores of neovascularization when we compared Group-1 with Group-3 and 4, and Group-5 with Group-8. Electron microscopic evaluation revealed a prominent increase both in neovascularization and collagen fibers in the samples taken from the groups received enoxaparine and hyperbaric oxygen treatment in combination.

CONCLUSIONS: These findings suggest that HBOT increases neovascularization and bursting pressures in ischemic colon anastomosis in contrast with enoxaparin.

Key Words:
Hyperbaric, Oxygen, Enoxaparin, Colon, Anastomosis.

Introduction

Wound healing might become a problem after a surgical intervention to the colon, especially after the surgery to the left colon. Most of the interventions to the gastrointestinal tract are completed with anastomosis and a leakage from the anastomotic site is a feared complication of the surgery. The risk of leakage is higher from the anastomosis in the distal part of the colon. It is known that anastomotic leakage is the major cause of morbidity and mortality in colorectal surgery[1-5]. One of the main reasons for high morbidity and mortality is the flora of the colon that contains many varieties of bacteria in very high numbers. The colon is also the part of the gastrointestinal track that carries high risk for obstructive malignancies, ischemic problems, bacterial infections and secondary problems to these conditions such as anemia, malnutrition, fluid-electrolyte imbalance, etc. Many instances of leaking or dehiscence in clinical situations (tension sutures, excessive devascularization of the ends) have in common the hypoperfusion of the anastomosis. On

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many occasions, minor degrees of ischemia leads to hypoperfusion at the ends of anastomosis[6].

Hyperbaric oxygen therapy (HBOT), in which a patient breathes 100% oxygen intermittently while the pressure in the treatment chamber is increased to a point higher than 1 ATA, which is the atmospheric pressure at sea level. HBOT is used widely as an adjunctive therapy to resolve certain recalcitrant, expensive, or otherwise hopeless medical problems.

When the tissue oxygen level falls below 30 mmHg, the host responses to infection and ischemia are compromised[7]. Specifically, oxygen dependent intracellular leukocyte bacterial killing becomes defective or nonexistent, and host repair processes such as fibroblast migration, fibroblast proliferation and collagen production are arrested [8,9]. Fibroblasts are the major producers of collagen in the repairing process. Hypoxia affects fibroblast activity by decreasing IGF-1 production, increasing TGF-β production, and increasing procollagen mRNA levels[10,11].

Maintenance of tissue perfusion and oxygenation around a wound is a basic requirement for wound healing. It is one of the goals of to the surgical interventions to prevent or reduce a possible ischemic damage in a tissue. Tissue hypoxia could be reversed by using HBOT[12,13]. Therefore, it might be presumed that HBOT may have beneficial effect on the healing process after the surgeries such as colon anastomosis.

Low molecular weight heparins are fragments of unfractioned heparin produced by controlled enzymatic or chemical depolymeryzation processes that yield chains with a mean molecular weight of about 5000[14]. These molecules are used either in prophylaxis or treatment of venous thrombosis[15,16], unstable angina[17], and ischemic stroke[18]. Beside this accepted indications, low molecular weight heparin (LMWH) were also studied in some different diseases[19-23]. In colorectal surgery, LMWH are widely used drugs, especially in patients with cancer.

In this study, we aimed to investigate the effects of HBOT and LMWH (enoxaparin) separately and in combination on healing process of, left colon anastomosis in rat model either in ischemic or non-ischemic conditions.

Materials and Methods

Eighty adult female Wistar albino rats weighing 200-240 g were divided into eight equal groups (n=10) as;

Group-1: Control group; Normal (non-ischemic) left colon anastomosis without HBOT and enoxaparin

Group-2: Normal left colon anastomosis + subcutaneous enoxaparin

Group-3: Normal left colon anastomosis + HBOT

Group-4: Normal left colon anastomosis + subcutaneous enoxaparin + HBOT

Group-5: Ischemic control group; Ischemic left colon anastomosis without HBOT and enoxaparin

Group-6: Ischemic left colon anastomosis + subcutaneous enoxaparin

Group-7: Ischemic left colon anastomosis + HBOT

Group-8: Ischemic Left colon anastomosis + subcutaneous enoxaparin + HBOT

The animals, which were in cages with free access to food (standard rat food) and tap water, were housed in an animal care facility with 12h: 12h light:dark cycle, at room temperature.

Surgery

The animals were anaesthetized by injection of ketamine hydrochloride (50 mg per kg of body weight) intramuscularly, after one night fasting. Abdominal access was achieved through a midline incision 4 cm long, and the left colon was diverted at 3 cm proximally to the peritoneal reflection. After the fecal contents had been milked out, a standardized end to end anastomosis was made by eight interrupted, inverting sutures of 6/0 polypropylene, after the resection of 0.3 cm segment of the colon out. The marginal vessels of the colon were preserved in non-ischemic first four groups. Marginal vessels were ligated and cut in the region two centimeters proximal and distal of the anastomosis and ischemia were performed in groups 5, 6, 7. We injected 2 cc 0.9% NaCl intraperitoneally and abdominal wall and skin were closed with 3/0 continuous trophylen sutures. Oral intake was allowed as water after 12 hours, and food after 24 hours.

Treatment

HBOT of the Group-3, Group-4, Group-7 and Group-8 was commenced immediately after the surgery performed four times a day for 4 days. The treatments were conducted in a small research chamber (0.4 m^3). The chamber was flushed with oxygen for 10 min. to vent the air inside before the compression, so that the animals could be pressurized with 100% oxygen. The HBO treatments were 80 min at 2.5 ATA including 10 min. compression and 10 min. decompression time. Group-1, Group-5 (controls) and Group-2 and Group-6 did not receive HBOT.

We administered enoxaparin (Clexane Aventis Pharma Pty Ltd) subcutaneously to the Group-2, Group-4, Group-6 and Group-8 once a day, with the dose of 1 mg/kg for four days.

Measurement of Anastomotic Bursting Pressure

The rats were sacrificed four days after the surgery. The abdominal incision was reopened and anastomotic sutures line was detected. About 4 cm segment of left colon was resected by preserving the adhesions, that includes 2 cm long proximal and 2 cm long distal part to the anastomotic line. A 16 gauge silastic catheter which was connected to a pump and a mercury manometer, was inserted into the proximal end of the segment and tied tightly by 2/0 silk. The distal end of the segment was closed by ligation with 2/0 silk. The segment was immersed in a water-filled glass case that allows reliable observation of bursting. Intraluminal pressure of the segment was increased by pumping air via the catheter with the flow rate of 5 ml/min. Bursting pressure was recorded as the peak pressure attained before rupture of the anastomosis.

Pathological and Histological Examinations

After measuring the bursting pressures, one sample from the tissues adjacent to anastomotic line for each group was fixed in a 2.5% phosphate buffered glutaraldehyde solution and post-fixed in 1% OsO4 for 1 h for ultrastructural examination[24]. The samples were dehydrated through a graded series of alcohol and embedded in Epon 812 (Fluka AG, Germany). The blocks were sectioned with an LKB Ultramicrotome (Stockholm, Sweden). Ultra thin sections were contrasted with lead citrate and uranyl acetate, and examined under an electron microscope (Jeol 100, Japan)[25].

Remaining colon tissues and other resected left colon segments were fixed with % 10 formaldehyde solution for at least 24 hours. Standard procedures were applied for specimens and paraffin blocks were prepared after the fixation. 3 μm sections were prepared and stained with hematoxylin-eosine. Specimens were undergone pathological examination with light microscope by a blinded pathologist. The pathologist evaluated the status of necrosis, epithelization, neovascularization, deposition of collagen and formation of granulation tissue during the pathological examination of the specimens. The parameters evaluated were scored in four levels such as; 0: absent, 1: mild, 2: moderate and 3: intense.

Statistical Analysis

First four (non-ischemic) and second four (ischemic) groups were evaluated separately. The groups were compared by using Kruskal-Wallis One-Way Anova analysis. Comparisons against control groups were done with Dunnett test. The level of significance was set at $p = 0.05$.

The experimental protocol of this study was approved by the local Ethic Committee of Istanbul University, Institute of Experimental Medicine Research.

Results

Bursting Pressure

Bursting pressures of the groups are given in Table I. No statistically significant differences were found between Group-1 and the other non-ischemic groups ($p > 0.05$). There were statistically significant differences between Group-5 and Group-7 ($p = 0.0159$) and Group-5 and Group-8 ($p = 0.0283$),

Table I. Bursting pressures of groups (mmHg).

Groups	N	Bursting Pressure ± Standart Deviation (mmHg)	Median
1	10	84.50 ± 24.35	77.50
2	10	60.40 ± 27.93	53.00
3	10	93.40 ± 24.82	83.00
4	10	95.00 ± 33.91	100.00
5	10	61.00 ± 21.94	59.50
6	10	57.00 ± 33.52	50.00
7	10	110.00 ± 37.61*	112.00
8	10	102.00 ± 23.78*	107.00

*Statistically significant differences between groups 5 and 7 ($p = 0.0159$) and 5 and 8 ($p = 0.0283$)

Table II. Mean values of the parameters in pathological analaysis.

Group	Necrosis	Epithelization	Granulation tissue form.	Collagenous tissue form.	Neo-vascularization
1	0.70±0.67	0.30±0.48	1.00±0.82	0.50±0.53	0.80±0.63
2	0.40±0.70	0.30±0.48	1.00±0.47	0.40±0.52	1.60±0.52
3	0.30±0.48	0.30±0.48	1.50±0.71	1.10±0.57	1.90±0.57*
4	0.80±0.92	0.40±0.52	0.90±0.74	0.90±0.74	1.90±0.88°
5	2.60±0.52	0.20±0.42	0.50±0.71	0.30±0.48	0.50±0.71
6	1.80±0.92	0.20±0.42	0.90±0.88	0.80±0.63	1.50±0.71
7	1.40±1.35	0.50±0.53	0.80±0.63	0.70±0.82	1.50±1.08
8	1.50±1.18	0.20±0.42	1.10±0.74	0.80±0.63	1.70±0.80‡

*Statistically significant difference between groups 1 and 3 ($p = 0.0100$)
°Statistically significant difference between groups 1 and 4 ($p = 0.0083$)
‡Statistically significant difference between groups 5 and 8 ($p = 0.0138$)

there were no statistically significant difference between Group-5 and Group-6 ($p > 0.05$).

Pathological Analysis of the Groups

The mean values of investigated parameters of groups pathological examinations were summarized in Table II. There were no statistically significant differences between groups concerning necrosis, epithelization, granulation tissue formation and collagen deposition ($p > 0.05$).

Statistically significant differences were found in the scores of neovascularization between Group-1 and Group-3 ($p = 0.0100$), Group-1 and Group-4 ($p = 0.0083$) and Group-5 and Group-8 ($p = 0.0138$) (Figure 1).

Electron Microscopic Evaluation

Prominent increment of neovascularization and collagenous fibers was evident in the electron microscopic evaluation of sample from Group-4. Some increase in neovascularization and collagenous fibers was also detected in Group-2 and Group-3, but it was not remarkable as much as in Group-4. In ischemic groups remarkable increment of neovascularization and collagenous fibers were observed only in Group 8 (Figure 2).

We observed lesser peritoneal adhesions around anastomotic sites in Group-2 and Group-6, which received only enoxaparin, than other groups (unmeasured observation).

Discussion

Anastomotic leakage after colorectal surgery occurs in 5-15% of patients undergoing this type of surgery and leads to a substantial morbidity and mortality[26]. Although there are some studies concerning the complication of anastomotic leakage after colorectal surgery, risk factors and preventive measures were not completely clarified yet. Probably infection and insufficient tissue oxygenation are the most important two factors among the un-

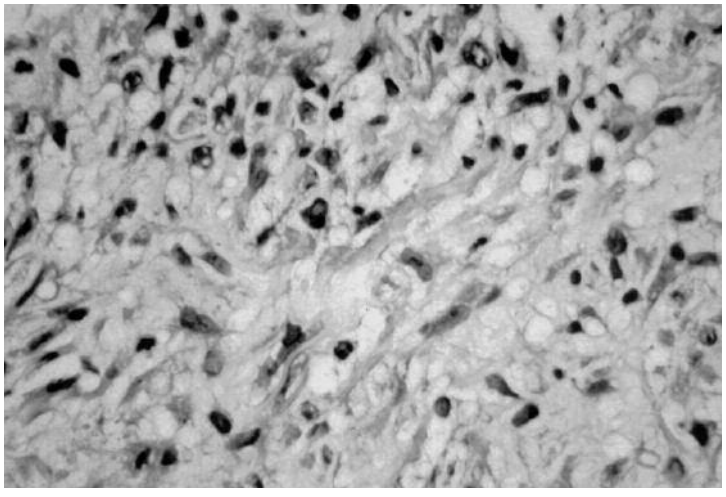

Figure 1. Fibroblastic proliferation, neovascularization and collagen deposition. Granulation tissue. (HE X500).

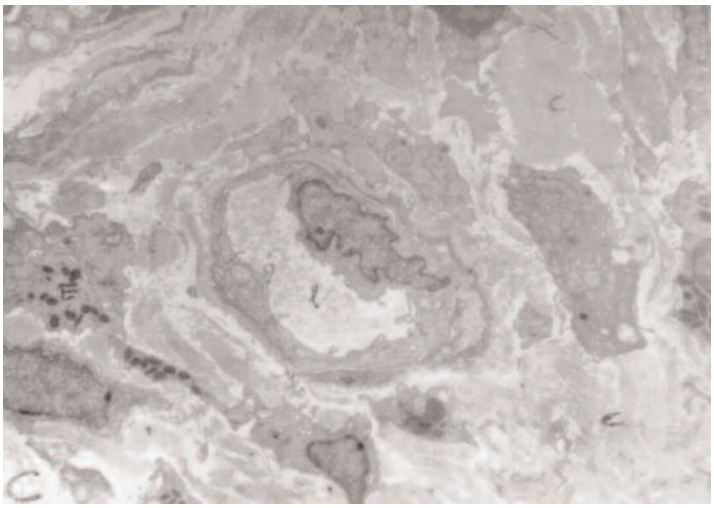

Figure 2. General view of lamina propria: Collagen fibers and neovascularisation are increased in connective tissue in the group that enoxaparin Na and hyperbaric oxygen were used together. C: collagen fibers, L: capillary lumen, E: eosinophil.

derlying reasons, which cause leakage from an anastomosis without any passage problem, after a standard surgery. The presence of ischemia and infection has hazardous effect on wound healing[27-29].

Numerous local and systemic factors affect anastomotic healing. The most important local factor is the perfusion and oxygenation of the site of anastomosis. To achieve safe anastomosis, intestinal blood flow should exceed 30%[30]. Oxydative function of neutrophils, leukocyte activation, fibroblast production, angiogenesis and epithelization can't be sufficient in wound healing if the tissue oxygenitation is inadequate. Collagen can't be synthesized in the absence of molecular oxygen since fibroblasts needs oxygen for their activity[31,32,33,24,34]. The increased oxygen tension that is achieved during HBOT can support poorly perfused and hypoxic tissues. In compromised wounds at normobaric conditions, watershed areas of ischemic tissue exist between individual blood vessels. Hyperbaric oxygen limits the hypoxic tissues since the diffusion pattern of oxygen is greatly increased by HBOT[35,36]. During HBOT, there will be enough oxygen dissolved in plasma besides hemoglobin-born oxygen[37]. Thus, oxygen tension in the wound tissue can be increased by HBOT which may enhance wound healing. Therefore, hyperbaric oxygen therapy is used in some problem wounds[38].

There is only one study in the literature concerning the effect of HBOT on colonic anastomosis[12]. Hamzaoglu et al reported that HBOT increased anastomotic healing of both normal and ishemic colon anastomosis. In an other study about the role of oxygen on anastomotic colon, Kirk and Irvin[39] demonstrated no significant role of 50% oxygen under normobaric condition, which is not a HBOT application.

After almost two decades of intensive researches, low molecular weight heparin has established its niche as an important class of antithrombotic compound. Recently it was reported that LMWH improved the outcome of chronic foot ulcers in diabetic patients with peripheral arterial occlusive disease[40,19]. These effects are due to the improvement of microcirculation[40]. It was also reported that LMWH accelerated gastric ulcer healing which was accompanied by an increase in mucosal regeneration and proliferation, angiogenesis and mucus content in the stomach[23]. In this study we also investigated the effects of enoxaparin, a LMWH compound on left colon anastomosis, individually and together with HBOT.

In many studies, investigators measured the hydroxyproline content of intestinal anastomosis since it was assumed that hydroxyproline level is an indicator for the collagen content. But in some studies there was no correlation between hydroxyproline concentration and mechanical strength of an anastomosis[40,41].

In our study, we measured the bursting pressure of the anastomosis, which is an evidence for healing level of an anastomosis. We also tried to obtain another objective measure for the healing process in the anastomotic site, with the pathological examination of the samples from anastomotic line, by light microscope and electron microscope.

The postoperative 4th day is the time, where the deposition of collagen was either at the beginning or still not increased in high amounts in a freshly constructed anastomosis[5,42]. Angiogenesis and epithelization are the main points deserving attention in this process[42]. According to the pathological examinations of the anastomotic line, we found a significant difference only in angiogenesis, in Group-3, 4 and 8, which can be at-

tributed to HBOT. Samples examined under electron microscope also supported this finding.

The value of bursting pressure was found higher in Group-3 and 4 than the Group-1 (control group), but the difference was not statistically significant. Probably the outcome we obtained is a normal result for an anastomosis which is not ischemic.

The values of bursting pressure of ischemic groups are significantly different from each other. The values obtained from Group-7 and 8 were significantly higher than Group-5. Therefore it is possible to say that HBOT might be much more beneficial in ischemic conditions and which makes sense.

Similar to the value between Group-1 and Group-2, Group-5 has higher pressure value than Group-6 and these differences have no significant meanings. These non significant findings might be due to another effect of therapies applied in this experimental model which is not a surprise for us. We formerly showed that enoxaparin had decreased the abdominal adhesions and HBOT had increased them[43].

The omental adhesions around the anastomotic sites in the Group-2 and Group-6 which received only enoxaparine were remarkably less than the other groups. Abdominal adhesions were much more dense, in groups which were underwent to HBOT, especially in Group-7 and 8 which were ischemic (unmeasured observation). Wounded sites probably gained much more capacity to form adhesions by the effect of ischemia and HBOT. Ischemic groups which didn't receive HBOT had the lowest pressure values, as they had probably the lowest healing capacity. Therefore the degree of the adhesions around the anastomotic site is a factor, which may affect the bursting pressure. Adhesions may have a potential benefit including neovascularization of ischemic structures such as anastomosis[44].

We did not detect any finding, which supports that enoxaparin increases angiogenesis, epithelization or bursting pressures.

Conclusions

HBOT increased angiogenesis that is known as a step in the phases of wound healing in our study. However, we could not detect any effect of enoxaparin on the process of wound healing. We showed that HBOT did not alter bursting pressure values of non-ischemic left colon anastomosis significantly, but HBOT increased bursting pressures in ischemic colon anastomosis.

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Conflict of interest

The Authors declare that they have no conflict of interests.

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