Journal of Plastic Surgery and Hand Surgery

ISSN: 2000-656X (Print) 2000-6764 (Online) Journal homepage: https://www.tandfonline.com/loi/iphs20

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To cite this article: Burak Sercan Ercin, Ahmet Bicer, Gurkan Yigitturk, Celal Cinar, Turker Cavusoglu, Yigit Uyanikgil & Tahir Gurler (2019) Who needs extra time? Amniotic membrane wrapped pauci-suture model for rapid anastomoses*, Journal of Plastic Surgery and Hand Surgery, 53:2, 89-96, DOI: 10.1080/2000656X.2018.1550419

To link to this article: https://doi.org/10.1080/2000656X.2018.1550419

Published online: 18 Jan 2019.

JOURNAL OF PLASTIC SURGERY AND HAND SURGERY
2019, VOL. 53, NO. 2, 89–96
https://doi.org/10.1080/2000656X.2018.1550419

ARTICLE

Who needs extra time? Amniotic membrane wrapped pauci-suture model for rapid anastomoses*

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ABSTRACT

An ideal anastomosis method will obtain the highest post-anastomotic vessel patency and will repair the vessel anatomically with minimal thrombosis in an easier, faster and cheaper fashion. To achieve these goals an anastomosis model using an amniotic membrane is introduced. The study was performed on the femoral arteries of 22 Wistar Albino rats (11 control group, 11 experimental group). In the experiment group, the microvascular anastomosis was completed with three sutures and a patch of amniotic membrane which was wrapped around the anastomotic site. The conventional anastomosis technique with eight sutures was performed in the control group. The effects of the model on the patency and histological structure of the vessels were evaluated. As a result, normal patency was determined radiologically and macroscopically in all of the anastomoses. No thrombosis or aneurysm was detected in any of the anastomoses. In the angiographic study, vessel patency was detected in both the control and experimental groups. The average time to complete the arterial anastomosis was 18.14 (±2.84) and 10.39 (±2.45) minutes in the control and the experiment groups respectively. In the histological studies, anti-eNOS staining revealed that endothelin levels were significantly higher in the experimental group. This method describes a new anastomosis model in microvascular surgery with promising results that call for additional experimental studies and further clinical implementations. We believe that this experimental technique can be put into clinical practice as an alternative to the conventional microvascular anastomosis technique.

ARTICLE HISTORY
Received 3 September 2018
Revised 5 November 2018
Accepted 12 November 2018

KEYWORDS
Microsurgery; amniotic membrane; anastomosis; experimental; anastomosis model

Introduction

Hundreds of anastomosis techniques have been described since the first microvascular anastomosis performed by Alexis Carrel [1,2]. The main emphasis has traditionally been on the speed, practicality and reliability of the anastomoses. A good anastomotic technique can be described as quick, atraumatic and easy to perform in order to limit ischemia time.

The microanastomosis technique is very well-established and is used in many centers as the gold standard. However, especially in the hands of inexperienced surgeons, it may turn out to be a time-consuming method. Moreover, every stitch passed through the vessel wall exerts an independent risk of starting the coagulation cascade. Therefore, the ultimate goal of many studies is to find a more practical and reliable method of anastomosis, including wrapping the anastomotic site with biologic or non-biologic sheaths. Though they generally hit the mark with necessitating the fewest number of stitches, they are typically used with an expensive tissue adherent as an adjunct to work effectively [3].

Amniotic membrane, as a cheap and readily available biological tissue, has proven to be beneficial in wound healing, ophthalmology, burns and nerve healing, as it contains many growth factors [4]. A search of the current literature reveals that amniotic membrane has many applications in plastic surgery. However, to the best of our knowledge, utilization of amniotic membrane as an adjunct in vascular anastomoses has not been studied before.

Our study is based on investigating the effects of amniotic membrane-aided vascular anastomosis on the vessels and the zone of tissue which the vessel in question perfuses, in an *in vivo* rat model utilizing histochemical, immunohistochemical and morphologic parameters.

Material and methods

Hypothesis

The hypothesis to be tested in this study was whether or not amniotic membrane is beneficial in vascular anastomoses performed with less (three) than the standard number of stitches (6–8), ending up with an anastomosis at least as patent as in the conventional anastomosis group in a shorter duration of time. An end-to-end anastomosis was performed on the rat femoral artery in the test and control groups. In the experimental group, a rolled sheath of amniotic membrane was planned to be wrapped

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*Details of presentations: 4th Asia-Pacific Reconstructive Microsurgery Congress - May 2018.

around the anastomotic line and completed with three stitches, while a conventional eight stitch anastomosis was performed in the control group. The intent was to compare the groups using objective parameters and methods.

Amniotic membrane preparation

Prior to operation, donors were screened for HIV, Hepatitis B and Hepatitis C. Human amniotic membrane was separated from the placenta and placed into the physiologic serum. The chorion layer was cleared. Amniotic membrane was cut with nitrocellulose membranes to fit 100 mm diameter sterile Petri dishes (Thermo Scientific, Waltham, MA). It was prepared by stretching it to the appropriate motility inserts prior to use in a six-well cell culture dish (Thermo Scientific, Waltham, MA). The prepared amniotic membrane was treated with 40 mM EDTA (Sigma, St. Louis, MO) for 120 min. The epithelium of the amniotic membrane was removed mechanically and treated in DMEM-F12 (Sigma, St. Louis, MO) solution at 37° C, 90% humidity and 5% CO_2 in an incubator overnight.

Surgical method

The study design had been planned to be performed on three groups and the three suture anastomosis model without amniotic membrane was initially part of the study. However, due to excessive bleeding from the anastomosis sites, the first two rats were lost immediately after anastomoses. Due to ethical concerns and the aim of reducing rat sacrifice, the third group was excluded from the study.

In the study group comprised of 11 male Wistar Albino rats, an oblique volar incision was made over the femoral triangle with the subjects in the supine position. The femoral arteries and veins were exposed and freed, while the branches encountered between the inguinal ligament and epigastric branches were cauterized. The artery and vein were carefully split off from each other. The femoral artery was freed further upon cauterizing the Murphy branch. Amniotic membranes were cut into 1×1 cm patches. Following the application of Acland type approximators, the vessels were sharply cut, and sufficient adventitiectomy was made. The lumens were flushed with heparin solution followed by delicate dilation with forceps. At this point, the timer was started. Three 10/0 Ethilon (Ethicon, Sommerville, NJ) stitches were made at 0, 120 and 240-degree positions to anastomose the vessel ends (Figure 1).

The prepared amniotic membrane patches were wrapped around the anastomotic site in a rolling fashion with utmost care taken for the smooth side (fetal side) to embrace the vessel (Figure 2). Due to its adhesive nature, the loose edge of amniotic membrane stick on itself and no stabilization methods (suture, fibrin glue etc.) were used.

The vessels were sprinkled with 1% lidocaine solution. Following the release of the approximator clamps, the epigastric fat pad was transposed upon the vessel. After 30 min of waiting, the anastomoses were tested with milking and tilting tests for detection of early failures. The inguinal incisions were closed with double running 4/0 Ethilon (Ethicon, Sommerville, NJ) sutures.

On the 21st postoperative day, the anastomoses were explored under general anesthesia using intramuscularly administrated ketamine HCl 100 mg/kg (Ketalar, Parke Davis, Morris Plains, NJ) and xylazine 10 mg/kg (Rompun, Bayer, Leverkusen, Germany).

Following repetition of initial patency tests, dynamic angiography was performed upon administration of intracardiac radiopaque solution (0.2 cc Gadopentetate Dimeglumine) (Magnevist, Bayer, Leverkusen, Germany). The femoral arteries and veins, including the anastomotic sites were biopsied while the rats were still alive. After biopsies, the rats were confirmed deceased.

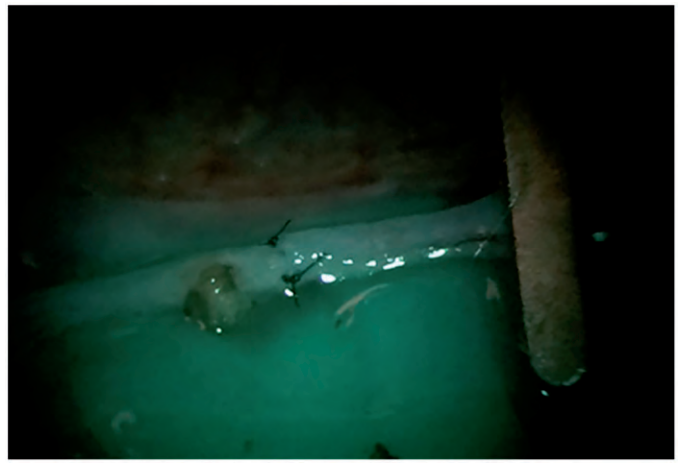

Figure 1. Three 10/0 nylon stitches were put at 0, 120, and 240 degrees positions to anastomose the vessel ends.

In the control group ($n = 10$, one rat was excluded due to perioperative cardiac arrest and eventual death), after standard (as in the study group) exploration and preparation, a conventional (0, 120 and 240 degree fashion) end-to-end eight-stitch anastomosis with 10/0 Ethilon (Ethicon, Sommerville, NJ) sutures was made at the left femoral arteries. Immediate postoperative patency was checked. After 21 day follow-up, patency tests were repeated. As in the study group, dynamic angiographies were performed and biopsies were obtained before the demise of the rats.

Results

The duration records of anastomoses are presented in Figure 3.

The distribution of the data was tested using the Kolmogorov–Smirnov test, which subsequently revealed normal distribution.

In the control group, mean anastomosis duration was 18.14 ± 2.84 min (minimum 15 min, 12 s; maximum 22 min, 8 s). In the study group, mean duration was 10.39 ± 2.45 min (minimum 8 min, 4 s; maximum 16 min, 8 s). The two groups were compared with each other utilizing the Student's *t*-test regarding the anastomosis duration. The difference between the groups was found to be statistically significant ($p < .001$) (Figure 4).

The patency of the vessels was clinically assessed by milking and lifting tests at post-anastomosis time 30 min (Figure 5) and again at postoperative day 21. The objective tests were performed on the 21st postoperative day via dynamic angiography. Statistical analyses were carried out using the chi-square (Fisher's exact) test, which yielded no statistically significant difference ($p = 1.000$) (Table 1). The patency rates between the groups at postoperative 30 min and 21 days were also not statistically significantly different ($p > .05$).

Histologic examinations were carried out on femoral artery/vein en-block biopsies taken on postoperative day 21. Macroscopically (and subjectively), dissection of the study group surgical sites was markedly easier. The statistical comparison of the groups regarding histological findings was carried out using the Kruskal–Wallis, and if any significant difference was

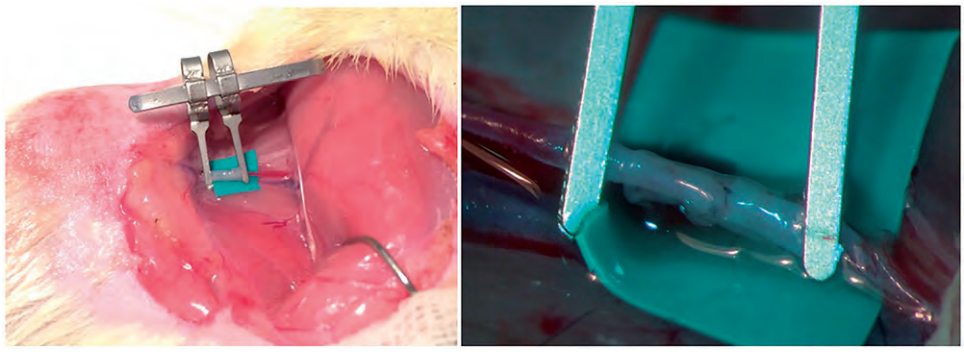

Figure 2. The prepared amniotic membrane patches were wrapped around the anastomotic site in a rolling fashion with utmost care been taken for the smooth side (fetal side) to embrace the vessel.

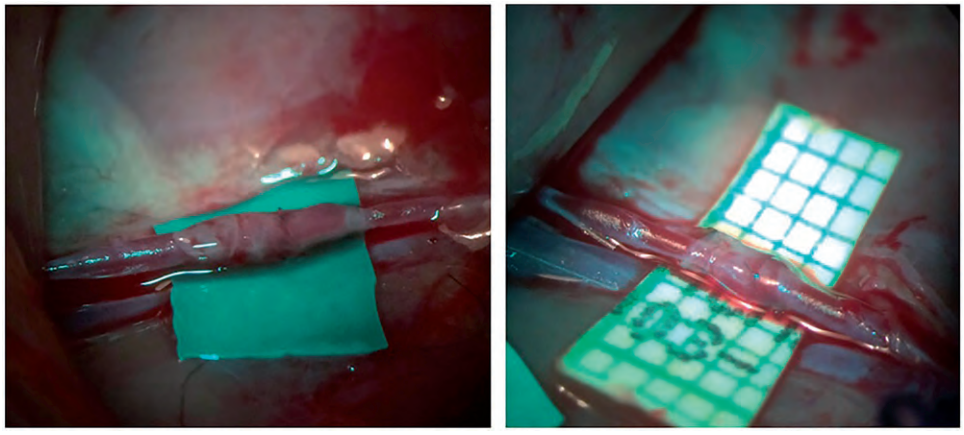

Figure 3. Thirty minutes after finishing anastomoses by using amniotic membrane vessel patency was seen.

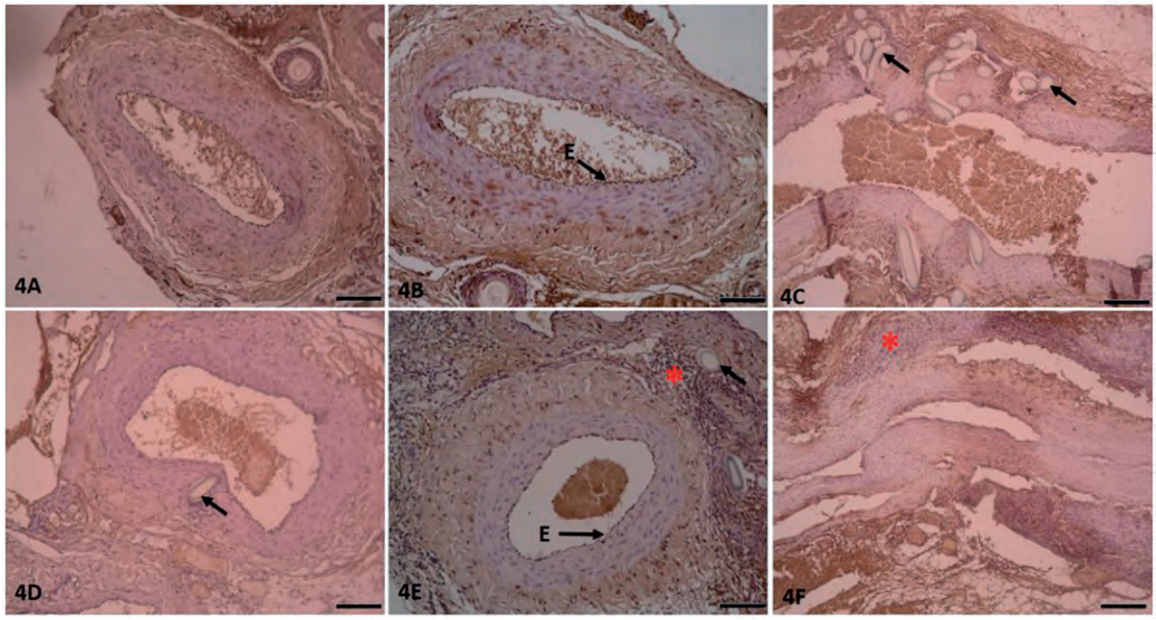

Figure 4. (A) Conventional group anti-eNOS staining, transverse tissue section ***Tm*** Tunica muscularis, ***L*** Lumen (×20 magnification, scale bar =250 μm). (B) Conventional group anti-eNOS staining, transverse tissue section ***E*** Endothel, ***yellow arrow*** Membrana elastica interna, ***L*** Lumen (×20 magnification, scale bar =250 μm). (C) Conventional group anti-eNOS staining, longitudinal tissue section ***L*** Lumen, ***black arrow*** Surgical suture materials. (D) Experiment group anti-eNOS staining, transverse tissue section, ***Tm*** Tunica muscularis, ***L*** Lumen (×20 magnification, Scale bar =250 μm). (E) Experiment group anti-eNOS staining, transverse tissue section ***E*** Endothel, ***yellow arrow*** Membrana elastica interna, (×20 magnification, Scale bar =250 μm). (F) Experiment group anti-eNOS staining, longitudinal tissue section. ***E*** Endothel, ***black arrow*** Surgical suture materials, ***red arrow*** *PMNL infiltration* (×20 magnification, Scale bar =250 μm).

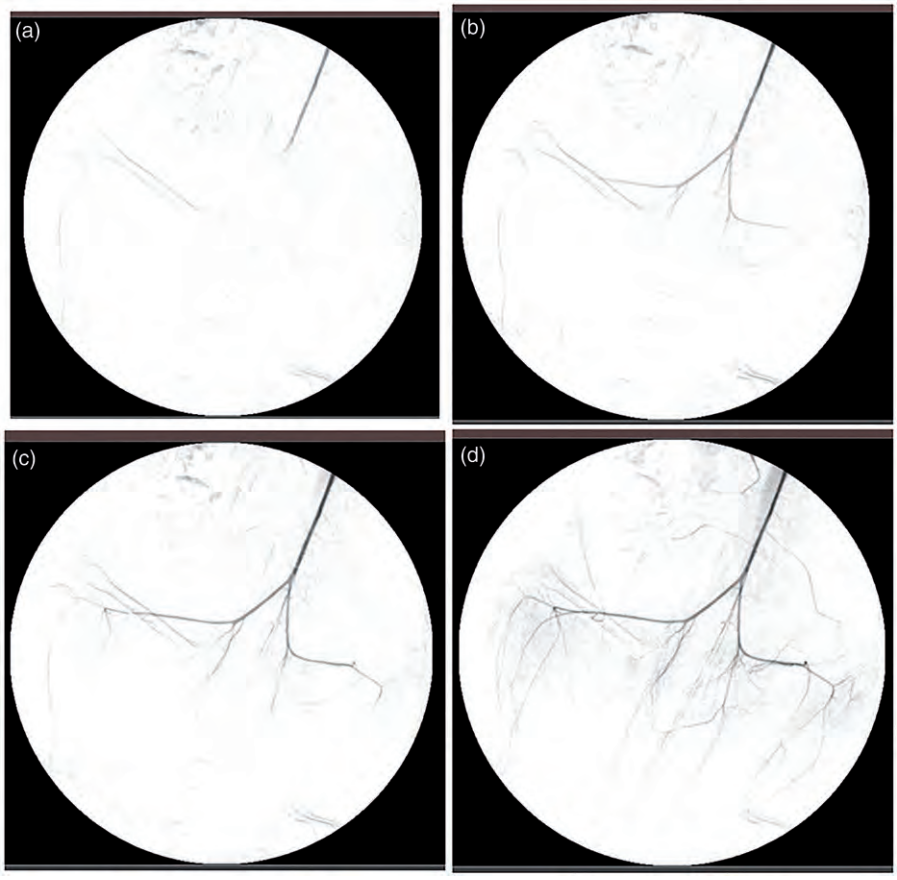

Figure 5. Dynamic angiography of left-sided anastomose from the conventional group.

Table 1. Patency rates of groups in terms of days.

	Conventional (*n*:10)	Experiment (*n*:11)	*p* value
30th minute			1.000
Patency (+)	10	11	
Patency (−)	−	−	
21st Day			1.000
Patency (+)	10	11	
Patency (−)	−	−	
Total			1.000
Patency (+)	10	11	
Patency (−)	−	−	

encountered, the Mann–Whitney *U*-Test was performed to break down the groups to compare two independent samples.

The histologic examinations were carried out by hematoxylin and eosin, and Mallory-azan stains, revealing identical intact luminal structures in both groups. The study group presented with less granulomatous tissue reaction due to fewer stitches.

A gradient method was used for scoring expressions of endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) (0–5). In the experimental group, the mean value of anti-VEGF staining intensity was 3.7 (minimum 3–maximum 5), while in the conventional group the mean value was 3.6 (minimum 3–maximum 5). Tissue VEGF expression was tested using anti-VEGF stains and found to be similar in both groups ($p > .001$) (Figure 6).

In terms of eNOS immunoexpression assessment in the experimental group, the mean value of anti-eNOS staining intensity was 4 (minimum 3–maximum 5). In the conventional group, the mean value was 2.9 (minimum 2–maximum 4). In the experimental group, eNOS levels were found to be increased compared to the conventional group. This increase was statistically significant ($p \leq .01$) (Figures 7,8).

After intracardiac infusion of 0.2 cc radio-opaque substance (Gadopentetate Dimeglumine) (Magnevist, Bayer, Leverkusen, Germany) in the left ventricle of the subjects on the 21st postoperative day, dynamic angiographic images were obtained (Figures 9,10).

Angiographic images revealed a patent femoral vascular tree, including the epigastric artery without any signs of pseudoaneurysm.

Discussion

A good anastomosis should basically have the following characteristics: quick, atraumatic and practical.

Most studies concerning vascular anastomosis focus on the search for an easier, speedier and more reliable technique. This search raises the question of their validity, as patency rates have reached up to 98% in free flap surgery [5].

Increasing demands on reconstructive microsurgeons in search of more reliable and faster anastomoses on smaller vessels are seen every day. In conjunction with 'supermicrosurgery' or 'supramicrosurgery' concepts, such as perforator to perforator anastomoses, free digit pulp transfers, tip replantations and lymphaticovenous anastomoses, there is a need for refined techniques and aids [6,7].

The major disadvantages of the conventional anastomotic technique, despite still being considered the gold standard, are as follows: being time-consuming, especially in the hands of inexperienced surgeons; being painstaking, especially in difficult surgical positions; having deep surgical fields and difficulty; and having risks with every pass of suture needles on tiny vessels [8–13]. As Acland et al. demonstrated, the granulomatous reaction

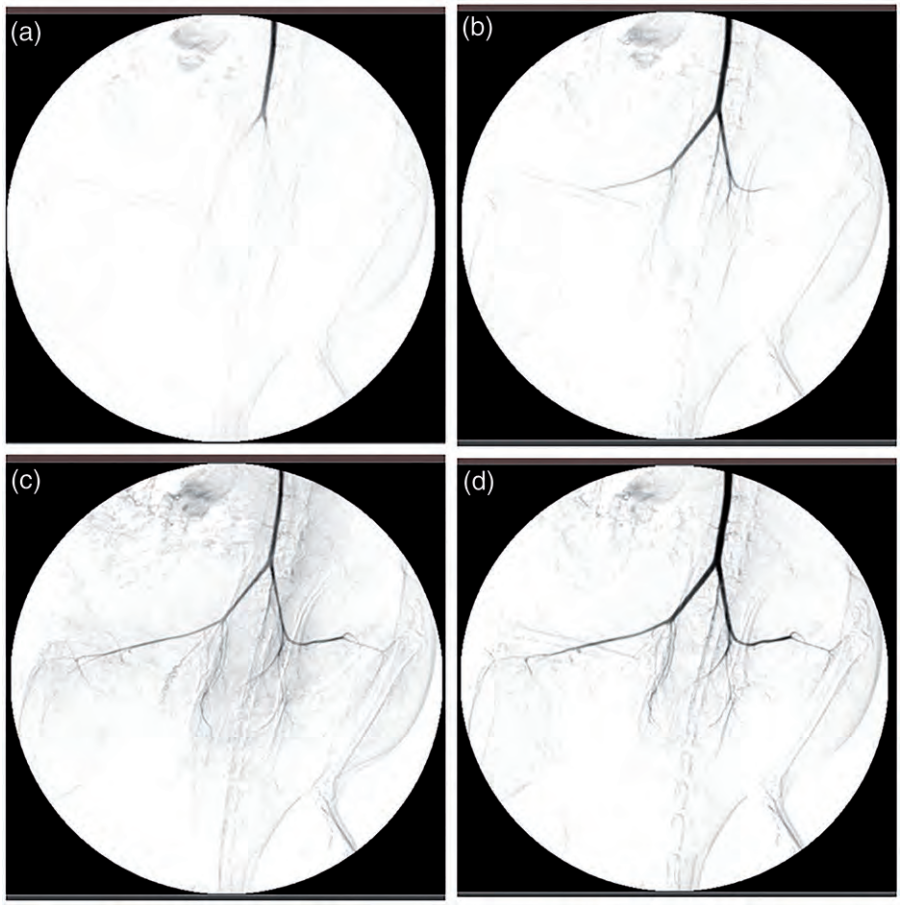

Figure 6. Dynamic angiography of left-sided anastomose from experiment group.

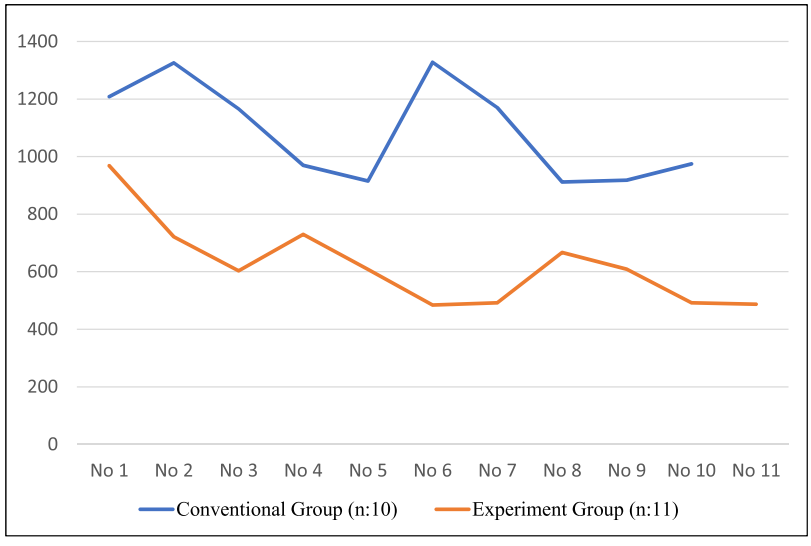

Figure 7. Durations of Anastomoses (seconds).

around every surgical knot tied around a vascular anastomosis suggests inadvertent damage to the tunica media and intima, despite the utmost care being taken to avoid damage [11].

Cost-effectiveness is another point to consider, especially in cases demanding multiple anastomoses. An additional consideration is the mishandling of delicate and expensive sutures in the hands of inexperienced microsurgeons [11]. Cutting these costs can only be achieved by reducing the need for suture material.

Another point to consider is decreasing the ischemia time in replantations [14]. This is especially critical as more and more critically severed extremities are being considered for revascularization, regardless of the level of injury and distance between the

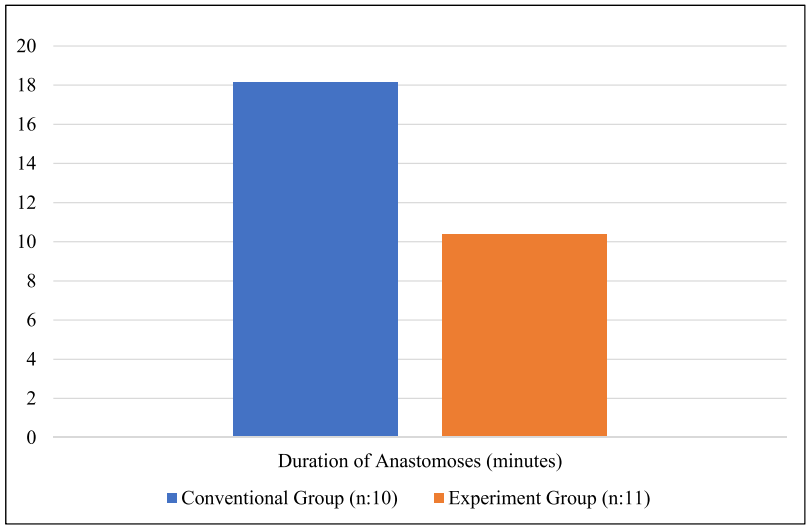

Figure 8. Comparison of the experiment and conventional group durations ($p < 0.001$).

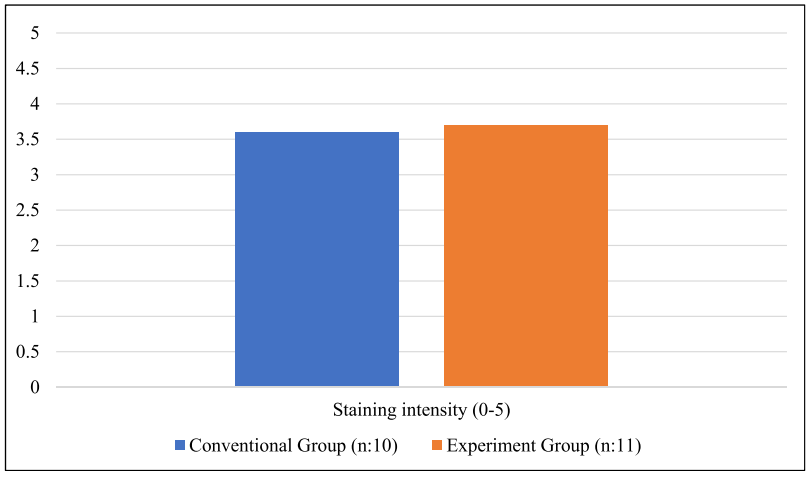

Figure 9. Relative intensity of Anti VEGF staining.

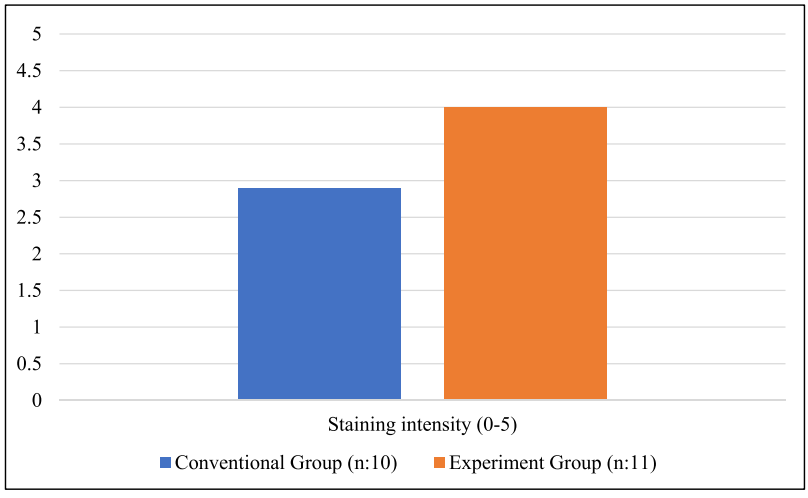

Figure 10. Relative intensity of Anti e-NOS staining.

injury location and a microsurgery center. The ischemia time is also a major critical factor in the success of composite tissue transfers.

Our study group consists not only yielded shortened anastomosis time but also every given anastomosis was completed with significantly fewer sutures.

Histologic studies demonstrated complete resurfacing of the endothelial lining. This is congruent with previous studies by Acland et al. who demonstrated completion of the endothelial lining by the 21st postoperative day following anastomosis [11].

The granuloma formation around the control group vascular anastomosis site should be considered as a direct consequence of the intensive use of suture material. In their study, Suguria et al. revealed that hyaline degeneration continues even after three weeks post-anastomosis. They considered this finding as an implication of an unfavorable outcome [15].

The comparison of eNOS levels in both groups revealed significantly higher values in the study group. This finding has several implications, as eNOS is the enzyme responsible for nitric oxide levels in vascular endothelium [16]. Nitric oxide, being one of the principle protective substances for the cardiovascular system [17], is an autacoid that effectively relaxes vascular smooth muscles and causes vasodilation [15]. Moreover, it inhibits thrombocyte aggregation [18]. Both of these effects are desirable following a microvascular anastomosis.

Aneurysms or pseudoaneurysms, neither macroscopically nor microscopically, weren't encountered in any of the subjects in either group. This may be perceived as an expected outcome because, in general, anastomotic aneurysms are encountered in vessels larger than 5 mm in a clinical setting [19]. However, studies with rats have revealed its incidence being not that low, in part due to more fragile vascular structure and higher pulse [20]. It may be postulated that the main reason for not having observed any signs of (pseudo)aneurysms in the study group, where considerably fewer sutures were used, is the supportive role of the wrapped amnion sheath, as proposed for venous sheath and fibrin glue by Sacak et al. in their study [3]. This protective role may be extended for its ability to withstand more external factors such as hematoma, kinking or torsion.

A search of the current literature yields several studies concerning the use of amniotic membrane in addressing various problems in plastic surgery [4]. Gray et al. proposed its use as a vascular conduit by laying amniotic sheets in a rolling fashion with care to place its fetal surface facing towards the vascular lumen [21]. The aforementioned study guided our decision on rolling the fetal side inwards. Although the proposed contact between the vessel lumen contents and the amniotic membrane was negligible, this configuration was thought to be safer.

The search for the holy grail of microvascular surgery led Lauritzen et al. to their definition of the telescopic method [22]. The disadvantages of this method, along with the difficulty of preventing leakage with just two sutures are well documented [23]. On the other hand, Sacak et al. successfully used sleeve vein graft as a sheath around the two suture anastomosis which was again reinforced by fibrin glue [3]. The major drawbacks of this technique may be its requirement of another donor site to yield the vein graft and the additional cost of fibrin glue. Human amniotic membrane, on the contrary, is cheap and readily available worldwide. It is a mere by-product of every healthy childbirth and is seen as a biological waste. Tens of sheets of the human amniotic membrane for use in microvascular anastomoses can be obtained from a single placenta.

The study design had been planned to be performed in three groups. A three suture anastomosis model without amniotic membrane had initially been included in the study. However, due to excessive bleeding from the anastomosis sites, the first two rats were lost immediately after anastomoses. As a limitation of this study, one may consider the lack of the third group (three suture anastomosis model without amniotic membrane). It was clearly observed that all members of the possible third group would have been lost due to excessive bleeding. Regarding the ethical issues, the third group was excluded from this study.

In summary, the following are features of the amniotic membrane wrapped three-suture anastomotic model: demands fewer sutures, cutting the total cost and time spent; is less traumatic, necessitating fewer maneuvers and trauma to the vessel wall; ends up in fewer foreign bodies inside the vascular lumen; yields less risk of back wall suturing (as all three sutures can be conveniently passed under direct vision); and has a theoretical benefit in early healing and recanalization under less vascular tone (with all the growth factors and eNOS), with nearly no production costs besides preparation. The disadvantages may be the risk of transmitting infections and steep learning curve, owing to difficulties in manipulating especially smaller-sized sheets with their sticky and viscous nature.

The limits of reconstructive microsurgery today are continuously pushed towards perfection with increasing finesse and precision. New studies are conducted for the purpose of finding safer and more practical methods of reconstruction.

At the very core of success in the field of microsurgical reconstruction lies microvascular anastomosis. Today, many centers use the conventional suture method. In this study, the authors propose their anastomosis method using human amniotic membrane that has gained and lost popularity in the past for different purposes. In this study, the amniotic membrane-wrapped paucisuture anastomotic model is shown to be comparable to the conventional method in terms of safety and convenience. However, it is shown to be faster and potentially more beneficial at the cellular level. The latter of these conclusive remarks obviates the need for large clinical and experimental series.

Ethical approval

The study had been carried out in the Ege University Research Center for Animal Experiments and Applications Laboratory following the approval of Ege University Local Animal and Clinical Research Ethics Committee (Number, 2016–056; Date, 27/07/2016).

Disclosure statement

No potential conflict of interest was reported by the authors.

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