Annals of Medical Research
DOI: 10.5455/annalsmedres.2019.10.604 2020;27(1):224-32

Original Article

The effect of amniotic membrane wrapping on colorectal anastomosis in rats undergoing pelvic radiotherapy

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Abstract

Aim: This study aimed to investigate the effect of amniotic membrane wrapped onto the anastomosis line following colon resection and primary anastomosis on anastomosis healing in rats undergoing pelvic radiotherapy.

Material and Methods: Fifty-six male Wistar albino rats were randomly allocated to four main groups. Group I, left colon resection and primary anastomosis; Group II, post-anastomotic amniotic membrane; Group III, preoperative radiotherapy; Group IV, preoperative radiotherapy and amniotic membrane. Radiotherapy was administered under general anesthesia eight and four days prior to surgery at a total of 20 Gy. In the first operation, all the rats underwent 1 cm left colon resection and primary anastomosis. The animals in each group were anesthetized to assess the clinical, mechanical, histologic, and biochemical parameters of anastomotic healing on the third and seventh postoperative days.

Results: In the third day groups, anastomosis line bursting was observed in only one subject in those undergoing amniotic membrane wrapping($p<0.05$) and in all rats in those with no amniotic membrane wrapping. No statistically significant difference was observed in any of the parameters on the histopathological examination of the day 3 groups. The amounts of granulation tissue and fibroblast were significantly higher in the post-anastomotic amniotic membrane group ($p<0.05$), and the macrophage amount was seen to be significantly low in both amniotic membrane groups ($p<0.05$).

Conclusion: Amniotic membrane application on the anastomoses in the intestines, either with or without radiotherapy, may contribute to wound healing by positively affecting the anastomosis health.

Keywords: Radiotherapy; colorectal anastomosis; anastomosis healing; amniotic membrane

INTRODUCTION

T3, T4, N^+ rectum cancer cases only undergoing surgery carry a high local recurrence risk. Preoperative radiotherapy (RT) applied in operable rectum cancer cases contributes to a significant improvement in survival by decreasing the local recurrence compared to surgical therapy alone (1). Together with this beneficial effect, preoperative RT continues to maintain its negative effect on postoperative anastomosis health due to tissue injury in the application site (2-5).

The rate of colorectal anastomosis leakage has been reported to be between 3% and 18% in the literature in patients undergoing sphincter preserving surgery following neoadjuvant chemoradiotherapy (CRT) for rectum cancer (6-8). Related mortality is between 6% and 22% (7,9).

Amniotic membrane (AM) constitutes the innermost fetal membrane. It is composed of three layers comprising a single epithelial layer, a thick basal membrane and an avascular mesenchyme. The basal membrane consists of growth factor and proteinase inhibitors. Studies have indicated AM to have antibacterial and low immunogenic properties, to reduce inflammation and scar formation and to accelerate wound healing and epithelization through permitting angiogenesis (10,11).

Received: 03.10.2019 **Accepted:** 22.12.2019 **Available online:** 18.02.2020
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Beginning from its first use for skin transplantation by Davies in 1917, AM has been used in many reconstruction operations until today (12).

In the literature, AM has been reported to have positive effects on anastomosis healing in conventionally sewn high risk colonic anastomosis in rats (13).

In our study, we aimed to investigate the effect of AM, which was wrapped onto the anastomosis line following colon resection and primary anastomosis, on anastomosis healing in rats undergoing pelvic RT.

MATERIAL and METHODS

This experimental study was carried out in the Multidisciplinary Test Animals Laboratory of Dokuz Eylül University School of Medicine (DEUSM) and ONKOMER Private Oncology Center after obtaining the Animal Tests Local Ethics Committee approval from the DEUSM.(Date 08.07.2011 No: 38/2011). All animals had free access to food and water ad libitum and were handled in accordance with the guidelines for care and use of laboratory animals established by the Ethical Committee of the DEUSM, Turkey, and the National Institutes of Health(U.S. publication no. 86-23, revised 1985).

At the beginning of the study, 56 male Wistar albino rats were randomly allocated to four main groups as Group I, left colon resection and primary anastomosis (NRT+NAM), Group II, post-anastomotic AM(NRT+AM), Group III, preoperative RT (RT+NAM) and Group IV, preoperative RT and AM (RT+AM). Half of all the rats in the groups were evaluated for analytic procedures on the third day and the remaining on the seventh day (Figure 1).

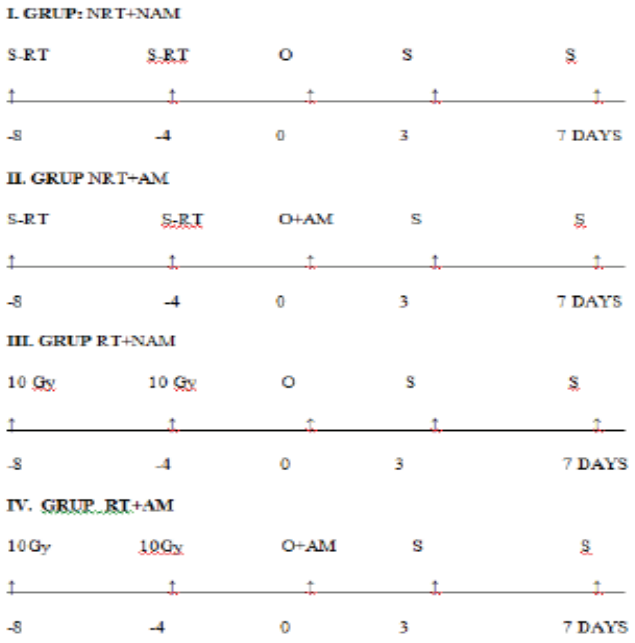

Figure 1. Protocols for the four groups of animals in this study. O = operation; S =sacrificed; AM =amniotic membrane; S-RT = sham radiotherapy.

The rats were operated under clean but non-sterile conditions in the Test Animals Laboratory DEUSM. Anesthesia was provided with intramuscular (IM) 50 mg/kg ketamine (Ketalar®, Pfizer) and 5 mg/kg xylazine hydrochloride (Rompun®, Bayer) mixture. Laparotomies were performed through a median incision. The bowels were covered with pads soaked in warm, sterile saline to reduce the heat loss from the tissues. A table lamp was used to keep the body temperature of the subjects at 37ºC. All the subjects underwent administration of 5 mL of ringer's lactate via the subcutaneous route to prevent postoperative dehydration.

Preparation of AM

AM was acquired from voluntary pregnant women who were controlled for infection (HIV, HBsAg, syphilis) and who did not experience premature rupture of the membranes, endometritis or meconium contamination after live births through caesarean section. The placentae were transferred to the laboratory at +4°C under sterile conditions. After the amnion and the chorion had been separated with a blunt dissection, they were rinsed with saline and 0.025% sodium hypochlorite solution for about 10 minutes. They were then stored in saline solution, which contained 2 million IU of penicillin and 2 gr of streptomycin per liter for 12-24 hours and prepared for use. The AM was stored at +4°C. Amniotic membrane that lost color was excluded from the study (14).

Irradiation technique

The rats in groups III and IV underwent RT twice 8 and 4 days before the operation. Anesthesia was provided by a mixture of intramuscular (IM) 50 mg/kg ketamine (Ketalar®, Pfizer) and 5 mg/kg xylazine hydrochloride (Rompun®, Bayer) during irradiation. The rats were placed onto “Styrofoam” ground as groups of 5 rats each under general anesthesia(The rats were irradiated through two ports at the antero-posterior and postero-anterior) (15). As a pilot study, diluted barium enema was applied to the first 6 rats, and the targeted areas were determined as 5x5 cm pelvic areas in the Simulator (SOMATOM Emotion Computed Tomography Simulator, Siemens-Germany). The rats were placed in an area of 5x35 cm as quintet groups on Styrofoam ground, and irradiation was applied. Irradiation was applied as isocentric source-axis distance (SAD) so as to be 80 cm in Co-60 teletherapy device(Theratron 780 E-Canada). The dose rate was 112.35 cGy/min. Each rat underwent a 20 Gy dose as 10 Gy on each day of the irradiation.

Operation technique

All the rats underwent laparotomy under general anesthesia 4 days after the completion of irradiation (or sham irradiation). The operative field was prepared with 10% povidone iodine solution after the skin had been shaved. A 3 cm midline skin incision was done. The linea alba and the peritoneum were opened, and the abdominal space was accessed. 1 cm of colon resection was performed 2-3 cm above the peritoneal reflection. Intestinal continuity was provided with 10-11 inverter sutures (6/0 monofilament polypropylene, Ethicon, England) as end-to-end anastomosis. In subjects with AM

application onto the anastomosis line, the AM was fixed with 6 sutures (6/0 monofilament sutures) so as to cover the anastomosis line at a 10 mm width. The abdominal muscle layer and the skin were closed separately with continuous sutures. Half of all the rats in the groups were re-operated under general anesthesia on the third day and the remaining on the seventh day for analytic procedures and were sacrificed through hemorrhage following in vivo assessment (2).

Analytic Procedures

The weights of all the rats were measured and recorded on the day of the first irradiation(or sham irradiation), 8 days before the operation, on the day of the second irradiation, 4 days before the operation, on the operative day and on the day of sacrification, on which the analytic procedures were performed.

Wound complications (infection and wound dehiscence), intestinal obstruction and anastomosis complications (presence of macroscopic abscess, anastomosis dehiscence and anastomosis stricture) were recorded. Furthermore, intraperitoneal adhesions were classified according to the grading system of Knightly et al. (Table 1) (16).

Table 1. Intraperitoneal adhesions were classified according to the grading system of Knightly et al. (16)

Grade 0	No adhesion.
Grade 1	A single, fine, easily detachable adhesion.
Grade 2	More spread-out, but easily detachable adhesion with traction.
Grade 3	Widespread visceral adhesions with parietal extensions.
Grade 4	Intense and widespread adhesions exending to the neighbouring mesentery, intestine, omentum and abdominal wall.

Anastomotic stricture was described as proximal/distal intestinal width ratio higher than 2.

Anastomotic integrity was investigated through measurement of the anastomotic bursting pressure (ABP) of each anastomosis. A fluid pump working with an infusion rate of 5 ml/min (Braun, fm-Germany) and a pressure transducer (Abbot, Monitoring Kit, Transpac II, Abbot Ireland Ltd, Sligo, Republic of Ireland) were used to measure the bursting pressures. The pressures were recorded through a monitor (PETAŞ KMA 450, Ankara, Turkey) as mmHg. A catheter that was placed through the anus of each rat was bound 2 cm below the anastomosis line and fixed. Similarly, the point 2 cm above the anastomosis line was also bound. During these procedures, attention was paid not to harm the anastomosis and the adhesions surrounding the anastomosis, if present. ABP and the bursting site (BS) were measured in-situ. Pressure changes and sudden pressure losses were observed from the monitor, and the BS was detected through a condensing lens. The value leading to bursting and the site of bursting were recorded. Afterwards, the anastomosis segment was isolated from the surrounding tissues and resected for in-vitro analytic procedures. Histological evaluation of wound healing in experimental intestinal anastomoses was performed and scored as described by De Roy van Zuidewein et al (17). The subjects were sacrificed through hemorrhage after these procedures had been completed.

Statistical Methods

The data are expressed as mean ± standard error of the mean. The Wilcoxon's signed-ranks test was used to analyze the body weight change of the groups at different times. The Kruskal-Wallis variance analysis was used to analyze the statistical differences between the study groups for adhesion scores, the bursting pressures and histological data. The comparisons between the groups were made using the Mann-Whitney U test. The differences between the groups on days 3 and 7 were evaluated by the Mann-Whitney U test.

RESULTS

Mechanical Analysis

While mortality occurred in no subjects, no statistically significant difference was found between the groups in terms of complications (Table 2). Adhesion formation was seen to a lower extent in AM-applied groups among those re-operated on the third day(p=0.012).

Body weight: When the groups that were operated on days 3 and 7 were analyzed, a significant weight loss was observed after the first operation in the group with no RT application (p=0.001). A statistically significant weight loss was seen in the RT-receiving group, which began after the second RT and continued until sacrification (p=0.001) (Figures 2 and 3).

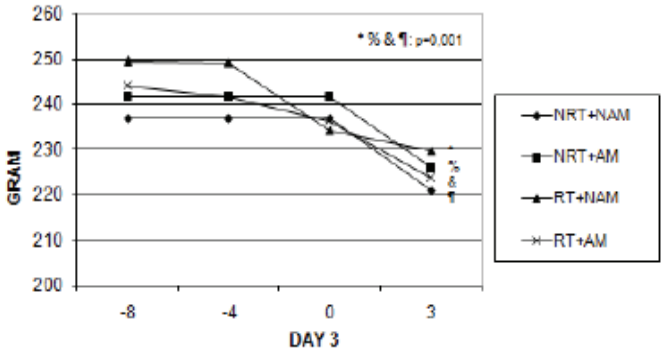

Figure 2. Weight changes during the experiment (Day 3)

Irradiated animals in (RT+NAM and RT+AM) had more pronounced weight loss, which started at the time of radiation, and weight loss was increased by the time of the operation (P = 0.001). Weight loss was not significant for any group after operation.

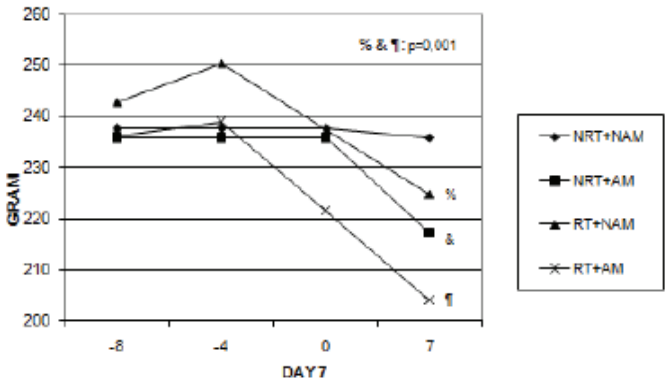

Figure 3. Weight changes during the experiment (Day 7)

Irradiated animals in (RT+NAM and RT+AM) had more pronounced weight loss, which started at the time of second radiation, and weight loss was increased by the time of the operation (P = 0.001). Weight loss was not significant for any group after operation

Anastomosis bursting pressure: The mean anastomosis bursting pressures(ABP) were found as NRT+NAM 68±31, NRT+AM 124±26, RT+NAM 75±29, and RT+AM 147±16 mmHg, respectively, on the third day, and this result was statistically significant(p=0.001) (Figures 4 and 5).

The difference between the ABP values of the NRT+NAM and NRT+AM groups was statistically significant on the third day(p=0.013). There was a statistically significant difference between the RT+NAM and RT+AM groups in terms of ABP (p=0.002).

In the seventh day groups, the mean ABP were found as 159±53 in the NRT+NAM, 202±30 in the NRT+AM group, 164±32 in the RT+NAM group and 209±16 mmHg in the RT+AM group, and there was a statistically significant difference(p=0.023). When the NRT+NAM and the NRT+AM groups were compared, there was a statistically significant difference in terms of ABP (p=0.047). There was a statistically significant difference between the RT+AM and RT+NAM groups in terms ABP (p=0.018).

Table 2. Details of Complications (NS: Not Significant)

	NRT+NAM		NRT+AM		RT+NAM		RT+AM		p	
Complications	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**	**Day 3**	**Day 7**
No. Of animals	7	7	7	7	7	7	7	7		
Wound infection	-	-	1	1	-	2	-	2	NS	NS
Wound dehiscence	1	-	-	-	-	-	-	-	NS	NS
Adhesion score (mean, SE)	1.71 ± 0.28(2)	1.57 ± 0.53(2)	1±0 (1)	1± 0(1)	1.42 ± 0.29(1)	2.28 ± 0.75(2)	1 ± 0(1)	2.28 ± 0.48(2)	0.012	NS
İntestinal obstruction	-	-	1	1	1	-	1	1	NS	NS
Anastomotic stenosis	-	-	-	-	-	-	-	-	NS	NS
Anastomotic dehiscence	-	-	-	-	-	-	-	-	NS	NS
Abdominal sepsis	-	-	-	-	-	-	-	-	NS	NS
Mortality	-	-	-	-	-	-	-	-	NS	NS

Anastomosis bursting site

All the BS were the anastomosis line in subjects that did not undergo, AM and the difference was statistically significant (p<0.001).

In the seventh day groups, when the BS was analyzed according to AM application, the BS was detected on the anastomosis line in 4(28.6%) subjects and found to be out of the anastomosis line in 10(71.4%) subjects, and these rates were 1(7.1%) and 13(92.3%), respectively, in the AM-applied group, and the difference was not statistically significant (p=0.146).

Histological Analysis (Table3)

Although there was no statistically significant difference between the groups in terms of necrosis on the third day (p=0.371), the amount of necrosis was higher in the RT group; however, no statistically significant difference was found when compared to the group that did not receive RT(p=0.133). Similarly, there was no statistically significant difference between the groups with and without AM application in terms of necrosis (p=0.716).

While granulation tissue formation did not show a significant difference in all the groups (p=0.071), it was to a higher extent in the NRT group, and there was no statistically significant difference. Although the granulocyte amount did not show a statistically significant difference in all the groups (p=0.192), it was not affected by RT application(p=0.548). The granulocyte amount was statistically significantly higher in the AM-applied groups compared to the NAM groups (p=0.069).

The macrophage amount did not show a statistically significant difference in all the groups (p=0.286). There was no statistically significant difference in terms of RT or AM application (p=0.892 and p=0.137, respectively).

No statistically significant difference was observed in the fibroblasts in all the groups (p=0.207). There was no statistically significant difference in terms of RT or AM application (p=0.304 and p=0.194, respectively).

When necrosis was evaluated in the seventh day groups, there was a statistically significant difference between the groups (p=0.001), and this difference had arisen from the

Table 3. Histologic Evaluation of wound healing in experimental intestinal anastomoses (scoring described by De Roy van Zuidewein et al.)(17)

	Day 3		Day 7	
	Mean±SEM (median)	P	Mean±SEM (median)	p
Mucosa		0.878		0.099
NRT+NAM	1.71± 0.18(2)		1.57±0.20(2)	
NRT+AM	1.85±0.14(2)		1±0.30(1)	
RT+NAM	1.57±0.29(2)		1.71±0.18(2)	
RT+AM	1.57±0.29(2)		1.85±0.14(2)	
Muscularis mucosa		0.878		0.099
NRT+NAM	1.71±0.18(2)		1.57±0.20(2)	
NRT+AM	1.85±0.14(2)		1±0.30(1)	
RT+NAM	1.57±0.29(2)		1.71±0.18(2)	
RT+AM	1.57±0.29(2)		1.85±0.14(2)	
Reepithelisation of mucosa				
NRT+NAM	5.42±0.42(6)	0.209	3.71±0.52(3)	0.424
NRT+AM	4.42±0.81(3)		3.71±0.77(3)	
RT+NAM	4.42±0.48(4)		4.57±0.57(4)	
RT+AM	3.71±0.35(4)		4.85±0.63(5)	
Regeneration of muscularis propria				
NRT+NAM	0.85±0.14(1)	0.168	0.71±0.18(1)	0.925
NRT+AM	0.28±0.18(0)		0.71±18(1)	
RT+NAM	0.71±0.18(1)		0.71±0.18(1)	
RT+AM	0.57±0.20(1)		0.57±0.20(1)	
Necrosis				0.001
NRT+NAM	0.14±0.14(0)	0.371	0.14±0.14(0)	
NRT+AM	0.28±0.18(0)		0.42±0.20(0)	
RT+NAM	0.71±0.28(1)		0.14±0.14(0)	
RT+AM	0.42±0.29(0)		1.42±0.20(1)*	0.007
Inflamatory exudate				0.008
NRT+NAM	1.71±0.18(2)	0.798	1.28±0.18(1)	
NRT+AM	2±0.30(2)		1.42±0.20(1)	
RT+NAM	1.71±0.28(2)		1.71±0.28(2)	
RT+AM	2±0.30(2)		2.57±0.20(3)*	0.004
Granulation tissue				0.028
NRT+NAM	1.42±0.35(1)	0.071	2.14±0.14(2)	
NRT+AM	1±0(1)		2.85±0.14(3)*	0.048
RT+NAM	1.14±0.14(1)		2±0.21(2)	
RT+AM	1±0(1)		2±0.30(2)	
Granulocytes				
NRT+NAM	1.42±0.20(1)	0.192	1.71±0.28(2)	0.355
NRT+AM	2.14±0.26(2)		2±0.30(2)	
RT+NAM	1.42±0.20(1)		2±0.30(2)	
RT+AM	1.85±0.34(2)		2.42±0.20(2)	
Makrophage				0.010
NRT+NAM	1.57±0.29(1)	0.286	1.71±0.28(2)	
NRT+AM	1.28±0.18(1)*		1±0(1)	0.024
RT+NAM	1.85±0.34(2)		2.28±0.28(2)	
RT+AM	1.14±0.14(1)		1.42±0.20(1)*	0.040
Fibroblasts				0.028
NRT+NAM	1.71±0.35(1)	0.207	2.14±0.14(2)	
NRT+AM	1.14±0.14(1)		2.85±0.14(3)*	0.048
RT+NAM	1.28±0.18(1)		2±0.21(2)	
RT+AM	1±0(0)		2±0.30(2)	

RT+AM group (p=0.002). When the RT and the NRT groups were compared, necrosis was statistically insignificantly greater in the RT groups (p=0.077). Necrosis was observed to a higher extent in the AM group (p=0.002).

A statistically significant difference was found between the groups in terms of inflammatory exudate formation (p=0.004).

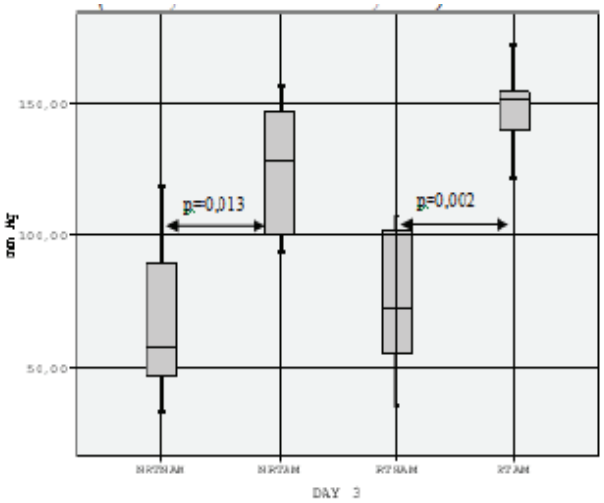

Figure 4. Anastomotic colon bursting pressures (mean±standard error) on the third postoperative days

Anastomotic colon bursting pressures (mean±standard error) on the third postoperative days. On day 3, AM wrapping animals in both groups had significantly higher pressure (P=0,013 and P= 0,002)

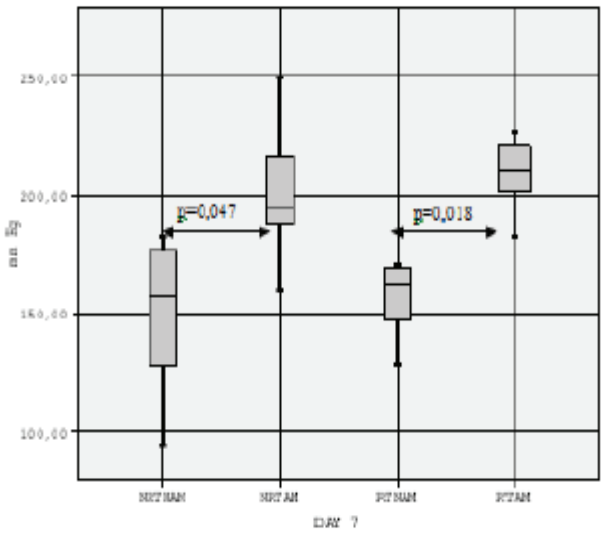

Figure 5. Anastomotic colon bursting pressures (mean±standard error) on the seventh postoperative days

Anastomotic colon bursting pressures (mean±standard error) on the seventh postoperative days. On day 7, AM wrapping animals in both groups had significantly higher pressure (P=0,047 and P= 0,018)

Granulation tissue formation showed a statistically significant difference among all the groups (p=0.028). It was seen to be significantly higher in the NRT+AM group. When the NRT+AM group was compared with the NRT+NAM and RT+AM groups, granulation tissue formation was found to be statistically significantly greater in the AM-applied groups and significantly lower in the RT-applied groups (p=0.010 and p=0.031, respectively).

The macrophage amount showed a statistically significant difference in all the groups(p=0.010).It was found to be statistically significantly low in the AM groups(p=0.005).

There was a statistically significant difference in terms of fibroblasts in the whole group, and this difference arose from the NRT+AM group (p=0.028) (Table3).

DISCUSSION

The main reasons for neoadjuvant radiotherapy application in rectum cancer patients are to reduce the extra-pelvic metastasis and pelvic recurrence risk from the cancer cells remaining after resection, to reduce the primary tumor size and lymph node metastases before resection, to enable the normally oxygenized cancer cells compared to the hypoxic cells in the tissues to be affected from radiotherapy to a higher extent and to prevent potential tumor spread during surgery (4).

In experimental studies, RT was shown in detail to delay wound and anastomosis healing2. Therefore, surgical therapy is delayed for 4-6 weeks and is aimed to avoid the negative effects of RT to overcome this problem in patients who receive RT and undergo surgery (18,19). Furthermore, a loop stoma is used to overcome the negative effect of neoadjuvant therapy at the early stage of anastomosis healing. Debate continues about whether a loop stoma is effective for the prevention of anastomosis leakage or for reducing the severity of anastomosis leakage (20).

Many studies have investigated anastomosis healing in colorectal surgery. In those studies, many substances such as vitamin A, zinc, besides isotonic, povidone-iodine,10% dextrose, short chain fatty acids containing solutions were used; however, very few took place in clinical practice (16).

Waninger et al. stated that short distance between sutures and loose suture tension was the best healing model. Furthermore, a water-resistant closure formed on serosal surface is essential for the healing of the luminal side of the intestine (21). The stratified layer of the intestinal wall significantly affects wound healing. Matching of the mucosa and the muscularis mucosa substantially depends on the anastomosis technique.

Both mucosa eversion and inversion delay wound healing. If the layers of the intestinal wall completely match, the anastomotic defect fully heals within three days (22). It was concluded in our study that these parameters were technically adequate and standard.

In our study, the method of Weiber et al. was adopted to prevent anesthesia-related complications by administering the radiation dose given in five days within 2 days as in previous experimental models (15).

A statistically significant weight loss was seen in RT-receiving groups, which began after RT and increasingly continued until sacrification following the operation ($p<0.05$).

Anastomosis dehiscence risk is maximum on the first postoperative day. Early integrity of the anastomosis depends on leak-proofing of the fibrin layer, which keeps water in the serosal region, and the suture-holding capacity of the intestinal wall for four days (22,23). However, in our study, the significant difference in the anastomosis bursting pressure in the AM groups was contributed to the adhesion of AM to the anastomosis line in the 3rd day and 7th day groups with or without RT. This association continued increasingly in the day 7 groups, and even the mean bursting pressures were similar to the 3rd day AM-applied groups when the NAM groups had reached the 7th day.

When the anastomosis BS was evaluated, while the bursting was seen to occur at a site out of anastomosis in all subjects undergoing AM ($p<0.001$), bursting was seen in the anastomosis line in groups with no AM application. This result suggested that AM was protective against the complications related to anastomosis dehiscence in the early period by providing a mechanical support onto the anastomosis. This result is consistent with those of previous studies carried out with AM (13).

Mucosa re-epithelization and muscularis propria regeneration did not differ in any of the groups when the healing period of anastomosis was evaluated. In a study by Terzi et al., they investigated the effect of luminal short chain fatty acids on colonic anastomosis and demonstrated its positive contribution to the improvement of the mucosal layer (16). Anastomosis and AM application were made intraperitoneally in our experimental study. While the effectiveness of AM was expected on the anastomosis line, the effect was considered to spread locally and intraperitoneally due to the application. In previous studies, AM was shown to decrease the extent of intraabdominal adhesions (26). This finding supported a small degree of adhesion on day 3 in the AM-applied groups in our study ($p=0.012$).

Resection of an intestinal segment leads to a vasodilation following an abrupt vasoconstriction and edema and swelling in the tissue with an increased permeability (24). Radiation-induced injury of endothelial microcirculation impairs this condition. Anastomotic sutures also negatively contribute, and ischemic necrosis may develop due to compression of the sutures in the swollen tissues. In addition to these findings, Hendrix et al. demonstrated that seromuscular blood flow decreased in the early period in colorectal anastomosis after radiotherapy (25). In our study, necrosis was found to be high in the RT groups among day 3 groups, although statistically insignificant, and this was not affected by AM application. There was a statistically significant difference in day 7 groups. While there was an insignificant elevation in the RT groups, it was greater in the RT+AM group ($p=0.002$). The higher degree of necrosis in the AM groups was considered to be associated with a reduction in inflammatory cells. This was related to the absence of an inflammatory cell increase in previous studies, and researchers stated that this decrease could not be explained (11,12,25). While the presence of an inflammatory exudate did not show a statistically significant difference in day 3 groups ($p=0.798$), this difference was significantly high in the RT groups among day 7 groups ($p=0.007$. This difference was statistically significant in the RT group among the AM groups ($p=0.010$). The short RT protocol was seen to be prominent in day 7 groups and not affected by AM application.

In a study by Kuzu et al., neovascularization was shown to lead to a delay in granulation tissue formation through being affected by RT (2). In our study, granulation tissue development showed a statistically insignificant difference in day 3 groups. The decrease in granulation tissue in the RT group did not differ significantly compared to the NRT groups. Similar results were seen in day 7 groups, and there was statistical significance. In the in-group paired comparison, AM application significantly increased granulation in the NRT groups. There were similar findings in the RT and NRT groups, and RT was seen to significantly hinder the progress of granulation tissue, and this condition was seen not to change based on whether AM was applied or not. Uludağ et al. reported that AM applied to high risk colonic anastomosis proportionally increased neovascularization, although not statistically, and thereby fibroblastic activity also increased in the anastomosis line (13). This opinion supports the granulation tissue increase in the NRT groups in our study. However, AM was seen not to effect the reduced granulation tissue in the RT groups. This was associated with the accuracy of the purpose of RT to reach endarteritis (26).

The secondary cell group that approaches the wound is macrophages. They are the cornerstone of a successful healing. Macrophages reach a significant amount within 48-96 hours. They release mediators such as TGF-α, VEGF, IGF and lactate and regulate cell proliferation, matrix synthesis and angiogenesis and remain in this region until the end of wound healing. While macrophages contribute to wound debridement, they provide microbial stasis through oxygen radicals and nitric oxide synthesis (27,28). In our study, the macrophage count did not show a significant difference in day 3 groups ($p=0.286$). In day 7 groups, the macrophage count was significantly high in the NAM groups ($p=0.005$). It was seen to be significantly low in the RT groups ($p=0.048$). How macrophages decrease in number due to AM is not clear. Similar results to AM application have been reported that could decrease inflammatory cell proliferation and prevent inflammation this way in acute corneal alkaline burns, persistent corneal ulcers with different etiology and gingival ulcers in humans. This result has also remained an unexplained

question in the study of Uludağ et al.(11,13).

Fibroblasts appear in the late phases of normal wound healing. They are responsible for the formation of collagen and structural extracellular matrix. The submucosa is responsible for holding the sutures that keep the intestinal borders together (22). While the fibroblast amount was significantly low in the RT-applied groups on day 7, the fibroblast amount increased in the AM groups, although statistically insignificant.

In addition, studies of Kang (2012) and Kim (2015), who examined the biological behavior of AM, show the interactions between breast cancer cells and AM in favor of the patient and will guide them to demonstrate similar interactions with other cancer cells in the future (29,30).

This experimental model has limitations that are not consistent with practice. As known, neoadjuvant RT is applied to middle and lower rectum cancers, and performing a very low anastomosis is attempted in these patients. This technique is difficult to perform in rats as the rectum is short. Thus, the study was carried out with intraperitoneal anastomosis following left colon resection as in previous studies (2,14,24,25,27). In addition, one of the tissues used for anastomosis is irradiated, and the other side is healthy in clinical surgical practice.

One of the limitations of the study was the lack of biochemical evaluation supporting or rejecting the histopathological aspect. This assessment may demonstrate the effect of AM on soluble and insoluble collagen storage changes in anastomotic wound healing, providing more detailed information on its benefit or harm (16).

CONCLUSION

This study was based on the prediction that AM, which has been used in various repair techniques for years, could reduce the effects of RT on intestinal anastomosis (8). The anastomosis bursting pressures were seen to increase with AM, both in the RT and the NRT groups, although wound and anastomosis complications did not show a significant difference in the study groups. However, the in-vivo and in-vitro findings after RT were positive, but not clearly consistent. It was seen that the tissue adhesion of the AM was good.

We consider that AM, which has been used successfully in wound healing for years, has antibacterial and low immunogenic properties and is known to accelerate wound healing through enabling angiogenesis, could be used for the prevention of early phase anastomosis leakage in high risk patients with or without RT. This way, loop stoma, which brings additional social and surgical morbidity, may be avoided. Whether this speculation will be realized will be determined through experimental studies that determine the amount of AM and its effects on long-term outcomes.

Competing interests: The authors declare that they have no competing interest.

Financial Disclosure: There are no financial supports.
Ethical approval: The Animal Tests Local Ethics Committee approval from the DEUSM(Date 08.07.2011 No: 38/2011).

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