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ORIGINAL CONTRIBUTIONS

The Positive Effects of the Human Amniotic Membrane on the Healing of Staple Line After Sleeve Gastrectomy Applied Long-Evans Rat Model

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Abstract

Background The staple line leakage is a dangerous complication of sleeve gastrectomy. Various strategies have been tried to reduce the leakage risk. The amniotic membrane (AmM) is the inner layer of the placental membranes and has anti-inflammatory, anti-fibrosis, and anti-scarring effects, and it also has lower immune characteristics which are another essential characteristic of AmM concerning its utility for grafting. In this study, we aimed to investigate the impact of AmM on the staple line healing process of sleeve gastrectomy model in rats.

Materials and Methods We used twenty-eight Long-Evans rats in this study. Sleeve gastrectomy was performed with tristapler. Fourteen rats served as controls, AmM was applied staple line of the other fourteen. Fourteen animals were sacrificed (seven from the AmM applied group and the other seven from the control group) on the third postoperative day. And, the other fourteen animals were sacrificed (seven from the AmM applied group and seven from the control group) on the seventh postoperative day. The tissue around the staple line was evaluated microscopically and macroscopically, bursting pressures and hydroxyproline levels were also measured.

Results The bursting pressure and hydroxyproline measurements of the AmM applied group was significantly higher on the seventh postoperative day ($p = 0.015$, $p = 0.012$) Fibroblast activity and neoangiogenesis of the AmM applied group was also significantly higher on the seventh postoperative day ($p = 0.004$, $p = 0.002$).

Conclusion This study showed that covering of staple line of sleeve gastrectomy model in rats significantly provided higher bursting pressures and increased hydroxyproline levels, fibroblast activity, and neoangiogenesis which may potentially lead a better staple line healing. We think further investigations are needed on this issue.

Keywords Sleeve gastrectomy · Amniotic membrane · Staple line healing · Leakage · Complication

Introduction

Staple line leakage is a potentially disastrous complication of sleeve gastrectomy procedure with an incidence of 0–5.7% [1], which is most commonly located at the proximal part of the stapler line near the angle of Hiss and esophagogastric junction [2]. Surgeons have utilized different reinforcement strategies to diminish the stapler line leakage after laparoscopic sleeve gastrectomy by oversewing of the staple lines, by applying buttressing material and tissue sealants like absorbable polymer membrane, bovine pericardial strips, fibrin glue, or cyanoacrylate, and by modifying the distance from the pylorus and the bougie size [3–6].

The amniotic membrane (AmM) is the inner layer of the placental-fetal membranes with a 0.02–0.5 mm wall thickness

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which includes a monolayer epithelium, a non-vascular stroma, and a dense basal lamina (made up of type IV, V, and VII collagen). These structures further consist of three adjacent but separate layers: the innermost compact sheet, middle fibroblast sheet, and the outermost spongy layer [7]. The membrane has momentous anti-inflammatory, antimicrobial, antifibrosis, anti-scarring, and enhanced wound healing effects, and also has lower immunogenicity which is another essential characteristic of AmM concerning its utility for grafting. The benefits of AmM have already been identified in such clinical circumstances including extensive application in patients with burn injury, local control of infection by surgical covering, re-epithelization, and ocular surface reconstitution [8]. But the mechanism of its curative influence remains ambiguous. Researchers have stated various mechanisms to clarify anti-inflammatory effects of the membrane within the upregulation of apoptotic activity or downregulation chemotaxis of neutrophils and macrophages by inhibiting the synthesis of the inflammation activating cytokines, interleukin-1α, 1β and 6, Tnf-α, and interferon-γ, and by increasing the synthesis of anti-inflammatory proteins IL-1 receptor antagonist and cytokine IL-10. And, AmM also reduces the peroxidation of fatty acids [9]. This low immunogenic membrane is a source of many tissue growth factors and receptors [10]. Proliferative growth factors provoke the proliferation of endothelial cells, fibroblasts, and collagen formation in the acute wound healing, thereby abridge the proliferation time of the wound healing process [11].

Theoretically, staple line reinforcement should increase its strength and help decrease the incidence of complications associated with staple lines. Many studies have investigated the safety and efficacy of staple line reinforcement. However, the results of these studies remain inconsistent rather than conclusive. And, a limited number of studies have investigated the beneficial effects of AmM in terms of early and late stages of the healing process in gastrointestinal tract surgery [7–9]. In this study, we specifically aimed to investigate the impact of AmM in order to reinforce the staple line with an experimental sleeve gastrectomy model in rats.

Materials and Methods

Selection of the Animals The researchers used twenty-eight female Long-Evans rats with a mean weight of 367–396 g obtained from the Biological Experiments with Living Animals laboratory of Uskudar University, Istanbul, Turkey. The rats were maintained on a regular laboratory rat diet and tap water with a 12-h day/night light cycle at a room temperature of 24–26 °C. We formed four groups containing seven rats. The rats were housed as 2/per cage and allowed to mobilize freely.

Randomization of the rats and experimental design Randomization was achieved using computer-generated random numbers. For the study, four groups were formed as follows: SG3 ($n = 7$) sleeve gastrectomy (control group), sacrificed on the third day; SG7 ($n = 7$) sleeve gastrectomy (control group), sacrificed on the seventh day; SGAM3 ($n = 7$) sleeve gastrectomy plus amniotic membrane administration, sacrificed on the third day; SGAM7 ($n = 7$) sleeve gastrectomy plus amniotic membrane administration, sacrificed on the seventh day. Groups SG3 and SGAM3 were sacrificed on the third postoperative day and SG7 and SGAM7 were sacrificed on the seventh postoperative day to investigate the early and late healing characteristics of the anastomosis. The evaluated endpoints were gross anastomotic healing, adhesion formation, mechanical strength, hydroxyproline content, and parameters of histopathological healing.

Preparation of the Amniotic Membrane Human amniotic membrane was obtained from human placenta provided from an elective cesarean section delivery after testing the patient for hepatitis B, hepatitis C, HIV, and syphilis. The placenta was directly transported to the laboratory in a sterile container including physiologic saline solution at 4 °C. At the laboratory, the amnion was separated from the rest of the chorion by blunt dissection. It was rinsed and soaked in saline and Dakin's solution (0.25 sodium hypochlorite solution) for 10 min to remove the blood and other contaminations. It was then stored in a saline solution containing 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin, and 2.5 μg/ml amphotericin B for 10 min. Finally, it was placed on sterile nitrocellulose paper, with the epithelial surface facing up. The paper with the adherent membrane was then cut into segments with lengths of 20 mm and widths of 10 mm. The prepared amniotic membrane segments were stored at − 80 °C until the experimental study begins. The stapler lines were covered and sutured with the amniotic membrane after removing the nitrocellulose paper.

Surgical Procedure All rats were fasted for 12 h before surgery which was performed under sterile conditions by a surgeon who is uninformed (blind) about experimental groups. General anesthesia was administrated by sevoflurane liquid 100% (Abdi Ibrahim Medical Drugs, Istanbul, Turkey) inhalation during all surgical procedure. Next, the surgeon exposed the peritoneal cavity with a 4-cm midline abdominal incision. After exploration of the abdominal cavity, the surgeon planned excluded the animals with gastrointestinal anomalies and intra-abdominal tumors from the study, but none of the animals had this kind of abnormality. The surgeon identified the gastroesophageal junction and the pylorus of the stomach. Following up the identification of the landmarks, sleeve gastrectomy was performed with an endo-Gia (45 mm long, 2.5 mm height) white cartilage tri- stapler

(Covidien AG, Dublin, Ireland) from the distal stomach to the gastroesophageal junction (Fig. 1a). After the sleeve gastrectomy was completed, the amniotic membrane was fixed to the staple line with three sero-serosal sutures with 6/0 polypropylene surrounding the stapler line (Fig. 1b). In order to prevent the amniotic membrane fixation sutures from being extra advantageous, three of the same sutures were also placed on the staple line of the control groups. After gastric sleeve creation with and without amniotic membrane application, the surgeon closed the abdominal wall with 3/0 silk sutures (Dogsan Surgical, Istanbul, Turkey) and injected 15 ml sterile saline subcutaneously for postoperative hydration at the end of the procedure. Animals were fasted for postoperative 24 hours, after which they were initiated on a standard diet and drinking water.

Sacrification of Rats A veterinary who is an expert on biological experiments on animals sacrificed rats by using a guillotine.

Determination of Sacritification Days Collagen amount around the staple line is the result of a balance between matrix metalloproteinase-mediated collagenolysis of existing collagen and formation of de novo collagen. The matrix metalloproteinase proteins are synthesized by neutrophils and macrophages manage the collagen volume in the first third day of the wound healing process. On the fourth day of the healing process, new collagen synthesis reaches its maximum rate. Between the fifth and seventh days following wound formation, these new synthesized, weak collagen fibers provide the stability of the healing wound. For this cause, we chose the third (early stage) and seventh (late stage) days to analyze wound healing process in the presented experimental rat model.

Evaluation of Gross Healing For gross assessment of staple lines, after relaparotomy following sacrification, the abdominal cavity and the stapling lines were examined macroscopically; the integrity of the stapling lines, the existence of operative site abscess, the existence of intestinal obstruction, and the adhesion formation were recorded.

Evaluation of Operative Site Abscess Formation Operative site abscess formation was scaled as present or absent at necropsy.

Evaluation of Adhesions Around the Staple Line Postmortem examination was conducted to grade adhesions on a scale from 0 to 3 according to the method introduced by van Deer Ham et al. [12].

Measurement of Gastric Sleeve Bursting Pressure The uninformed (blind) surgeon made bursting pressure measurements within 3 min of sacrifice. First, the abdominal cavity and mediastinal cavity was exposed for complete visualization of the upper gastrointestinal tract including esophagus and stomach. The stapler line integrity was determined by exposing the stapling line and removal of the adhesions in the surrounding tissues. Next, two cuts, one from mid-esophagus, and the other were 2 cm distal to pylorus, which were made to obtain a full stomach segment including all stapler line. Content of the stomach was removed with physiologic saline solution. The distal end of the removed stomach segment was ligated with small titanium clip (Ethicon, Somerville, New Jersey, USA) after an infusion set connected to a sphygmomanometer was inserted into the proximal end of the esophagus. The surgeon put the excised part of the stomach in a beaker filled with a physiological saline solution and inflated with air. The intraluminal pressure was increased in 5-mmHg increments and maintained for 10 s. The appearance of air bubbles was used to record the bursting pressure in mmHg. Notably,

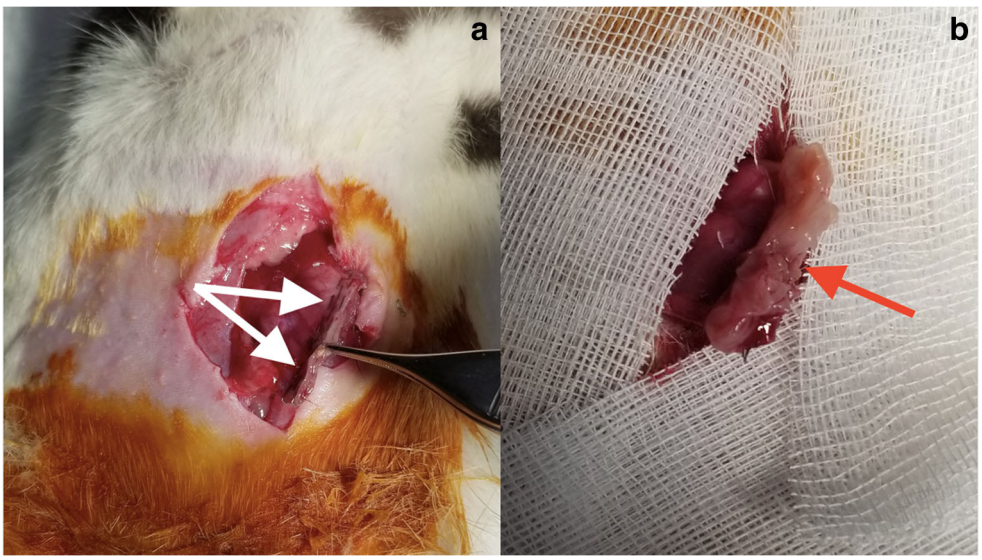

Fig. 1 **a** Gastric sleeve and staple lines. **b** Staple line after application of amniotic membrane

bursting occurred at the stapler line in all samples. The leakage or dehiscence on the stapler line was determined if the bursting pressure of the stomach was measured as 0 mmHg or if the significant findings of leakage/dehiscence (abscess formation in the peritoneal cavity) observed; these animals were excluded from the study. Next, the stomach was cut longitudinal axis and divided into two, one half was placed in 10% formalin solution for histopathological evaluation, and the other half was stored at − 80 °C for hydroxyproline measurement.

Histopathological Evaluation The 10% formalin-fixed stomach segments were sectioned, stained with hematoxylin and eosin, and analyzed with light microscopy at × 200 magnification by the same pathologist who was blinded to the treatment groups. Infiltration of inflammatory cells (leukocyte count), the activity of fibroblast cells, neoangiogenesis, and collagen content were measured using the modified Ehrlich and Hunt scale by Philips et al. [13].

Tissue Hydroxyproline Assay The tissue concentrations of hydroxyproline, which represent perianastomotic collagen levels, were measured using the Rat hydroxyproline ELISA Kit (MyBioSource, San Diego, California, USA). The values were expressed as nanogram amount of hydroxyproline per milliliter of tissue (ng/ml).

The Detection Principle of Rat Hydroxyproline ELISA Kit This experiment uses a double-sandwich ELISA technique and the ELISA Kit provided is typical. The pre-coated antibody is rat hydroxyproline monoclonal antibody, and the detecting antibody is a polyclonal antibody with biotin-labeled. Samples and biotin-labeling antibody are added into ELISA plate wells and washed out with 1.5 ml of phosphate-buffered saline. Then, avidin-peroxidase conjugates are added to ELISA wells in order, using tetramethylbenzidine substrate for coloring after reactant thoroughly washed out by phosphate-buffered saline. Tetramethylbenzidine turns into blue in peroxidase catalytic and finally turns into yellow under the action of the acid. The color depth and the testing factors in samples are positively correlated.

Power Analysis Power analysis was conducted by using G*Power (v3.1.9.2) program, to define the number of samples. The power of the study was confirmed as 1 (= probability of type II error) and was set at 80%. Based on the adhesion scores of previous studies [8], estimated from the level of intra-abdominal abscess formation, we determined our clinical significance at a minimum of 1 unit with 0.7 units of standard deviation (SD). The resultant calculated effect size was $d = 1.319$. To obtain 20% type II error (power is 80%) at = 0.05 level, it was decided to involve at least seven rats in each group.

Statistical Analysis For statistical analysis, the NCSS (Number Cruncher Statistical System) 2007 (Kaysville, Utah, USA) program was used. Descriptive statistical methods (mean, standard deviation, median, frequency, ratio, minimum, maximum) were used when evaluating the study data. The Mann-Whitney *U* test was used to compare two groups of data that did not show normal distribution; Kruskal-Wallis test and Bonferroni-Dunn test were used in double comparisons. Fisher-Freeman-Halton exact test and Fisher's exact test were used to compare the qualitative data. Wilcoxon-signed ranks test was used for intragroup comparisons of non-normally distributed parameters. Significance was evaluated at least $p < 0.05$.

Ethical Approval All procedures in the study performed by following the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 83-26, revised 1985, Bethesda, MD, USA) and the Guidelines of the Animal Welfare Act. And, the Uskudar University, Board of Biological Experiments with Living Animals approved the experimental protocol (April 26, 2018–15).

Results

The weights of the rats while the beginning of the experiment ranged between 367 and 396 g (380.57 ± 8.35).

Macroscopic Evaluation There is no statistical difference noticed according to groups on the macroscopic evaluation of anastomosis dehiscence, abscess formation in the peritoneal cavity. Moreover, adhesion score of SGAM groups is lower than SG groups on day 3 and day 7 ($p = 0.048$; $p = 0.003$, respectively) (Table 1).

Bursting Pressure and Hydroxyproline Values Measurements of the bursting pressures of the anastomoses are demonstrated in Table 2. Significant anastomotic leakage suggestive findings (gastrointestinal content contamination in the abdominal cavity, anastomotic dehiscence) were not observed. The bursting pressure measurements of the SGAM7 group were significantly higher than the SG7 group ($p = 0.015$; $p < 0.05$). The hydroxyproline measurements of the SGAM/group were significantly higher than the SG7 group ($p = 0.012$; $p < 0.059$). The bursting pressure and hydroxyproline measurements of SGAM3 were higher than the SG3 group, but there was no statistically significant difference ($p = 0.214$) (Table 2).

Histological Evaluation The histological evaluation measurements are shown in Table 3. Inflammatory cell infiltration rate of SGAM7 was significantly lower than SG7 ($p = 0.013$;

Table 1 Macroscopic evaluation (evaluation of anastomosis dehiscence, intra-abdominal abscess, and adhesion score)

			SG ($n = 7$) n (%)	SGAM ($n = 7$) n (%)	p
Anastomoses dehiscence	Day 3	No	4 (57.1)	5 (71.4)	[a]1.000
		Yes	3 (42.9)	2 (28.6)	
	Day 7	No	6 (85.7)	7 (100)	[a]1.000
		Yes	1 (14.3)	0 (0)	
		[a]p	0.559	0.462	
Intra-abdominal abscess	Day 3	No	5 (71.4)	4 (57.1)	[a]1.000
		Yes	2 (28.6)	3 (42.9)	
	Day 7	No	5 (71.4)	7 (100)	[a]1.000
		Yes	2 (28.6)	0 (0)	
		[a]p	1.000	0.192	
Adhesion score	Day 3	1	0 (0)	1 (14.2)	
		2	0 (0)	3 (42.8)	
		3	3 (42.9)	2 (28.6)	
		4	4 (57.1)	1 (14.2)	
	Median (Q1–Q3)		4 (3–4)	2.5 (2–3.25)	[b]*0.048**
	Day 7	1	1 (14.2)	5 (71.4)	
		2	1 (14.2)	2 (28.6)	
		3	3 (42.8)	0 (0)	
		4	2 (28.6)	0 (0)	
	Median (Q1–Q3)		3 (2.75–4)	1 (1–2)	[b]*0.003***
		[b]p	0.302	*0.006***	

[a] Fisher's exact test

[b] Mann-Whitney U test

*$p < 0.05$

**$p < 0.01$

$p < 0.05$). Fibroblast activity of SGAM7 is significantly higher than SGAM3 ($p = 0.004$; $p < 0.05$). Also, neoangiogenesis in SGAM7 is significantly higher than SG7 and SGAM3 ($p = 0.002$; $p = 0.018$, respectively, $p < 0.05$).

Discussion

Bariatric surgery has proven to be the most beneficial long-term therapy for obesity [14, 15], and sleeve gastrectomy is one of the most performed operations for morbid obesity. Although SG has recently taken the place of the Roux-en-Y gastric bypass as the most preferred bariatric surgical procedure, staple line leakage has been propagated as the most dangerous complication with varying rates between 0 and 8% [2, 16, 17]. The upper third of the staple line is the specific leakage site in 89.9% of all cases [2]. This complication is the second most common cause of mortality (0–1.4%) after a bariatric surgical procedure [18]. The wound strength is fragile in the beginning days following surgery. Furthermore, the peril of wound dehiscence is highest throughout the first postoperative days, even if the operation is implemented under optimal circumstances [19]. Various determinants contribute to this healing process, such as adequate blood perfusion, the tension on the staple line, the patient's comorbidities, and progression rate of inflammation [20]. Staple line reinforcement has been suggested to minimize the risk of leakage and can be obtained with different alternatives: oversewing the staple line with a running absorbable suture, buttressing it with specific materials or roofing the staple line [21]. But the use of a biological material to ensure the strength of the staple line of the gastric sleeve is a new idea. The AmM can be easily obtained and is widely used as an acellular biological material by ophthalmologists and urologists as a reconstruction material in operations for a long time [2, 22]. In the presented study, sleeve gastrectomy with 2.5-mm height staple, from the distal stomach to gastroesophageal junction model, was used to study whether the AmM can be useful in preventing the staple line leakage. The results revealed AmM decreased the inflammatory cell infiltration and improved neoangiogenesis, as well as the hydroxyproline concentrations in the tissue nearby the staple line at the late stages of wound healing. The bursting pressure values were improved significantly. The exact mechanism by which the AmM stimulates wound healing needs to be further investigated.

Table 2 Bursting pressures assessment verus hydroxyproline measurements

		SG ($n = 7$)	SGAM ($n = 7$)	[b]p
Bursting pressure				
Day 3	Min-max (median)	0–30 (10)	0–30 (22.5)	0.214
	Ort ± Ss	10.71 ± 11.70	19.17 ± 12.01	
Day 7	Min-max (median)	120–180 (140)	140–300 (240)	*0.015**
	Ort ± Ss	148.57 ± 22,68	234.29 ± 65,03	
	[b]p	*0.001***	*0.003***	
Hydroxyproline measurement (ng/ml)				
Day 3	Min-max (median)	0.01–2.61 (0.64)	0.04–3.19 (0.51)	0.886
	Ort ± Ss	0.93 ± 0.85	1.21 ± 0.90	
Day 7	Min-max (median)	0.002–1.46 (0.15)	0.06–12.89 (1.40)	*0.012**
	Ort ± Ss	0.57 ± 0.41	4.60 ± 2.69	
	[b]p	0.225	0.317	

[b] Mann-Whitney U test

*$p < 0.05$

**$p < 0.01$

The dehiscence of the staple line is the most reliable marker of future complications which may cause mortality and morbidity and poor long-term outcome [23]. In our study, the staple line leakage rate in the SG3 and SG7 groups increased to 42.9% and 14.3% respectively, where the rate mentioned above was not significantly high when compared to the SGAM3 and SGAM7 (14.3% and 0%, respectively) groups. Since the staple line surface strength is very limited in the first days of surgery, the risk of anastomotic dehiscence is of importance utmost, even if the surgical process is performed under optimal circumstances [13]. Therefore, the early days of surgery are critical for cut surface healing. Many published data presented, however, that covering high-risk anastomoses or cut surfaces with AmM makes significant decreases in the dehiscence rates [8, 23].

In daily clinical practices, the physical strength is not an ideal enough parameter to assess the healing of staple line; nevertheless, bursting pressure can be used as an indirect method to analyze mechanical strength of the staple line [24]. The mean value in the SGAM3 and SGAM7 groups was higher than the SG3 and SG7 groups, and the difference was statistically significant on the seventh day in the presented study. Tissue damage and wound healing process head to the extensive itemization of collagen and this temporarily reduced total collagen concentration could be a significant contributing factor for anastomotic dehiscence/leakage. Determining the degree of stabilizing crosslinks between collagen molecules may also give an idea for structural support of the healing tissue. Hydroxyproline measurement is a way to assess the stability of collagen [25]. The mean hydroxyproline concentrations in the SGAM3 and SGAM7 groups were higher than the SG3 and SG7 groups, and the difference was statistically significant on the seventh day. Collagen amount around the staple line is the result of a balance between matrix metalloproteinase-mediated collagenolysis of existing collagen and formation of de novo collagen. Study of Koob TJ et al. advocated that human AmM has matrix metalloproteinase-inhibiting factors which may reduce the degradation of existing collagen [26]. Rinastiti et al. also showed the collagen increasing effect of the AmM [27]. In the presented study, collagen content in the group SGAM 7 is higher than SG7. Increased collagen and hydroxyproline concentration and improved bursting pressure measurements show AmM improves the staple line healing process in experimental sleeve gastrectomy model, thus enhances the mechanical strength of the staple line.

Neutrophils are the earliest migrating cells into the wound during the inflammation. Neutrophils also have an essential responsibility in the process of wound healing [28]. Inflammatory mediators such as TNF-α, IL-1, IL-6, and reactive oxygen metabolites located around the wound site stimulate neutrophils at the beginning of the wound healing process [29]. These inflammatory mediators are found in systemic circulation and even in higher concentrations in peritoneal exudate [30]. Rico et al. proved that extreme inflammation weakens and delays intestinal healing [31]. Since the inflammatory and chemoattractant characteristics of the wound healing process, stimulated neutrophils in the systemic circulation and the intraperitoneal cavity quickly grow around the wound surface, hence amplifies the inflammatory reaction [32]. In our study, it is demonstrated that SGAM7 has lower inflammatory cell infiltration than SG7. Many studies showed the anti-inflammatory influence of AmM which reduces the inflammatory cell infiltration [33, 34]. Specific semipermeable composition of the AmM also blocks the invasion of inflammatory cells and cytokines and allows diffusion of

Table 3 Evaluation of inflammatory cell infiltration, fibroblast activity, neoangiogenesis, collagen deposition

			SG ($n = 7$) n (%)	SGAM ($n = 7$) n (%)	[b] p
Inflammatory cell infiltration	Day 3	1	1 (14.3)	1 (14.3)	
		2	4 (57.1)	3 (42.9)	
		3	2 (28.6)	3 (42.9)	
	Median (Q1–Q3)		2 (2–3)	2.5 (2–3)	0.335
	Day 7	1	1 (14.3)	1 (14.3)	
		2	0 (0)	4 (57.1)	
		3	6 (85.7)	2 (28.6)	
	Median (Q1–Q3)		3 (3–3)	2 (2–3)	*0.013**
		[b] p	*0.013**	0.335	
Fibroblast activity	Day 3	1	4 (57.1)	6 (85.7)	
		2	3 (42.9)	1 (14.3)	
		3	0 (0)	0 (0)	
	Median (Q1–Q3)		1 (1–2)	1 (1–1.25)	0.327
	Day 7	1	2 (28.6)	0 (0)	
		2	4 (57.1)	5 (71.4)	
		3	1 (14.3)	2 (28.6)	
	Median (Q1–Q3)		2 (1.75–2.25)	2 (2–3)	0.379
		[b] p	0.108	*0.004***	
Neoangiogenesis	Day 3	1	4 (57.1)	5 (71.4)	
		2	2 (28.6)	2 (28.6)	
		3	1 (14.3)	0 (0)	
	Median (Q1–Q3)		1 (1–2)	1 (1–2)	0.619
	Day 7	1	3 (42.9)	0 (28.6)	
		2	4 (57.1)	2 (28.6)	
		3	0 (16.7)	5 (71.4)	
	Median (Q1–Q3)		2 (1.75–2.25)	4 (2.5–4)	*0.018**
		[b] p	0.244	*0.002**	
Collagen deposition	Day 3	0	0 (0)	2 (28.6)	
		1	7 (100)	3 (42.9)	
		2	0 (0)	2 (28.6)	
	Median (Q1–Q3)		1 (1–1)	1 (0.75–2)	0.498
	Day 7	0	5 (71.4)	3 (42.9)	
		1	2 (28.6)	2 (28.6)	
		2	0 (0)	2 (28.6)	
	Median (Q1–Q3)		1 (1–2)	2 (1–3)	0.268
		[b] p	0.111	0.194	

[b] Mann-Whitney U test

*$p < 0.05$

**$p < 0.01$

oxygen to the wound area [34]. This peculiarity of AmM inhibits the local effects of over-activated inflammatory cells and exaggerated inflammation cytokines in the peritoneal cavity. Besides, AmM owns antimicrobial attributes and decreases the bacterial counts by the antibodies and lysozyme enzyme found in its structure and supports the healing of infected wounds [8]. Neoangiogenesis is a highly active part of wound healing that is harmonized by different kinds of growth factors and many various mediators. Studies prove that human AmM includes a vast amount of angiogenic growth factors such as angiogenin, angiopoietin-2, EGF, bFGF, HB-EGF, HGF, PDGF-BB, PlGF, and VEGF which are vital determinants of new blood vessel generation required for endothelial cell migration [26]. Impaired neoangiogenesis process

causes hypoxia, and thus impaired wound healing. It is clearly shown that the application of VEGF to chronic diabetic wounds improves neoangiogenesis and wound healing [35]. In the current study, new vessel formation is significantly higher in amniotic membrane applied group on the seventh day. But in contrast with many studies including the presented one, corneal transplantation of AmM diminishes the neovascularization process by reducing FGF [36, 37]. This discrepancy implies that the AmM shows different behavioral characteristics according to the anatomical site in which it is transplanted. However, this issue would be clarified in further, detailed, perhaps molecular or cellular studies.

Despite all the potential positive effects, the widespread use of the AmM in the clinic remains a challenge because of the high cost for preserving freshly prepared AmM [38]. AmM can be frozen at − 80 °C and stored for use up to 6 months. However, facility of freezing at − 80 °C is only available in research laboratories and is not readily available in hospitals where AmM may be required at a short notice. But, the results of the study of Ashraf et al. showed storage of amniotic membrane for 6 weeks at − 20 °C which is available most hospital blood banks is as safe and efficacious as that at − 80 °C [39]. At − 20 °C, storage may lead to commercial use of the AmM in future times.

In conclusion, the presented experimental study revealed that covering of sleeve gastrectomy staple line with AmM significantly prevented the destructive effects of staple line leakage and granted a safer and healthier staple line than stapling alone through the late phases of wound healing in sleeve gastrectomy procedure. Although further investigations are needed to clarify the mechanism of AmM's ability in healing the sleeve gastrectomy staple line, our results show a promising future for the clinical use of AmM to enhance the safety of sleeve gastrectomy procedure.

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Authors' Contributions MFF, TK, KS, and SI collected the information, reviewed the literature, and wrote the manuscript. AcK, GV, and MFF collected the information. AIF and AK critically reviewed the manuscript and approved the final form. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures in the study performed by following the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 83-26, revised 1985, Bethesda, MD, USA) and the Guidelines of the Animal Welfare Act. And, the Uskudar University, Board of Biological Experiments with Living Animals approved the experimental protocol (April 26, 2018–15).

Informed Consent Statement For this type of study formal consent is not required.

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