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Effects of Concentrated Growth Factor and Hyaluronic Acid in an Experimental Model of Acute Traumatic Tympanic Membrane Perforation

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Abstract

Objective: The aim of this study is to investigate the efficacy of concentrated growth factor (CGF) and hyaluronic acid (HA) application on the healing of acute tympanic membrane (TM) perforation.

Methods: A total of 30 male albino rats were included in the study. The animals were randomly divided into three groups with 9 rats in each group (A, B, C). A CGF was applied to the perforated TMs of rats in group A. A gelfoam particle soaked in 1% hyaluronan was applied to the perforations in group B. The rats in group C were left to heal spontaneously. All rats were kept under control for 21 days. TM healing was evaluated. On the 21st day, all rats were decapitated for histopathological evaluation.

Results: In group A, 18 of the 18 TM perforations were closed and the mean healing time was 12.11 days. In group B, 18 of the 18 TM perforations were closed and the mean healing time was 15.05 days. In group C, 16 of 18 TM perforations were closed and the mean closure time was 17.11 days. There was a statistically significant difference in the mean TM closure time in all groups at the end of the 21st day (p=0.001).

Conclusion: In this study, we used autologous CGF and HA in the treatment of acute TM perforations in rats. We observed that the application of both products was a more effective method than the control group. We think that using these methods, which are easy, inexpensive, and have low patient morbidity, as graft or graft support in chronic TM perforations will be beneficial and successful results will increase.

Keywords: Concentrated growth factor, hyaluronic acid, tympanic membrane

INTRODUCTION

Tympanic membrane (TM) perforation often has different causes, such as trauma and infection. If the perforation is not healed, important problems such as chronic otitis media and hearing loss may occur. The reported incidence of TM perforation ranged from 1% to 4% (1). Perforations often heal spontaneously unless chronic inflammation develops; however, with chronic perforation, surgical repair is usually required (2). In traumatic perforations, especially when less than a quarter of the membrane is perforated, approximately 90% of them heal spontaneously, while grafting is often required to repair large perforations (3).

Healing of perforated TM after acute trauma includes epithelial proliferation and migration, fibroblast proliferation, angiogenesis, and tissue remodeling. The epithelial layer first forms a bridge over the wound, the surface of the fibrous layer

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is closed, and the TM takes its final form (4). Different cytokines and growth factors are released from the wound site during the acute stage of wound healing. Many otolaryngologists perform microsurgical procedures to accelerate the healing process. These include early patching, surgery, and use of biomaterials such as hyaluronate, epidermal growth factor (EGF), fibroblast growth factor, platelet-derived growth factor, and transforming growth factor (5,6). Autogenous materials may be used more frequently in TM repairs because of their positive effects on wound healing quantity and quality. Various animal experimental studies have examined the effects of these growth factors on wound healing. These studies have helped us learn the healing process of TM perforations in humans.

This study aimed to investigate the effectiveness of concentrated growth factor (CGF) and hyaluronic acid (HA), which can be obtained by a simple and inexpensive method, on the healing of acute TM perforation.

METHODS

This study was approved by the Animal Research Ethics Committee of Bezmialem Vakif University Medical Faculty (approval date: 21.03.2019, approval no. 2019/91). Thirty male albino rats with bilateral normal TM (weight, 250-300 g) breeding in the multidisciplinary research laboratories were examined and included in the study.

Concentrated Growth Factor Preparation

Of the 30 animals, three were used for CGF preparation. Then, 4 mL of intracardiac blood were collected from the three rats and placed in test tubes without anticoagulants. These tubes were centrifuged (MEDIFUGE TM, Silfradent Srl, S. Sofia, Italy) at 2700 rpm for 2 min, 2400 rpm for 4 min, 2700 rpm for 4 min, and 3 min at 3000 rpm. The aim was to obtain a dense fibrin matrix containing growth factors at a higher rate by centrifuging the samples at different speeds and times. After the procedure, four layers were obtained from bottom to top: Red blood cell layer, growth factor and stem cell layer (CGF), buffy coat layer, and serum layer. Then, the CGF gel layer was separated using sterile surgical scissors and placed over the target site (7).

Surgical Procedure

The animals were randomly divided into three groups with nine rats in each group (groups A-C). Ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (5 mg/kg) were administered intraperitoneally to anesthetize all animals. In the otomicroscopic examination, a perforation was created using a pike in the posterosuperior quadrant of both TMs in all rats. CGF was applied to the perforated ears in group A. A 1.5-2 mm-diameter gelfoam particle soaked in 10 µL of 1% hyaluronan (Healon, Pharmacia AB, Sweden) was applied to the perforations in group B. No intervention was made to the perforated ears in group C, and they were left to heal spontaneously. All rats were observed in their groups in cages with access to water and food. The animals were examined microscopically daily under inhaled isoflurane anesthesia until the animals were sacrificed or 21 days had passed to define the time of perforation closure.

Histopathological Examination

On day 21, after intraperitoneal pentobarbital injections (80 mg/kg), all rats were decapitated. Their external ears were separated from the osteocartilaginous connections, and each bulla was opened. TMs were removed together with the bone annulus. All ear samples were stored in 10% formaldehyde solutions and sent to the pathology laboratory for histological evaluation. In total, 52 surgical specimens fixed in formaldehyde were decalcified in formic acid. Sections were embedded in paraffin. Tissue blocks were cut into 5-mm-thick slides, treated with hematoxylin and eosin, and then examined under a light microscope. Fibrosis, neovascularization, inflammation, and edema in tissue samples were evaluated in histopathological examination. All examinations were performed by the same pathologist without knowing which tissue sample belonged to which group. Changes in parameters were scored as (-), (+), (++), or (+++). Negative showed no change; (+), (++), and (+++) scores showed mild, moderate, and significant changes, respectively. No rats died during the study period.

Statistical Analysis

In the evaluation of the study findings, IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA) program was used for statistical analysis. While evaluating the study data, the conformity of the parameters to the normal distribution was evaluated with the Shapiro-Wilk test. In addition to statistical methods (mean, standard deviation, and frequency), the Kruskal-Wallis test was used for the comparison of the parameters that did not show a normal distribution in the comparison of quantitative data, between more than two groups. The chi-square test was used to compare qualitative data. Significance was evaluated at the level of $p<0.05$.

RESULTS

The closure process of TMs was examined microscopically for 21 days in a total of 27 rats. In group A, 18 of the 18 TM perforations in which CGF was applied were closed, and the mean healing

time was 12.11 days. In group B, 18 of the 18 TM perforations in which HA was applied were closed, and the mean healing time was 15.05 days. In group C, whose perforations were left to spontaneous heal, 16 of 18 TM perforations were closed, and the mean closure time was 17.11 days (Table 1). One of the rats in the control group, which was left to heal spontaneously, did not heal in both TMs.

The mean closure times were evaluated in all groups after 21 days, and a significant difference was observed (p=0.001) (Table 1). When the difference in the mean closure time between the groups was compared, it was shorter in group A than in groups B and C, and the differences were found significant (p=0.004, p=0.001, respectively). It was also slightly shorter in group B than in group C (p=0.037) (Table 1). Histopathological evaluation initiated after 21 days of TM recovery evaluation revealed no significant difference between all animal groups in terms of edema, neovascularization, fibrosis, and inflammation parameters (Table 2).

DISCUSSION

TM perforations in humans appear in various sizes depending on the causes, such as infections, accidents, explosion, slap injury, or instrument-induced injury. Even a very small perforation can negatively affect people's daily quality of life. Approximately 90% of TM perforations heal spontaneously within 7-21 days, depending on their size (4). Following an acute trauma to the TM, an exudative reaction begins first at the edges of the perforation. After a while, a keratin migration starts from the external squamous epithelial layer of the TM to the center of the perforation. Crust layer formation protects the underlying tissue, providing a suitable basis for cell migration and the healing process. Closure of the TM defect initially occurs through the keratin layer, then squamous epithelial cells fuse, and a supporting connective tissue closes the perforation (8).

At present, myringoplasty is an effective procedure commonly used to repair TM perforations. Many graft types are used in myringoplasty. These grafts should be biocompatible and easy to obtain. In perforation repair, while autografts (temporal muscle fascia, tragal cartilage, and fat tissue), gel films, and paper patches are used for stromal support, silver nitrate cauterization and trichloride acetic acid can be applied to the perforation borders. The TM structures of humans and rats have similar histological features (9). Considering that similar results can be obtained in humans, rats are generally used in experimental studies on TM. Animals studies have shown the presence of stem cells in the annulus and manubrium malleus of TM. These cells are involved in the repair of TM owing to their high proliferation properties (4). Platelet-rich products are used in many areas in graft and wound treatment, as they promote accelerated angiogenesis, chemotaxis, mitosis, and proliferation of stem cells (10). To benefit from the effects of these products in wound healing, many types of platelet concentrate products have been used for years. These products take various names depending on their contents, centrifugation methods, and presence or absence of an anticoagulant in the production techniques. Platelet-rich plasma (PRP), platelet-rich fibrin (PRF), and CGF are some of these products. Studies on topical HA, EGF, PRP, PRF, and many other product applications have been reported in the treatment of traumatic TM perforations (11). Yeo et al. (12) reported that mouse TM perforations showed better closure than the control group with the application of a 2-µg platelet-derived growth factor. Mondain et al. (13) showed that the application of fibroblast growth factor to mouse TM perforation resulted in faster closure. By contrast, O'Daniel et al. (14) studied pigs and showed that EGF receptors are present in all three TM layers, especially the squamous epithelial layer.

PRP and PRF were first defined by Dohan et al. (15) by dividing them into two different groups according to their production methods and contents. El-Anwar et al. (16) performed tympanoplasty with conchal cartilage for dry and large perforated TM 64 patients. In half of the cases, they applied PRP during the procedure. They observed a higher graft retention rate and a lower infection rate in the PRP group. Another similar study showed better graft success rates using PRP-impregnated gel foam in tympanoplasty (17). Erkilet et al. (18) applied PRP to perforated TMs in rats and reported that PRP had positive effects on healing and shortened the recovery time. The main

Table 1. Closure time of the study and control groups

	Closure time (day)						
	n (ear)	Minimum	Maximum	Mean	SD	p	Pairwise comparisons of the groups
Group A (CGF)	18	9	15	12.11	1.94	0.001	Group A-B; p=0.004 Group B-C; p=0.037 Group A-C; p=0.001
Group B (HA)	18	11	17	15.05	1.55		
Group C (control)	16	12	20	17.11	1.68		

Kruskal-Wallis test p<0.05, Pairwise comparison: Mann-Whitney with Bonferroni correction, SD: Standard deviation, CGF: Concentrated growth factor, HA: Hyaluronic acid

generations of autologous platelet concentrate produced by centrifugation of venous blood at different rates are PRP, PRF, and CGF. CGF, which is the newest autologous platelet concentrate, was first described by Sacco (19). The rationale for this product is related to the *in situ* administration of multiple autogenous growth factors, as well as specific fibrin scaffold formation. Lei et al. (20) conducted a comparative study between advanced PRF (A-PRF), a variant of PRF, and CGF. They showed that A-PRF had a looser fibrin network than CGF, and stimulation of a sustained release of growth factor for 10 days was comparable in both products. Another comparative study showed that platelet and growth factor concentrations in A-PRF were comparable to CGF, but A-PRF and CGF had higher platelet and growth factors than PRP and plasma-rich growth factors (21). Contents of PRF and CGF are similar, but CGF is rich in growth factors and has a denser fibrin matrix. These materials have properties that increase soft tissue and bone healing (22). Membranes obtained from these biomaterials can be used as graft material in otological applications such as TM repair. CGF or PRF is preferred over PRP because no anticoagulants are added, and their membranes provide better biomechanical resistance. This event is valuable in terms of graft survival.

Ensari et al. (23) used PRF as graft material in rats and reported that the mean recovery time of TM perforation was 10.3±2.18 days in the study group and 17±2.40 days in the control group ($p<0.05$). Gür et al. (24) applied PRF in 30 of 60 patients with acute TM trauma and applied paper patches to the other 30 patients. They observed perforation closure in 93% of the patients in the PRF group and 83% of the patients in the paper group. They did not observe a significant difference in the healing rates of TMs. They reported 7.8 as the mean closure time of TMs in the PRF group. In addition, Habesoglu et al. (25) reported that the use of PRF in acute traumatic TM perforations resulted in significantly better recovery rates and times. In our study, we examined the healing process of TM perforations we have created in 18 rats for 21 days to observe the effect of CGF on traumatic TM perforation. Subsequently, 18 of the 18 TM perforations in which we applied CGF were closed, and the mean recovery time was 12.11 days. In the control group, whose perforations were left to heal spontaneously, 16 of 18 TM perforations were closed, and the mean closure time was 17.11 days (Table 1). A significant difference was found between the two groups in terms of mean recovery times of TM ($p=0.001$).

Table 2. All rats were sacrificed on day 21: Histopathological evaluation of TMs

	Group A (CGF)		Group B (HA)		Group C (control)		
	n	%	n	%	n	%	p
Edema							
-	11	61.1	15	83.3	15	83.3	0.052
+	6	33.3	-	-	2	11.1	
++	1	5.6	3	16.7	1	5.6	
+++	-	-	-	-	-	-	
Neovascularization							
-	16	88.9	17	94.4	18	100	0.347
+	2	11.1	1	5.6	-		
++	-	-	-	-	-		
+++	-	-	-	-	-		
Fibrosis							
-	-	-	5	27.8	6	33.3	0.113
+	11	66.7	10	55.6	9	50	
++	4	22.2	3	16.7	3	16.7	
+++	3	11.1	-	-	-	-	
Inflammation							
-	18	100	18	100	17	94.4	0.361
+	-	-	-	-	1	5.6	
++	-	-	-	-	-	-	
+++	-	-	-	-	-	-	

Chi-squared test $p<0.05$, TMs: Tympanic membrane, CGF: Concentrated growth factor, HA: Hyaluronic acid

CGF, like other platelet concentrate products, accelerates wound healing, because it contains growth factors and cytokines. Since it is an autologous product, it does not pose a risk of infection and immune response. It also slowly releases growth factors into the environment through the fibrin network. It is easy and inexpensive to obtain (26). Recent studies with CGF are mostly on dental operations and bone tissue, and there are very few studies on TM. Sohn et al. (26) used CGF for maxillary sinus augmentation as well as implant surgery. They showed that CGF has an accelerating effect on new bone formation and soft tissue healing. In another case report, sinus perforation was treated with CGF, and new bone formation was demonstrated in the control evaluation performed with radiological examinations 6 months later (27). Talaat et al. (28) applied PRP, PRF, and CGF to the defects in 20 patients with bone defects caused by mandibular lesions. As a result, they reported that CGF is an economical and safe product that accelerates new bone formation. Topkara et al. (29) performed an animal study and showed that the viability of chopped cartilage covered with fascia, which is one of the important camouflage materials in rhinoplasty surgery, will increase when used with CGF. Zhao et al. (30) performed repeated local injections of CGF in patients with septal mucosal defects after rhinoplasty, and they observed complete closure in all patients, indicating that this technique is an easy and convenient procedure. Many surgeons have used adipose tissue as a graft, especially in the repair of small TM perforations. Fat graft myringoplasty is also a cost-effective, simple, and non-invasive surgery for TM perforations. However, fat grafting is a surgical procedure, albeit a minor one. CGF membrane application does not require any surgical procedure, and a blood sample from the patient is sufficient. In addition, CGF has a protective effect against infections.

Since fibrin obtained from platelet-rich materials contains proinflammatory cytokines, an increase in inflammation and fibrosis rates is expected in the areas where it is applied (31). Hu et al. (32) added PRP, PRF, and CGF to adipose tissues taken from rats and examined histologically. They observed richer vascularity and less fibrosis in the CGF added group compared with the PRP and PRF groups. Erkilet et al. (18) evaluated the improvement of TM perforations in rats in which PRP was applied, although it was not significant, fibroblastic reaction and neovascularization were higher in the study group on day 7. Ensari et al. (23) evaluated the perforated TMs of the rats to which they applied PRF, and they reported that neovascularization was more frequent days 3, 5, and 7, although it was not significant in the study group ($p>0.05$). Herein, we performed the histopathological evaluation in the last week of our study. When the tissues were taken for histopathological examination on day 21, no significant difference was found between all animal groups in terms of edema, neovascularization, fibrosis, and inflammation parameters (Table 2). In other studies, significant differences were found compared with the control groups in terms of these parameters; no difference was found between the groups because our histopathological evaluation was performed at a later stage.

HA, which is routinely used in ophthalmologic surgery because of its viscoelastic properties, is involved in various processes of early wound healing such as cell migration, organization of granulation tissue in cell proliferation, moderation of the inflammatory response, and angiogenesis (33,34). Local application of HA has positive effects in closing TM perforations in tympanoplasty and in reducing the length of hospital stay and cost (35). These effects regulate the healing of the fibrous layer by preventing dehydration of the perforation margins, providing a supportive environment for the keratin and hyperplastic epithelium that occurs during the healing process, and regulating functions such as mobility and phagocytic properties of polymorphonuclear leukocytes (36). Ozturk et al. (37) performed an experimental animal study with 24 male rats; they divided the rats into three groups as control rats, MeroGel rats, and daily topical HA rats. Perforations were closed in 70.8%, 91.7%, and 100% in the control, MeroGel rats, and daily HA rats, respectively. They reported that the main role of HA in repairing the TM is to provide a moist, wound-healing environment to aid in the healing process. Kaur et al. (38) used topical application of 1% sodium hyaluronate to repair human chronic TM perforations and reported that 86.7% (26/30) of the patients responded positively to the treatment. Rivas Lacarte et al. (39) reported that with the topical application of 1% sodium hyaluronate in 16 patients with chronic TM perforations, the perforation size reduced in 12 (75.0 %) patients; 6 (37.5 %) of whom showed complete healing, and 4 (25.0 %) showed no healing. Güneri et al. (6) evaluated the improvement of TM perforations with HA in another experimental animal study. While the mean closure time of TM perforation was 8.6 ± 2 days in the HA-treated group, it was 15 ± 2 days in the control group ($p=0.0432$). Similarly, in our study, all TM perforations were closed in HA rats, but the mean closure time was 15.5 days. In our control group, this period was 17.11 days ($p=0.037$). We think that these differences in mean recovery times are attributed to the number of rats used, localization of the perforations on the TM, and amount of HA applied. These findings obtained from rat studies suggest that HA application will have positive effects on the healing of acute traumatic TM perforations. In

addition, a study on wound healing has shown that a moist wound environment accelerates the healing of both acute and chronic wounds and promotes the growth of new tissue (40). Therefore, generally speaking, topical application of HA in the repair of traumatic TM perforation may exhibit a better healing state, thanks to the moist environment it provides.

CONCLUSION

Few studies have focused on the use of PRF or CGF in the repair of TM perforations. In our study, we used autologous CGF membrane and HA in the treatment of acute perforations in rats. The application of both products was a more effective method than the control group. With these methods, which are easy, inexpensive, and have low patient morbidity, as graft or graft support in chronic TM perforations will be beneficial and promote successful results. The development of biological materials can assist or, in appropriate cases, replace conventional myringoplasty in repairing chronic TM perforations.

Ethics

Ethics Committee Approval: This study was approved by the Animal Research Ethics Committee of Bezmialem Vakif University Medical Faculty (approval date: 21.03.2019, approval no. 2019/91).

Informed Consent: Animal experiments.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: H.S., Y.A., Y.U., Design: H.S., Y.U., Data Collection or Processing: Ö.İ.B., S.K., Analysis or Interpretation: Y.A., S.Ş.E., D.H., G.B., Literature Search: S.K., D.H., Y.U., Writing: H.S., Y.A.

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