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Amniotic membrane covering for facial nerve repair☆

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Abstract

Amniotic membranes have been widely used in ophthalmology and skin injury repair because of their anti-inflammatory properties. In this study, we measured therapeutic efficacy and determined if amniotic membranes could be used for facial nerve repair. The facial nerves of eight rats were dissected and end-to-end anastomosis was performed. Amniotic membranes were covered on the anastomosis sites in four rats. Electromyography results showed that, at the end of the 3rd and 8th weeks after amniotic membrane covering, the latency values of the facial nerves covered by amniotic membranes were significantly shortened and the amplitude values were significantly increased. Compared with simple facial nerve anastomosis, after histopathological examination, facial nerve anastomosed with amniotic membrane showed better continuity, milder inflammatory reactions, and more satisfactory nerve conduction. These findings suggest that amniotic membrane covering has great potential in facial nerve repair.

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Key Words

neural regeneration; peripheral nerve injury; tissue engineering; amniotic membrane; facial nerve injury; electromyography; nerve anastomosis; latency; amplitude; neuroregeneration

Research Highlights

- (1) In the past, the placenta, the source of amniotic membranes, was discarded after parturition. Currently, besides ocular and dermal applications, amniotic membranes are also used in regenerative medicine.
- (2) In this study, amniotic membrane was used to cover the anastomosed facial nerve to determine if this method facilitates facial nerve repair.
- (3) Compared with simple facial nerve anastomosis, facial nerve anastomosed with amniotic membrane showed better continuity, milder inflammatory reaction, and more satisfactory nerve conduction. These findings suggest that amniotic membrane covering has great potential for use in facial nerve repair.

INTRODUCTION

The amniotic membrane is an avascular membrane that is composed of an epithelial layer and an inner mesodermal tissue, which can reduce potent proinflammatory cytokines^[1-3]. In the past, the placenta, the source of the amniotic membrane, was discarded after parturition^[4]. In recent years, human amniotic membrane-derived mesenchymal stem cells have been isolated from the amniotic membrane^[5]. Besides use in regenerative medicine^[6-10], the amniotic membrane and human amniotic membrane-derived mesenchymal stem cells are also used for their anti-inflammatory properties in ocular and dermal applications, including burn lesion treatment, surgical wound covering^[11], and ocular surface reconstitution^[12-13]. As human bone marrow-derived mesenchymal stem cells have low efficiency for transdifferentiation^[14], their efficacy is still limited^[15]. However, non-bone marrow-derived mesenchymal cells such as menstrual blood-derived mesenchymal cells^[16], umbilical cord blood-derived mesenchymal stem cells^[17], and placental chorionic plate-derived mesenchymal cells^[18] have higher transdifferentiation efficiency. Recently, these mesenchymal stem cells have been used for clinical applications, and their safety and feasibility in placenta-derived extraembryonic mesodermal cell based therapy have been demonstrated^[19]. However, these cells are used as allografts, and problems of immunological rejection arise.

It was formerly believed that, because mesenchymal stem cells do not express HLA-DR6-9^[20], they resisted immunologic rejection to some extent. Subsequently, it was understood that only human amniotic membrane-derived mesenchymal stem cells do not express the major histocompatibility complex class I molecule, and thus may be expected to show immunologic tolerance. Differentiated human amniotic membrane-derived mesenchymal stem cells and human amniotic epithelial cells from full term pregnancy have low immunogenicity and have been successfully transplanted into allogeneic recipients^[21]. Human amniotic membrane-derived mesenchymal stem cells are multipotent and can differentiate into various cell types, including neural cells^[22].

Synkinesis and axonal misrouting are the common complications after facial nerve repair. Thus, it is essential to choose a suitable surgical modality to provide satisfactory results considering both aesthetics

and functionality. The surgical modalities may be nerve grafts, regional muscle transfers, primary neuroorrhaphy, free tissue transfers, static procedures, and nerve transfers^[23]. The gold standard technique is primary neuroorrhaphy, but there is a long delay period between the injuries and repair. Nerve grafts, regional muscle transfers, and free tissue transfers are the preferred alternative techniques^[23].

We hypothesize that covering the amniotic membrane around the anastomosed facial nerve segment has a better capacity to recover the function of injured facial nerves. We performed this study to verify this possibility.

RESULTS

Quantitative analysis of experimental animals

Eight rats were equally divided into two groups: (1) anastomosis group ($n = 4$), in which, only facial nerve anastomosis was performed; (2) anastomosis with amniotic membrane coverage group ($n = 4$), in which, the amniotic membrane was used to cover the facial nerve anastomosed segment. The operated left facial nerve sides of rats were examined as the experimental group ($n = 8$) and the non-operated right facial nerve sides of rats were examined as the control group ($n = 8$). All eight rats were included in the final analysis.

Measurement of facial nerve latency in rats

In the anastomosis group, the latency values in the 3rd week after electromyography of the facial nerve on the operated sides were significantly greater than those recorded from the control sides ($P < 0.05$). The latency values of the second electromyography performed in the 8th week were significantly greater than those obtained from the control sides ($P < 0.05$). The decrease in the latency values at the 8th week when compared with those obtained at the end of the 3rd week for the study group was found to be statistically significant ($P < 0.05$). However, there was no significant difference in the latency value on the control sides between the end of the 3rd and 8th weeks ($P > 0.05$) (Table 1).

For rats with anastomosis with amniotic membrane coverage, the latency value of the 3rd week electromyography of the facial nerve in the experimental group was significantly greater than in the control group ($P < 0.05$). However, there was no significant difference in the latency value of the 8th week electromyography of the facial nerve between experimental and control groups ($P > 0.05$). Similarly, there was no significant

difference in the latency value observed in the control group between the 8th week and the 3rd week electromyography of the facial nerve ($P > 0.05$) (Table 1). The latency values obtained in the 3rd week electromyography of the facial nerve on the operated sides were significantly greater in the anastomosis group than in the anastomosis with amniotic membrane coverage group ($P < 0.05$). The latency values in the 8th week were also significantly greater in the anastomosis group than in the anastomosis with amniotic membrane coverage group ($P < 0.05$) (Table 1).

Measurement of facial nerve amplitude

For anastomosis rats, the amplitude values of the 3rd week and 8th week electromyography of the facial nerve were significantly lower in the experimental group than in the control group ($P < 0.05$). There was no significant difference in the increased amplitude values of the operated or non-operated facial nerve between the 8th week and 3rd week of electromyography ($P < 0.05$) (Table 2).

For anastomosis with the amniotic membrane coverage rat group, the amplitude values of the 3rd week electromyography of the facial nerves in the experimental group were significantly lower than in the control group ($P < 0.05$). There was no significant difference in amplitude values of the 8th week electromyography of the facial nerve between the experimental group and control group ($P < 0.05$). Significant differences in the increased amplitude values of the operated sides were found between the 3rd week electromyography and the 8th week electromyography ($P < 0.05$). However, there was no significant difference in the increased amplitude values of the control sides between the 3rd week electromyography and the 8th week electromyography ($P > 0.05$) (Table 2).

The amplitude values of the 3rd week and 8th week electromyography of the operated facial nerves were significantly greater in the anastomosis with amniotic membrane coverage group than in the anastomosis group ($P < 0.05$).

Table 1 Evaluation of latency periods (ms) of the operated and non-operated facial nerve by electromyography in the postoperative 3rd and 8th weeks

Group		Operated side	Control side	P
Anastomosis	3 rd week	2.30±0.08	1.32±0.03	0.019 ^a
	8 th week	1.87±0.06	1.41±0.02	0.018 ^a
	3 rd week–8 th week; ^b P value	0.048 ^a	0.066	
Anastomosis with amniotic membrane coverage	3 rd week	1.81±0.05	1.44±0.05	0.019 ^a
	8 th week	1.42±0.07	1.41±0.06	0.278
	3 rd week–8 th week; ^b P value	0.066	0.577	
Evaluation of anastomosis–anastomosis with amniotic membrane coverage				
	3 rd week	0.019 ^a	0.019 ^a	
	8 th week	0.020 ^a	0.741	

Data are expressed as mean ± SEM with eight rats in the operated/control sides and four rats in the anastomosis and anastomosis with amniotic membrane coverage groups. ^a $P < 0.05$ (Mann Whitney *U* test); ^bWilcoxon Signed Ranks test.

Table 2 Evaluation of the amplitude (mV) values of operated and non-operated facial nerve by electromyography in the postoperative 3rd and 8th weeks

Group		Operated side	Control side	P
Anastomosis	3 rd week	1.34±0.05	3.92±0.17	0.020 ^a
	8 th week	2.96±0.04	3.86±0.16	0.019 ^a
	3 rd week–8 th week; ^b P value	0.039 ^a	0.461	
Anastomosis with amniotic membrane coverage	3 rd week	1.62±0.17	4.05±0.21	0.021 ^a
	8 th week	3.85±0.31	3.97±0.29	0.306
	3 rd week–8 th week; ^b P value	0.036 ^a	0.461	
Evaluation of anastomosis–anastomosis with amniotic membrane coverage				
	3 rd week	0.028 ^a	0.381	
	8 th week	0.020 ^a	0.559	

Data are expressed as mean ± SEM with eight rats in the operated/control sides and four rats in the anastomosis and anastomosis with amniotic membrane coverage groups. ^a $P < 0.05$ (Mann Whitney *U* test); ^bWilcoxon Signed Ranks test.

Histopathological examination of the facial nerves

As demonstrated by electron microscopy, facial nerve anastomosis sites wrapped with amniotic membrane revealed almost complete end-to-end attachment and continuity of the nerve fibers. In contrast, scant and scattered nerve fibers with irregular and incomplete connections were observed at the nerve anastomosis sites without amniotic membrane.

In addition, foreign body reactions and inflammatory responses were lower in the amniotic membrane-wrapped anastomosis sites as compared with the control group without anastomosis (Figure 1).

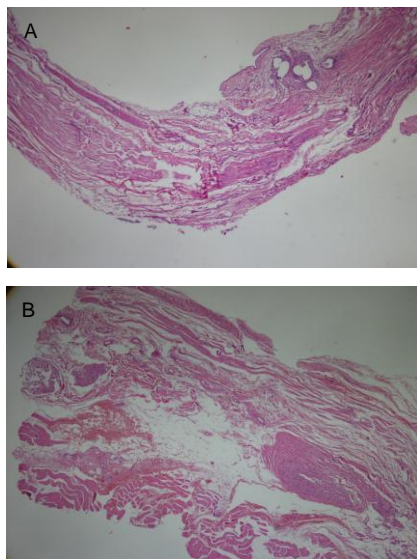


Figure 1 Histopathological observation of facial nerve anastomosis side with (A) or without (B) amniotic membrane covering (hematoxylin-eosin staining, $\times 100$).

(A) Almost complete end-to-end attachment and continuity of the facial nerves are observed. Besides foreign body reaction to the suture material, minimal inflammatory response is seen.

(B) Short and scattered nerve fibers without any connection and intervening muscle and fibrous tissue with inflammatory reaction can be seen.

DISCUSSION

Human amniotic mesenchymal stem cells are multipotent and can differentiate into various cell types, including neural cells^[22] and chondrocytes^[24], and they have low immunologic antigenicity^[25-26]. A recent study investigating the effects of a mixed cell population consisting of human amniotic epithelial cells and human amniotic membrane-derived mesenchymal stem cells

reported a reduction in fibrosis in lungs of bleomycin injured mice^[27]. Human amniotic membrane-derived mesenchymal stem cells have also been successfully used in cell therapies for injured nerve^[28] and stroke^[29]. In the present study, amniotic membrane was used for facial nerve regeneration and repair due to its anti-inflammatory effects^[21], and as a multipotent stem cell source for regeneration^[3]. The histopathological examination of facial nerves was made at the end of the 8th week. Two cases of facial nerves were either covered or uncovered with the amniotic membrane, to determine the anti-inflammatory and facial nerve regeneration effects of the amniotic membrane. Histopathological examination of the facial nerve anastomosis sites which were wrapped with amniotic membrane revealed almost complete end-to-end attachment with continuity of nerve fibers. In contrast, there were scant and scattered nerve fibers with irregular and incomplete connections observed at the nerve anastomosis sites without amniotic membrane. In addition, foreign body reaction (especially to the suture materials) and inflammatory responses were lower in the amniotic membrane wrapped anastomosis sites as compared with the group in which anastomosis was performed without amniotic membrane.

The human amniotic membrane was used as a source of human amniotic membrane-derived mesenchymal stem cells, and the effects of amniotic membrane on regeneration and repair of facial nerve injury were investigated. It was determined that the amplitude values in electromyography of the facial nerves, which were performed in the 3rd and the 8th weeks of the operated sides, where amniotic membrane of appropriate shape and size was used to cover the anastomosed facial nerve segment, were significantly greater than when only facial anastomosis was performed. Similarly, the latency values in electromyography of the facial nerve which was performed both in the 3rd and the 8th weeks for the operated sides, where only facial anastomosis was performed, were significantly longer than the values obtained in the group in which amniotic membrane was used to cover the facial nerve. The role of growth factors or stem cells in facial regeneration^[23] will be determined in future studies.

In conclusion, this study investigated the effects of amniotic membrane on facial nerve regeneration. The higher amplitude, shorter latency, complete end-to-end attachment, and good continuity of nerve fibers demonstrated that the use of amniotic membrane is safe, simple, and effective for facial nerve repair.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

This study was performed at Animal Laboratory of Haydarpaşa Numune State Hospital for Research and Training and, also at Department of Neurology in GATA Haydarpaşa State Hospital for Research and Training, İstanbul, Turkey for 8 weeks between December 2010 and January 2011.

Materials

To exclude the gender effects on nerve generation, eight female rats (*Rattus Norvetikus* of a Wistar Albino strain) with an initial body weight of 200–300 g, aged 3–6 months, were included in this study. All rats were kept under the same conditions throughout the study and were given the same amount of food and water every day.

Methods

Surgical anesthesia and premedication

Surgical anesthesia was achieved *via* intraperitoneal injection of 100 mg/kg ketamine (Pfizer Inc., Peapack-Gladstone, NJ, USA) and 10 mg/kg xylazine hydrochloride (Bayer, Berlin, Germany). Before surgery, a bolus dose of 250 mg cefamandole (Lilly, Indianapolis, IN, USA) was intramuscularly administered for a single dose preoperative prophylactic antibiotic treatment.

Surgical technique

On the left side of each rat, the cutaneous and subcutaneous tissues were cut open through 1.5–2 cm oblique incisions extending from the inferior part of the ear to the mandibula. Following dissection of the surrounding tissues, the parotid gland was discarded and the facial nerve was presented (Figure 2).



Figure 2 Exposure of the facial nerve (arrow).

The facial nerve was cut open through an oblique dissection using No. 15 scalpel blades. Then, the incision was sutured with 9.0 Prolene sutures and edge-to-edge anastomosis was performed. Similar procedures were applied for all operated rat facial nerves. In the anastomosis group, the parotid gland was placed and the cutaneous and subcutaneous tissues were sutured with 8.0 Prolene sutures following edge-to-edge anastomosis of the facial nerve. However, for anastomosis with amniotic membrane coverage, amniotic membrane (obtained from the Bank of Ophthalmology) was prepared by cutting into an appropriate shape and size. After edge-to-edge anastomosis of the facial nerve, the anastomosed segment was only covered by the amniotic membrane without any sutures between the membrane and nerve (Figures 3 and 4). At the end, the parotid gland was placed and the cutaneous and subcutaneous tissues were sutured similar to those in the anastomosis group.



Figure 3 The amniotic membrane was prepared by cutting in appropriate shape and size.

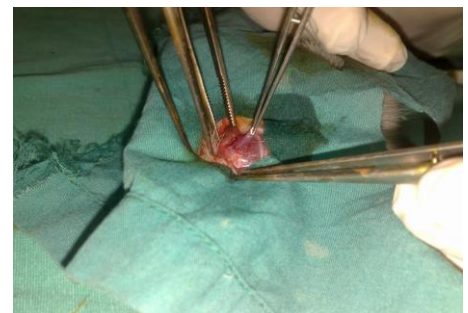


Figure 4 After edge-to-edge anastomosis of the facial nerve, the amniotic membrane covered the anastomosed segments.

Electromyography evaluation of the facial nerve

Rats were kept under observation for 8 weeks with two electromyography evaluations made on the 3rd and the 8th weeks at the Department of Neurology at GATA Haydarpaşa State Hospital for Research and Training,

Istanbul, Turkey. Electromyography records were done by placing recording electrodes on nasal tips of rats after required anesthesia with intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine hydrochloride. The grounding electrode was placed on the sternocleidomastoid muscle. Bipolar electrodes were used for neural stimulations. The main trunk of the facial nerve was stimulated through the nerve trace in the parotid section. The stimulus threshold was increased from 0.1 mA until the motor unit action potentials were obtained from the orbicularis oris and buccal muscles; meanwhile, the whisker and muscle movements were also observed (Figure 5).

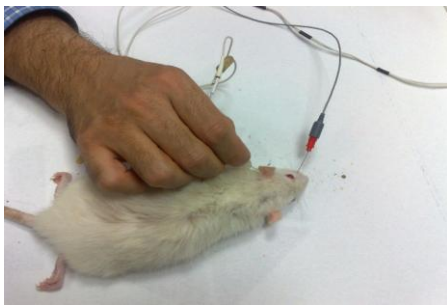


Figure 5 Electromyography records were made by placing the recording electrode on nasal tips of rats.

The grounding electrode was placed on the sternocleidomastoid muscles. Bipolar electrodes were used for neural stimulations. The main trunk of the facial nerve was stimulated through the nerve trace in the parotid section.

The facial nerve latency and amplitude values of operated and control sides were recorded. The latency was the elapsed time between the first initiation of the stimulus and the appearance of the first remarkable wave. The amplitude was the distance between positive and negative peaks of the obtained potential (Figure 6).

The histopathological examination of the facial nerve

At the end of the 8th week, the anastomosed facial nerve segments were removed for histopathological examination (Figures 7, 8). Both proximal and distal ends of the neural specimens were precisely marked. The neural specimen was fixed with 10% formalin and embedded into paraffin blocks. Sections with a thickness of 4 μ m were stained for immunohistochemical analysis. During histopathological examination, the presence of inflammatory reactions and the nerve structures were evaluated by observing complete end-to-end attachment and the continuity of the nerve fibers and inflammatory cells. Finally, rats were euthanized by intraperitoneal injection of 200 mg/kg pentobarbital.

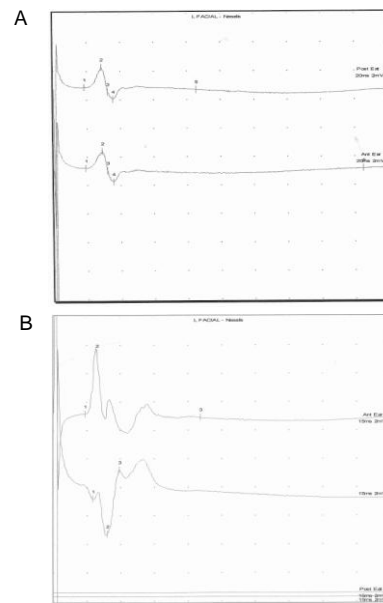


Figure 6 Electromyography recordings of anastomosed facial nerve covered with amniotic membrane at the 3rd and 8th weeks (A, B), showing the latency and amplitude values.



Figure 7 At the 8th week, the anastomosed facial nerve segment (arrow) was exposed for an incisional biopsy.



Figure 8 After 8 weeks, the anastomosed facial nerve segment (arrow) was removed for histopathological examination.

Statistical analysis

Data were statistically analyzed using NCSS (Number

Cruncher Statistical System) 2007 and PASS 2008 Statistical Software (Kaysville, UT, USA). During the evaluation process, the Mann Whitney *U* test was used to compare the parameters that were not normally distributed, as well as for the comparison of statistical parameters (mean \pm SD). Furthermore, Wilcoxon Signed Ranks test was used for intragroup comparisons. A value of $P < 0.05$ was considered as statistically significant.

Author contributions: Murat Karaman, Arzu Tuncel, Mehmet Güney Şenol, Murat Hakan Karabulut, and Nihan Karaman were responsible for conception and design of the study, data collection, analysis, and interpretation of the data. Murat Karaman, Arzu Tuncel, Shahrouz Sheidaei, Ildem Deveci, and Nihan Karaman wrote the manuscript and revised the manuscript for intellectual content. Murat Karaman, Shahrouz Sheidaei, Mehmet Güney Şenol, and Murat Hakan Karabulut participated in statistical analysis, and provided technical or material support. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The animal study was approved by the Ethical Committee of Marmara University Animal Laboratory, İstanbul, Turkey.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

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