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Original Article

Efficacy of Concentrated Growth Factors in Treating Tympanic Membrane Perforation in Guinea Pigs

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BACKGROUND: Less invasive and cost-effective alternatives are needed to manage tympanic membrane perforation (TMP). Therefore, the effectiveness of concentrated growth factors (CGF) in promoting tympanic membrane regeneration in guinea pig models of eardrum perforation was invetigeted.

METHODS: Large TMPs were created in 34 guinea pig ears using a CO_2 laser and divided into 3 groups: CGF-gelatin sponge (with-CGF group), saline-gelatin sponge (without-CGF group), and untreated group. In the with-CGF group, CGF and gelatin sponges were implanted into the perforations, while the without-CGF group received gelatin sponges impregnated with saline. Eardrums were observed under a light microscope on days 14 and 28, and tympanic membranes were examined histologically with hematoxylin and eosin staining.

RESULTS: On day 14, 8 of 14 (57.1%) ears in the with-CGF group achieved perforation closure, while no closures were observed in the without-CGF or untreated groups. The closure rate was significantly higher in the with-CGF group compared to both without-CGF and untreated groups ($P < .001$). By day 28, 12 of 14 (85.7%) ears in the with-CGF group and 8 of 14 (57.1%) ears in the without-CGF group had closure. No closures were noted in the untreatedgroup. Although the closure rates between the with-CGF and without-CGF groups were similar ($P = .07$), the with-CGF group showed a significantly higher rate than the untreated group ($P < .001$). Histological analysis revealed that the regenerated tympanic membrane was thicker in the with-CGF group compared to the without-CGF group.

CONCLUSION: Concentrated growth factor effectively promotes tympanic membrane regeneration and provides a promising, minimally invasive treatment option for TMP.

KEYWORDS: Guinea pig, intercellular signaling peptides and proteins, platelet-rich fibrin, therapy, tympanic membrane perforation

INTRODUCTION

Tympanic membrane perforation (TMP), commonly treated by otorhinolaryngologists, is caused by trauma, ventilation tubes, or chronic suppurative otitis media. Almost all acute and small TMPs heal spontaneously. However, large TMPs fail to heal when left untreated. Tympanic membrane perforations cause various disorders, such as hearing loss, recurrent otorrhea, and cholesteatoma formation.[1] Therefore, patients may benefit from TMP repair. Patients with TMP can undergo surgical treatments, including tympanoplasty or myringoplasty. However, these surgical treatments have disadvantages, such as skin incisions and harvesting of autologous tissue under general or local anesthesia, resulting in high medical costs. Therefore, novel, simple, and minimally invasive treatments for TMP should be explored.

Regeneration of tissues and organs requires manipulating cells, scaffolds, and regulatory factors.[2] External exogenous growth factors, including basic fibroblast growth factor (bFGF),[3] epidermal growth factor (EGF),[4] and platelet-derived growth factor (PDGF),[5] are effective in treating TMP. Several studies of tympanic membrane regeneration in rodents have demonstrated high closure rates and early closure with the use of growth factors.[3-6] Among these, tympanic membrane regeneration with bFGF has been

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clinically applied in several basic studies and is a convenient and versatile option.[7] However, the closure rate of TMPs associated with this method is reportedly 57.5%-70%, with a final effective closure rate of 40% and a reperforation rate of 30%.[8,9] Treatment with bFGF is effective for TMP, but the efficacy of these interventions for TMP remains debatable, especially given their healing rates and reperforation risk.

Platelet-rich fibrin (PRF), a platelet concentrate, has been used in regeneration research and has demonstrated effectiveness against TMPs in animal studies.[10] Concentrated growth factors (CGFs) are the latest platelet concentrates reported by Sacco [11] and are used in various regenerative therapies.[12,13] This most recently generated complex comprises a fibrin matrix enriched with growth factors, as well as plasma and leukocyte cytokines.[11] Concentrated growth factors can be produced quickly and easily by centrifuging blood samples using a custom centrifuge device and is rich in various growth factors, such as bFGF, EGF, PDGF, and insulin-like growth factor-1 (IGF-1).[14] Concentrated growth factor is a larger, denser, and richer growth factor fibrin matrix than PRF[15,16] and can be expected to be effective in TMP. However, only a few studies have reported on the application of CGF to TMP. Therefore, this study aimed to investigate the effectiveness of CGF for treating TMP in a guinea pig model.

METHODS

The study protocol was approved by the Ethics Committee for Animal Experiments of Ehime University (approval no: 05HI87-4, date: 2021/1/26). All experiments were performed following the Institutional Animal Care and Use Committee of the Ehime University School of Medicine and the guidelines and the Guidelines for the Proper Conduct of Animal Experiments. Efforts were made to minimize the number of animals used and their suffering. This study was conducted following the ARRIVE guidelines, and the relevant regulations and guidelines were adhered to.

MAIN POINTS

- Concentrated growth factor (CGF) treatment significantly improves the closure rate of tympanic membrane perforations (TMP) in a guinea pig model, with 57.1% closure on day 14 and 85.7% on day 28, compared to no closure in untreated group.
- The tympanic membrane regenerated with CGF was notably thicker (36.1 ± 9.9 μm) compared to the group without CGF group (21.4 ± 8.4 μm), indicating more effective tissue regeneration and possibly reducing the risk of reperforation.
- Concentrated growth factor application represents a minimally invasive alternative to traditional surgical treatments for TMP, offering a simpler and cost-effective option with a high rate of closure and regeneration.
- Histological analysis revealed that the tympanic membrane treated with CGF showed thicker regenerative tissue and greater connective tissue hyperplasia and angiogenesis compared to those without CGF treatments.
- This study supports the potential of CGF as a novel and effective treatment for TMP, suggesting it could be a valuable addition to current treatment options, reducing the need for more invasive surgical interventions.

Experimental Animals

Hartley guinea pigs (weight range: 400-550 g) were obtained from Japan SLC (Shizuoka, Japan) and housed in cages (1 animal per cage) in a laboratory at a controlled temperature (21-23°C) under a 12 h light/12 h dark cycle.

Preparation of Concentrated Growth Factors

Four guinea pigs were anesthetized using intraperitoneal injections of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (7.5 mg/kg). Approximately 9 mL of blood was collected from the heart into a glass test tube (no additive tube was used). The tube was immediately centrifuged in the centrifugal machine (Medifuge MF200, Silfradent srl, Forli, Italy) under the following conditions: 30 seconds of acceleration, 2 minutes at 2700 rpm, 4 minutes at 2400 rpm, 4 minutes at 2700 rpm, 3 minutes at 3000 rpm, and 36 seconds of deceleration. The centrifuged blood samples had 3 separate layers: an upper transparent layer of serum, a middle white turbid layer of CGF, and a lower layer of blood cells (Figure 1). Only the middle layer containing CGF was extracted and was cut and used for experiments. All the guinea pigs were euthanized by decapitation after blood collection.

Tympanic Membrane Perforations Model

Following the procedures described by Hakuba et al,[17] tympanic membrane perforations were created using a CO_2 laser (Lezawin II, MORITA, Japan). The guinea pigs were anesthetized using intramuscular injections of medetomidine (0.25 mg/kg), midazolam (1.0 mg/kg), and butorphanol (0.25 mg/kg). After anesthesia, the tympanic membrane was observed under a microscope, and perforations were created in the guinea pigs using a CO_2 laser (3 W, pulse duration of 0.3 seconds, single use) with an attachment for 4 W. The created TMP was large, and it covered all quadrants. The

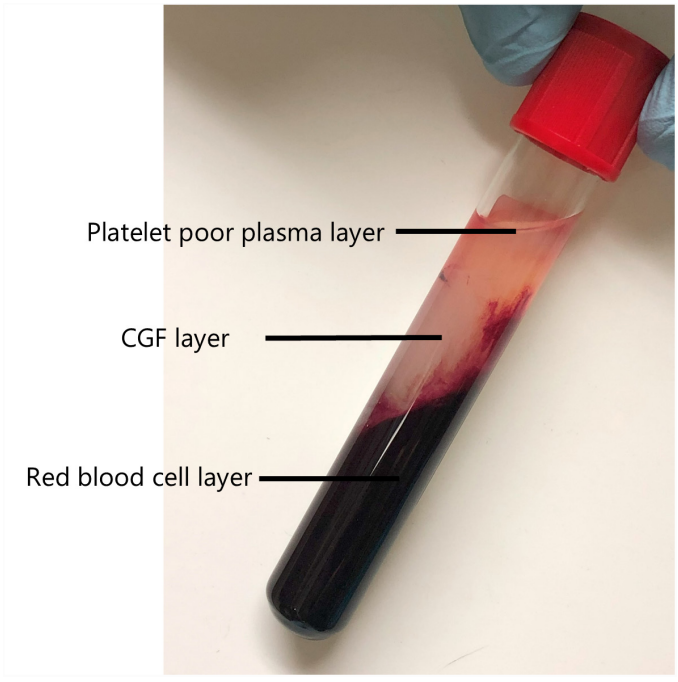

Figure 1. Concentrated growth factors (CGF). After centrifugation, 3 layers, which comprised an upper transparent layer, a middle white turbidity layer of CGF, and a lower layer of red blood cells, were observed in the tube.

laser was applied 2-3 times to each tympanic membrane. Tympanic membrane perforations spanning all the quadrants were created (Figures 2 and 3). The perforated ears were grouped into 3 groups: the CGF-gelatin sponge (Spongel, LTL Pharma, Japan) (with-CGF group), saline-gelatin sponge (without-CGF group), and no treatment (untreated group).

For the with-CGF group, shredded CGF was implanted to fill the space from the eardrum to the external auditory canal. They were covered with gelatin sponges to prevent drying and fibrin glue (Beriplast P combi-set tissue adhesion, CSL Behring, Australia) for stabilization. For the without-CGF group, saline and gel foam sponges were inserted into the tympanic cavity to cover the TMP, and fibrin glue was used to stabilize them. Tympanic membrane perforation was not administered to the untreated group.

Microscopic Observation and Histological Study

Tympanic membrane perforations were observed under a microscope on days 14 and 28, and the tympanic perforation was assessed for complete closure at each time point (Figures 4 and 5). On day 28, after observation of the tympanic membrane, the animals were euthanized by decapitation, and their temporal bones were obtained. They were immersed in 4% paraformaldehyde for 1 day (approximately 24 hours), washed in a stream of water for 30 minutes, and decalcified in ethylenediaminetetraacetic acid (10%, pH 7.0) for approximately 2 weeks. Subsequently, each sample was dehydrated using a series of graded alcohol concentrations, embedded in paraffin, cut into sections with a thickness of 4 μm, and stained with hematoxylin and eosin. The thickness of the regenerated eardrum was measured at 3 arbitrary points, and the average value was defined as the thickness of the tympanic membrane.

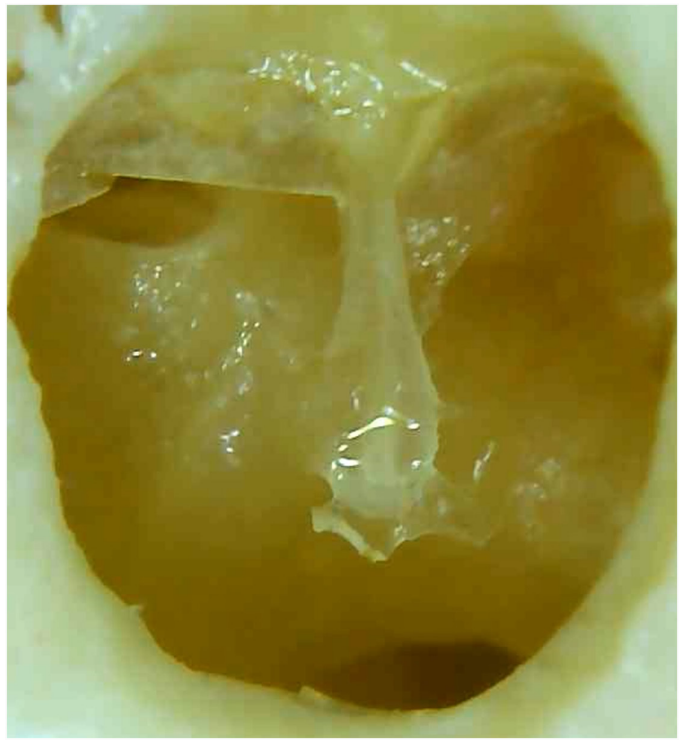

Figure 3. A tympanic membrane perforation.

Statistical Analysis

The parameters were compared for the groups using Fisher's exact test or the Mann–Whitney *U* test using JMP for Macintosh (SAS Institute Inc.; Cary, NC, USA). Statistical tests were based on a significance level of $P < .05$.

Figure 2. A tympanic membrane before creating perforation.

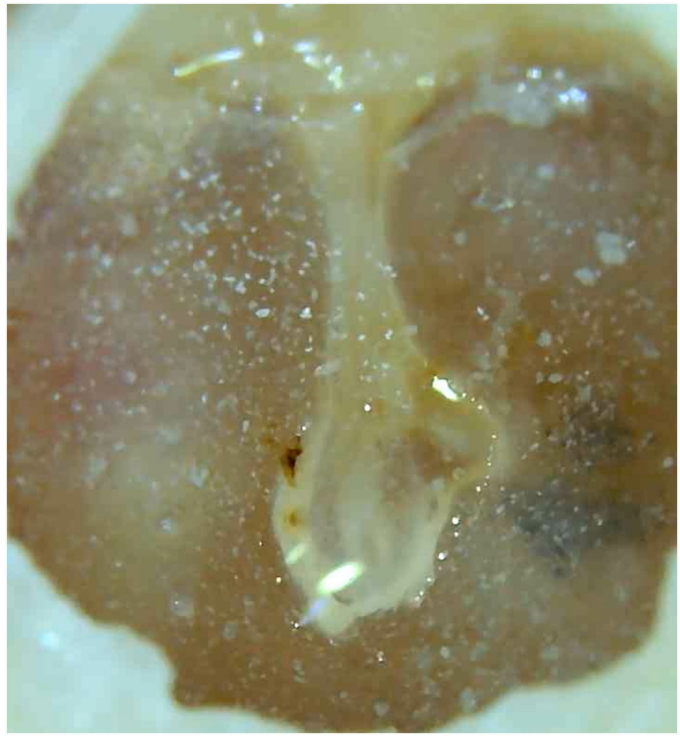

Figure 4. Closed case.

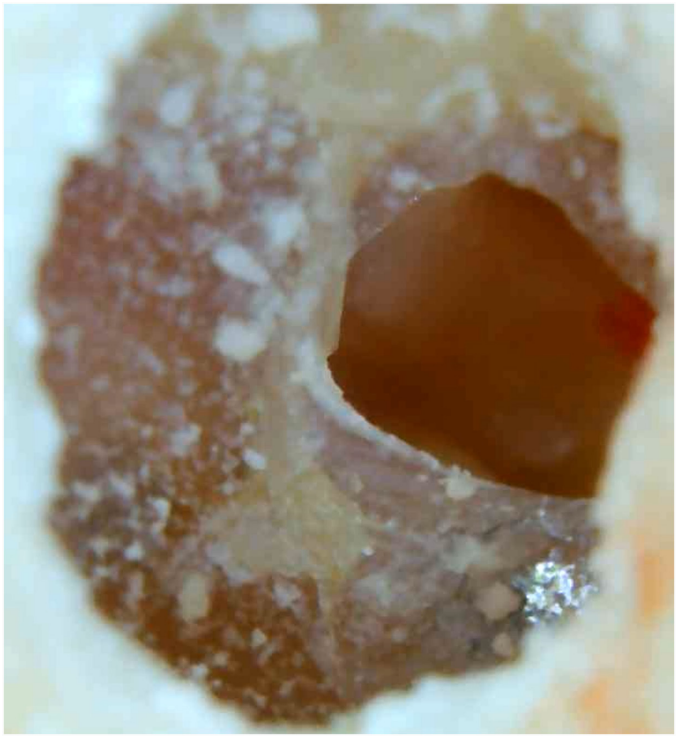

Figure 5. Unclosed case.

RESULTS

Closure Rate of Tympanic Membrane Perforations

Figure 3 shows the closure rate of the TMP on days 14 and 28. Overall, 8 (57.1%) of the 14 ears in the with-CGF group demonstrated closure of the TMP on day 14 (Figure 6), whereas no closure was observed in the without-CGF or untreated groups. The closure ratio was significantly higher for the with-CGF group than for the untreated group ($P = .002$). On day 28, 12 (85.7%) of the 14 ears in the with-CGF group and 8 (57.1%) of the 14 ears in the without-CGF group showed TMP closure (Figure 7), whereas no closure was observed in the untreated group. The closure rate ratio was not significantly different for the with-CGF and without-CGF groups ($P = .21$); however, it was significantly higher for the with-CGF group than for the without-CGF group ($P < .001$). Figure 3C shows the time course for the tympanic perforation closure. Significant closure of the tympanic perforation was achieved by day 14 in the with-CGF group, indicating an earlier closure.

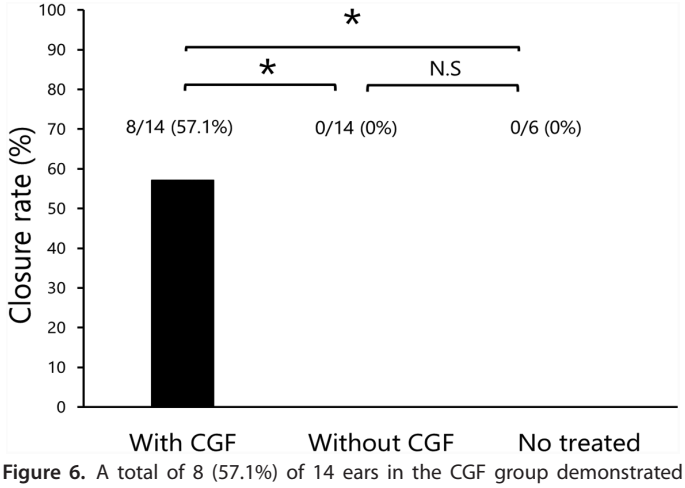

Figure 6. A total of 8 (57.1%) of 14 ears in the CGF group demonstrated closure of the TMPs on day 14, whereas no ears in the without-CGF and untreated groups showed closure of the TMP on day 14. The ratio of closure was significantly higher in the without-CGF and untreated groups ($P = .002$).

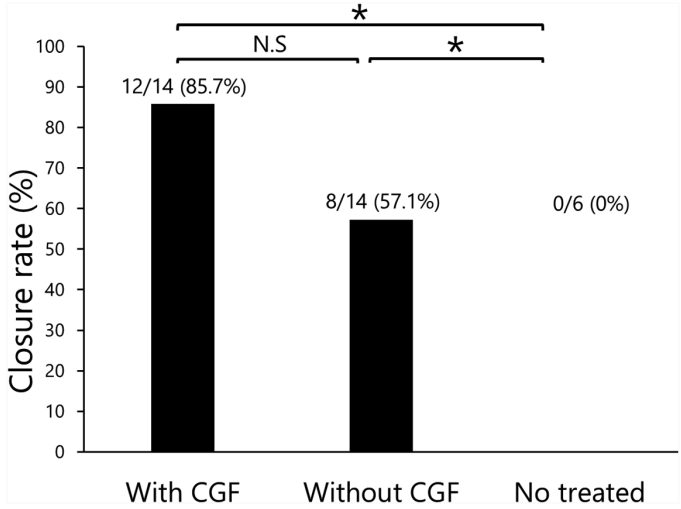

Figure 7. On day 28, 12 (85.7%) of the 14 ears in the with-CGF group and 8 (57.1%) of the 14 ears in the without-CGF group showed closure of the TMP, whereas no closure was observed in the untreatedtreated group. The ratio of closure rate was not significantly different for the with-CGF group and the without-CGF group ($P = 0.21$), whereas it was significantly higher for the with-CGF group than for the without-CGF group ($P < .001$). The time course of tympanic perforation closure rates.

Histological Observation

Figure 4 shows the results of the histological analysis of the regenerated tympanic membrane. The regenerative tympanic membrane was thin, and the fibrous layer was absent in the without-CGF group (Figure 8). In contrast, the regenerative tympanic membrane was thick in the with-CGF group, and connective tissue hyperplasia and angiogenesis were observed in the middle layer (Figure 9). Figure 2E shows the thickness of the regenerated tympanic membrane in the with-CGF and without-CGF groups. The average thickness of the regenerative tympanic membrane was 36.1 ± 9.9 μm for the with-CGF group and 21.4 ± 8.4 μm for the without-CGF group. The thickness of the regenerated tympanic membrane was significantly higher for the with-CGF group than for the without-CGF group ($P = .005$) (Figure 10).

DISCUSSION

We investigated the effect of CGF on a persistent traumatic TMP model using a guinea pig. Our results suggest that the TMP closure rate was significantly higher for the with-CGF group than for the without-CGF and untreated groups on day 14. While the closure ratio was not significantly different between the with-CGF and without-CGF groups on day 28, the regenerative tympanic membrane was substantially thicker for the former than for the latter. These results suggest that CGF promotes tympanic membrane proliferation and may be a novel treatment option for TMP.

Tympanic membrane perforation is generally treated surgically using a graft to reconstruct the tympanic membrane. Tympanoplasty and myringoplasty are frequently performed, and the outcomes after surgery are generally favorable, although they may vary depending

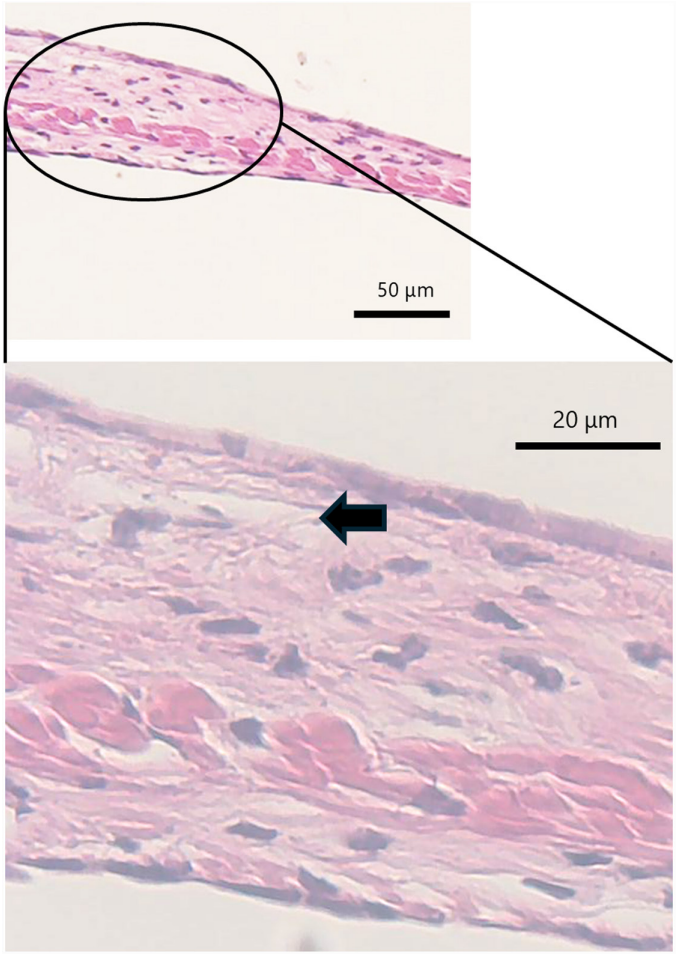

Figure 8. Histological study of the regenerated tympanic membrane. With-CGF group. Arrow shows neovascularization.

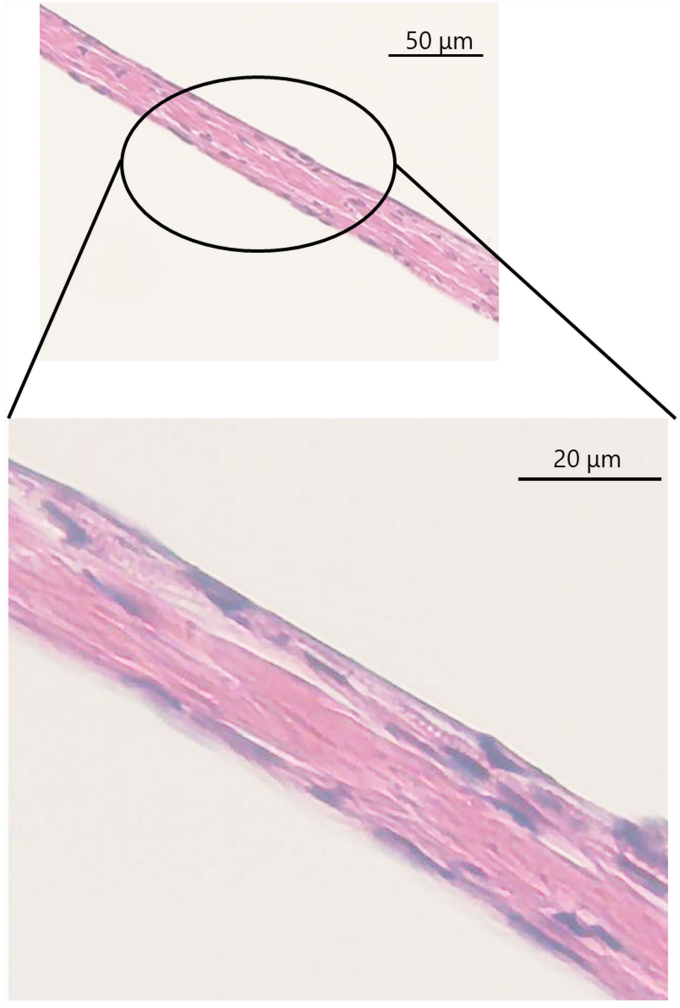

Figure 9. Histological study of the regenerated tympanic membrane. Without-CGF group.

on the healthcare facility.[18] These surgical procedures are invasive; they encompass the retrieval of autologous tissue via skin incisions, potential requirements for general anesthesia or hospitalization, and risk of surgical complications, leading to elevated healthcare expenses. Therefore, minimally invasive treatments are needed. The method used in this study is simple and reasonable.

Several attempts have been made to develop new treatments for TMP, with regulatory factors playing essential roles in promoting regeneration. Several studies have demonstrated the efficacy of TMP treatments, including bFGF, EGF, and PDGF.[3-5] A potent growth factor, bFGF, stimulates the proliferation and differentiation of endothelial cells, fibroblasts, and keratinocytes.[19] Studies comparing patients treated with bFGF to those who received no intervention, placebo, or alternative treatment have reported that bFGF treatment improved closure rates, shortened closure times, or both.[19-21] However, Santos et al[8] reported no significant differences in the closure rates or hearing improvement with bFGF treatment relative to placebo in a phase II trial involving 54 patients. Compared with traditional myringoplasty in patients with TMP secondary to chronic otitis media, bFGF treatment has a remarkably lower overall closure rate.[1] Epidermal growth factor, a 53-amino acid mitogenic polypeptide present in many mammalian species, has been investigated for its potential to accelerate the healing of TMP.[22] In clinical studies, EGF is considered a topical treatment for traumatic TMPs. These studies revealed that the topical application of EGF improved the closure rate and shortened the closure time of TMP.[23,24] However, evidence on the efficacy of EGF in chronic TMP secondary to chronic otitis media is lacking.[1] Platelet-derived growth factor, a family of closely related proteins, exists as approximately 30-KD disulfide-bonded dimers with A and B chains (PDGF-AA, AB, and BB). They act as potent mitogens for connective tissue cells and fibroblasts and promote the synthesis of fibronectin

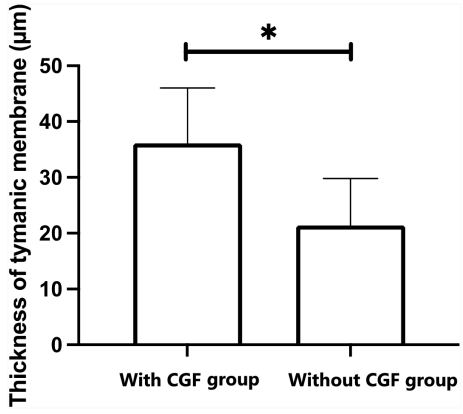

Figure 10. The thickness of the regenerated tympanic membrane in the with-CGF group was significantly higher than that of the without- group.

and hyaluronic acid. Additionally, it triggers the release of transforming growth factor β (TGF β), which initiates collagen production in fibroblasts and is secreted by various cell types, including platelet α-granules, endothelial cells, fibroblasts, smooth muscle cells, and macrophages.[5,25,26] Although Yeo et al reported that PDGF shortened the closure time of TMP in an animal study,[5] other reports have stated that it did not affect TMP treatment.[25,27] As mentioned above, regulatory factors, including bFGF, EGF, and PDGF, are effective for tympanic membrane regeneration. However, regeneration using only 1 factor has certain limitations.

Platelet-derived substances have been increasingly used in regenerative medicine. They have several advantages, including high growth-factor levels and safety. Since platelets are collected from patients, they are unlikely to cause allergies, thereby eliminating the possibility of rejection. Concentrated platelet preparations are first divided into platelet-rich plasma (PRP) and PRF, and CGF is a more advanced version of PRF. Platelet-rich plasma is an autologous biological product containing higher amounts of platelets compared to circulating blood and hence includes high concentrations of the following growth factors known to modulate the proliferation of various tissues and promote wound healing: PDGF, EGF, FGF, transforming growth factor-β1, insulin-like growth factor-1, and vascular epithelial growth factor.[28] Platelet-rich plasma is a novel and effective substance increasingly used for TMP repair. However, generating PRP is complicated and requires additives, anticoagulants, and bovine serum albumin.[29] In contrast, PRF, a platelet concentrate, requires no anticoagulant or thrombin but only centrifuged autologous blood.[30] Platelet-rich fibrin has been proven effective against TMP in animal studies.[10] In a clinical study of tympanoplasty for chronic dry eardrum perforation, the rate of graft survival was higher in the cartilage graft plus PRF group than in the cartilage graft without PRF group.[30] Concentrated growth factor is an advanced form of PRF in which no additives are required. It is easy to prepare and manipulate and is inexpensive. Compared with PRF, CGF contains a denser and richer growth factor-fibrin matrix. Furthermore, CGF has a 3D fibrin network in which growth factors bind to each other.[15,16] This network provides a slow release of growth factors and facilitates wound healing.[16,31] The duration of growth factor release was greater than 8 days, and growth factors were continuously released for 14 days in vitro.[11] Concentrated growth factor significantly outperformed PRP and PRF in promoting osteogenesis during the later stages.[16] This may be because the insoluble fibrin network provides a scaffold for cells and serves as a substrate for the continuous release of growth factors. Furthermore, cells are exposed to fibrin molecules that exhibit 3-dimensional cell–cell interactions. Compared with PRF, CGF has a higher fibrinogen level and a more stable fibrinogen network, which can prevent plasma-mediated degradation. This is attributed to the unique centrifugal process used for CGF.[16] Moreover, each growth factor contained in CGF demonstrates more than one effect and controls cellular processes such as cell migration and proliferation, ECM remodeling, and angiogenesis, which provide an ideal environment and promote wound healing. These properties are expected to create more favorable conditions for tympanic membrane regeneration.

In our histological findings, the regenerated tympanic membrane for the with-CGF group was significantly thicker than that for the without-CGF group, suggesting that the regeneration of the middle (fibrous) layer may have been accelerated. Although immunostaining was not performed in this study, the histological images showed a thickness clearly different from that of the without-CGF group. Growth factors have different properties for the regeneration of the tympanic membrane. Basic fibroblast growth factor is involved in the proliferation of the middle layer,[5] EGF in the proliferation of the epithelial layer,[17] and PDGF also promotes the regeneration of the middle layer.[5] Concentrated growth factor functions as a growth factor,[15] and its interaction may lead to earlier and thicker tympanic membrane regeneration. Spontaneously healed TMs tend to be thin and dimeric because of the incomplete formation of the fibrous layer,[4,32,33] resulting in a higher risk of reperforation, loss of stability, stiffness, and efficient vibration. Santos et al[8] found that within 2 months, 30% of patients (7/23) treated with bFGF and 20% of patients (2/10) who received a placebo initially experienced complete closure of their TMPs and subsequently developed reperforation. The thickness of the regenerated tympanic membrane is considered to be related to the reperforation of the tympanic membrane after closure of the perforations.[8] In our study, gelatin sponges were used to enhance the differences between the CGF and saline groups and maintain a better, wet environment for regeneration. Gelatin can be a good scaffold for the regeneration process and may have influenced tympanic membrane regeneration in this study.[34] Gelatin sponges are biomaterials commonly used in middle ear surgery and have been shown to be effective for TMP repair.[35] Gelatin has been shown to shorten the duration of closure in the gelatin and spontaneous closure groups but does not contribute to the closure rate.

This study has some limitations. First, the present study focused on a model of acute traumatic perforation of the tympanic membrane; therefore, chronic perforation of the tympanic membrane requires further investigation. Second, re-perforation was not considered in this study and requires further investigation, including long-term follow-up. Third, this study used gelatin for tympanic membrane regeneration, and the effect of CGF alone on TMP was not tested. An ideal TMP closure has the necessary elements: it is a simple procedure that is less invasive, provides early closure, has a high closure rate, is safe, improves hearing, and is inexpensive. We found that CGF is a simple, minimally invasive, and early closure technique. Therefore, it is considered to be more effective for TMP.

CONCLUSION

In conclusion, CGF can induce the regeneration of the tympanic membrane, especially the fibrous layer, and can be used to conservatively treat TMP.

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: This study was approved by the Ethics Committee of Ehime University (approval no.: 05HI87-4; date: January 26, 2021).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – T.H., M.O., N.H.; Design – T.H., M.O.; Supervision – M.T., N.H.; Resources – T.H., M.O.; Materials – T.H., M.O., T.N.; Data Collection and/or Processing – T.H.,T.N., E.N., S.A.; Analysis and/or Interpretation – T.H., M.O.; Literature Search – T.H., M.O., M.T.; Writing – T.H., M.O., M.T.; Critical Review – N.H.

Declaration of Interests: The authors have no conflicts of interest to declare.

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