Regeneration of chronic tympanic membrane perforation using 3D collagen with topical umbilical cord serum

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ABSTRACT

Chronic tympanic membrane (TM) perforation is one of the most common otology complications. Current surgical management of TM perforation includes myringoplasty and tympanoplasty. The purpose of this study was to evaluate the efficacy and feasibility of three dimensional (3D) porous collagen scaffolds with topically applied human umbilical cord serum (UCS) for the regeneration of chronic TM perforation in guinea pigs. To achieve this goal, we fabricated porous 3D collagen scaffolds (avg. strut diameter of 236 ± 51 μm, avg. pore size of 382 ± 67 μm, and a porosity of 96%) by using a 3 axis robot dispensing and low temperature plate systems. Guinea pigs were used in a model of chronic TM perforation. In the experimental group (n = 10), 3D collagen scaffold was placed on the perforation and topically applied of UCS every other day for a period of 8 days. The control group ears (n = 10) were treated with paper discs and phosphate buffered saline (PBS) only using the same regimen. Healing time, acoustic-mechanical properties, and morphological analysis were performed by otoendoscopy, auditory brainstem response (ABR), single-point laser Doppler vibrometer (LDV), optical coherence tomography (OCT), and light microscopic evaluation. The closure of the TM perforation was achieved in 100% of the experimental group vs. 43% of the control group, and this difference was statistically significant (p < 0.034). The ABR threshold at all frequencies of the experimental group was significantly recovered to the normal level compared to the control group. TM vibration velocity in the experimental group recovered similar to the normal control level. The difference is very small and they are not statistically significant below 1 kHz (p = 0.074). By OCT and light microscopic examination, regenerated TM of the experimental group showed thickened fibrous and mucosal layer. In contrast, the control group showed absence of fibrous layer like a dimeric TM.

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1. Introduction

Chronic tympanic membrane (TM) perforation due to otitis media or trauma has long been regarded as the most common complication of otological diseases. Repeated exposure to pathogens can lead to recurrent, acute otitis media with consequent permanent alteration of the middle ear and the sound-transmitting mechanism, and/or active, chronic otitis media with otorrhea that is refractory to treatment [1]. The main goal of closing a chronic tympanic membrane (TM) perforation is to prevent recurrence of active disease or to improve the hearing.

The majority of acute TM perforations can spontaneously recover; however, chronic TM perforations are known to be incapable of spontaneous healing without surgical intervention. Autologous tissue tympanoplasty (fascia or perichondrium) increases the success rates, but requires more invasive technique and greater operative time. Many materials have been used as a scaffold, including paper patch [1], gelfoam [2], and Alloderm [3], silk fibroin scaffold, and porcine-derived acellular collagen [4] in acute TM perforation. However, it is difficult to treat a chronic TM perforation due to decreased regenerative activity of the TM at the margin of the perforation. In an effort to overcome this drawback, the use of the novel adjuvant scaffolds such as collagen [5], calcium alginate [6], silk [7], and chitosan [8–11] have been tried as patch. However, only bioscaffold showed a limited rate of healing in a chronic TM perforation study, similar to the rate of the paper patch technique. To enhance the healing of chronic TM perforations, biomolecules including growth factors [12,13], hyaluronic acid [14], and pentoxifylline [15] have been used.
together with good results. To date, chemically recombinant growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet derived growth factor (PDGF) have been mainly used combined with bioscaffold for TM regeneration [16,17].

Recently, Kakehata et al. reported good results by using a new treatment for closing chronic TM perforation using autologous serum [18]. Both autologous serum and umbilical cord serum owe their efficacy to the presence of various growth factors, like EGF, acidic and FGF, PDGF, hepatocyte growth factor, vitamin A, transforming growth factor (TGF)-β, substance P, insulin-like growth factor (IGF)-1, nerve growth factor (NGF), fibronectin, and serum antiproteases, such as o2-macroglobulin [19–21]. The concentrations of EGF, TGF-β, and NGF are several times higher in umbilical cord serum than peripheral blood serum [22,23]. Vajpayee et al. [20] used the first trial of umbilical cord serum (UCS) therapy for persistent corneal epithelial defects. Their results revealed that umbilical cord serum induced faster healing compared to autologous serum.

According to Hakuba et al. [13], collagen scaffold supplemented with basic fibroblast growth factor was effective for closing chronic TM perforations. Collagen is well known to be the most promising natural biomaterial and has been examined for many applications in tissue engineering because of its abundance, ubiquity, and biocompatibility [24]. Although collagen is an excellent candidate material for closing TM perforation, its low shape-controllability (pore size, pore interconnectivity, porosity, etc.) remains important limitations. To address these problems, a new processing method of collagen should be necessary. In general, the 3D scaffolds should play the role of the extracellular matrix (ECM); as a result, they need various biological and physical properties to obtain successful tissue regeneration [25,26]. In the TM regeneration, 3D scaffold has been rarely used, only Kim et al. [10] used 3D chitosan scaffolds for the first time. The 3D chitosan scaffolds showed good TM cell viability in vitro with acceleration TM regeneration in vivo.

In this study, we fabricated 3D collagen scaffolds having precisely controlled pore structures (pore size, pore interconnectivity, and porosity), which are consisted of successive layers of perpendicular collagen struts, by using a 3-axis dispensing and innovative low temperature plate system. In particular, pore structure of the scaffold can highly affect the rate and depth of cellular in-growth in vitro and in vivo, cell morphology, and even phenotypic expression [27,28]. From this reason, the 3D collagen scaffold having a homogeneous pore size was tried to regenerate TM. In addition, we also chose a combination of 3D collagen scaffold and umbilical cord serum as a regulation factor. This work can present the first trial of hUCS combined with a 3D collagen scaffolds for closing chronic TM perforation in guinea pigs.

2. Materials and methods

2.1. Fabrication of 3D collagen scaffolds

Type-I collagen (Matrixen-PSP; Bioland, South Korea) was used for scaffold fabrication. A collagen solution was prepared at a fixed concentration of 4.5% (w/v) in 0.05 M acetic acid (pH 3.2). 3D collagen scaffold was fabricated using a 3D dispensing machine (AD-3000 C; Ugin-tech, Siheung, South Korea) supplemented with a low temperature plate. In Fig. 1, a schematic for the fabricating apparatus was described. To fabricate pore-structure controlled collagen scaffold, the collagen solution was extruded by computer-driven dispensing apparatus on the low temperature plate (Fig. 1). The temperature of the dispensing plate was fixed at −10 °C. The collagen solution was laid with a layer-by-layer manner through a 300 μm-diameter plotting needle moving at 10 mm s⁻¹. To extrude the solution, applied pneumatic pressure (135 ± 7 kPa) was used. After dispensing the solution, the fabricated collagen scaffolds were immediately placed in a freeze-dryer (FD, SFDSM06, Samwon, South Korea) at −75 °C for 2 days and then those were cross-linked in 50 mM 1-ethyl-(3-3-dimethylaminopropyl) hydrochloride (EDC) solution in 95% ethanol for 12 h at room temperature. The final collagen scaffold was washed several times with PBS solution.

2.2. Characterizations of collagen scaffold

The morphology of the collagen scaffolds was observed under an optical microscope (BX FM-32; Olympus, Tokyo, Japan) equipped with a digital camera and by scanning electron microscopy (Sirion; FEI, Hillsboro, OR). Before scanning electron microscopy observation, the scaffolds were sputter-coated with gold. Sample preparation and measurements were performed according to the manufacturer’s instructions. The scaffold porosity was calculated using the equation 1 – M/ρs, where M is the mass of the scaffold, ρ is the collagen density, 1.3 g/cm³, h is the scaffold thickness, and s is the surface area of the scaffold. The final geometry of the scaffold was measured using a digital caliper micrometer. The scaffold was weighed with a precise balance (AD-4 autobalance; Perkin-Elmer, Waltham, MA).

The mechanical properties of the collagen scaffolds were measured using the tensile test mode. The scaffolds were cut into small strips (10 mm × 20 mm × 1.2 mm). For the scaffold, five samples were obtained from different sites. The uni-axial test was characterized using a UTM (Top-tech 2000; Chemilab, Suwon, South Korea). The stress–strain curves of the collagen scaffold were recorded at a stretching speed of 2 mm s⁻¹. All values are expressed as the mean ± standard deviation (n = 5).

Fourier-transformed infrared spectra (FT-IR) were measured before and after the cross-linking process of the collagen scaffold. The infrared spectra of the collagen were measured using the attenuated total reflection mode on a 6700 FT-IR spectrometer (Nicolet, West Point, PA). Data were acquired over the range of 4000–400 cm⁻¹ for 30 scans at a resolution of 8 cm⁻¹.

2.3. In vitro cell culture

Collagen scaffolds for use with cell cultures, 10 mm × 10 mm × 1.3 mm, were sterilized with 70% EtOH and...
UV light, and placed in culture medium overnight. Normal human dermal fibroblast cells (NHDFs, MCTT, Korea) were used to observe cell viability in the scaffolds. The fibroblasts were cultured in fibroblast growth medium (FGM) containing 2% fetal bovine serum (FBS), 0.1% insulin, recombinant human fibroblast growth factor-B (rhFGF-B), and 0.1% GA-1000 antibiotic solution (Lonzan, USA). The cells were maintained up to passage 2 and collected by trypsin-EDTA treatment. The cells were then seeded onto the scaffolds at a density of $1 \times 10^5$ cells per sample and incubated in an atmosphere of 5% $\text{CO}_2$ at 37°C.

2.4. Creation of chronic TM perforation

Experiments were performed in twenty five male guinea pigs (weighing 250 g each, purchased from Samtaco Bio Korea, Osan, Korea) with normal tympanic membranes and Preyer’s reflexes. The guinea pigs were housed in rooms with a constant temperature of 22°C, humidity of 50%, and an ambient noise level <40 decibels (dB). All animal experiments were performed in accordance with local ethical committee at the Research Center for Resistant Cells, Chosun University.

The guinea pigs were anesthetized by the intraperitoneal injection of zollettil1 (1:1 combination of tiletamine and zolazepam) and xylazine hydrochloride. Ear canals and the tympanic membranes were examined using an operating microscope. Ear infection was excluded by the examination of the external auditory canal and the tympanic membrane. Our method is a modification of the method originally described by Cui et al. to create chronic TM perforations [29]. Bilateral TM perforations of 60% in size were created at the anterior area of pars tensa under the surgical microscope using thermal myringotomy with monopolar needle tip (30 W, Electro-surgical unit, ITC, Daejeon, South Korea) in twenty guinea pigs (40 ears). Five guinea pigs (10 ears) were preserved as normal control. Following thermal myringotomy, a small mitomycin-C-soaked piece of gelfoam (0.2 mg/mL) was placed for 5 min over the perforation edges, and thereafter removed. Postoperatively, 100 µL of hydrocortisone with ciprofloxacin (CIPROCIN HC otic Suspension, Han Lim Pharm Co., Seoul, South Korea) was topically dropped using a syringe twice per day for 7 days. At 3 weeks, ears were reinspected and further cautery was applied if any degree of closure had occurred to maintain 60% sized central perforation. TM perforations were left undisturbed for a period of 6 weeks, with no further sign of healing evidence. After 6 weeks, 95% (2/40 ears) of the perforation were still open.

2.5. Application of 3D collagen scaffold with umbilical cord serum

Guinea pigs were randomly divided into 2 groups: control group ($n = 10$, paper patch) and experimental group ($n = 10$, 3D collagen with umbilical cord serum). Prior to insertion of the collagen patch, the perforation edges were freshened sharply by curved pick for the prevention of avoiding folding the epithelial edge into the middle ear. In the experimental group, 3D collagen was placed over the perforation and human umbilical cord serum (kindly donated from JB Stem Cell Research Center, Gwangju, South Korea) 0.2 ml was topically applied at 2, 4, 6, and 8 days post-patch in the experimental group. The control group ears were treated with paper discs after trimming of perforation edges and topically applied 0.2 ml phosphate buffered saline (PBS, pH 7.4, Gibco), only using the same regimen.

2.6. Otoendoscopic observation

To examine the healing process, each TM was observed by otoendoscopy every other day under the intraperitoneal injection of zollettil1 (1:1 combination of tiletamine and zolazepam) and xylazine hydrochloride; if necessary, external auditory canal was infiltrated using lidocaine HCl and epinephrine (1:100,000, Xylocaine®, USP, USA).

2.7. Hearing testing

Hearing sensitivities were measured using auditory brainstem response (ABR) thresholds. ABRs were assessed preoperatively before, and 4 weeks after application of TM scaffold. The animal was placed in a soundproof booth and anesthesia was performed using intraperitoneal injection of cocktail (Zollettil/xylazine), owing to the need to isolate the guinea pigs in a sound proof field. Auditory brainstem response (ABR) was recorded using an evoked potential system (Tucker-Davis Technologies, Alachua, FL, USA) and a Samsung computer. Stimuli were digitally synthesized using Sigen® software and were presented through an ER-2 insert earphone (Etymotic Research, Elk Grove Village, IL, USA). Acoustic stimuli consisting of click (low frequencies less than 4 kHz) and 4, 8, 16, and 32 kHz tone bursts were produced. Tone bursts consisted of a 3 ms envelope consisting of a 1 ms ramp onset, 1 ms plateau, and 1 ms decay. ABR was recorded through Grass® stainless steel needle electrodes placed subcutaneously at the vertex (active), right cheek (inverting), and left cheek (common). The resulting signal was band-pass filtered (100–3000 Hz), amplified (10,000×), and digitized by a TDT Bioamp (Tucker Davis Technologies). Responses were collected and averaged at 30 presentations per second for up to 512 times. The stimulus was presented at 90 dB SPL and progressed downward in 10 dB steps until no response was identifiable. A separate model was used for each frequency. The ABR threshold shift at each frequency was compared among the groups, using one-way analysis of variance (ANOVA) and a 2-tailed t test was used to identify differences between the individual groups. $p < 0.05$ defined as statistical significance.

2.8. In vivo single point laser Doppler vibrometer (LDV)

Regenerated tympanic membrane vibration was measured by LDV. We measured the umbo velocity. Stimuli were frequency sweeps from 0.1 to 10 kHz at a 90 dB sound presentation level (SPL). An earphone of a TDT workstation (ABR/OAE Workstation, Tucker-Davis Technologies, Alachua, FL USA) was the sound source and was placed within 2–3 mm of the eardrum, through the opening of a special designed sound coupler that had two openings. Ear canal pressures were measured with an ER-7 C microphone (Etymotic Research, Elk Grove Village, IL USA) with the probe tube situated 2 mm from the tympanic membrane through the opening of the sound coupler. The outer surface of the speculum was covered by a glass cover slip. Umbo velocity was measured with a LDV (CLV-700 sensor head, HLV-1000 vibrometer controller, Polytec, Waldbronn, Germany). The LDV was mounted onto a Leica microscope and was used to focus a helium–neon laser onto the target. The targets were 0.5 mm² of foil with reflective polysyntrene microbeads, weighing less than 0.05 mg (3 M, Minneapolis, MN USA). Output voltages of the vibrometer and the microphone were analyzed using FFT (Fast Fourier Transform) analyzer software (SigCal, Tucker-Davis Technologies, Alachua, FL USA) on a computer. Mann–Whitney U test was performed.

2.9. Ex vivo optical coherence tomography (OCT)

In SD OCT system, a broadband light source having a center wavelength of 840 nm and a spectral bandwidth of 62 nm were used (Superlum SLD-35-HP). The light was split into reference and sample beams by using a 50:50 optical coupler (Thorlabs FC850-40-50-APC). Sample and reference beam are reflected at reference mirror and scattered from sample, respectively. Then the two
beams are combined and create interference pattern that deliver to home-made spectrometer and form a spectral interferogram on the CCD (Atmel Avila SM2 CL 2014). After performing the Fast Fourier Transform (FFT), OCT image was obtained. The experimental axial resolution is 8 μm.

2.10. Light microscopic examination

For histological analysis, healed TMs were obtained from guinea pigs and were analyzed by a light microscope (Olympus, Japan). The thickness of the regenerated TM was measured by image measurement system (Image inside, Focus Tech, Daejon, Korea).

3. Results

3.1. Morphological characterization of 3D collagen scaffold

Fig. 2(a and b) shows the surface and cross-sectional SEM images of the finally fabricated scaffold, respectively, which had a strut diameter of 236 ± 51 μm, a pore size (distance between struts) of 382 ± 67 μm, and a porosity of 96%. As shown in the images, the layer-by-layered structure of the collagen micro-strut was definitely well designed and pore size was homogeneously fabricated. The magnified images of Fig. 2(a) show that the surface of the collagen scaffold was fully roughened through the freeze-drying process. The cross section of a collagen strut demonstrates the highly porous structure of the collagen struts (Fig. 2(b)).

3.2. FT-IR results before and after cross-linking of collagen

Fig. 3 shows FT-IR spectra before and after cross-linking the collagen scaffold. In the spectrum, the N–H stretching vibration peak for the amide A is approximately 3324 cm⁻¹ and amide I band (1630 cm⁻¹), amide II bands (1543 and 1452 cm⁻¹) and amide III bands (1211, 1235 and 1268 cm⁻¹) are visible. As shown in the IR spectra, the positions of peaks before and after cross-linked collagen are located in the completely same points, but the area (or intensity) of the IR bands for cross-linked collagen is much smaller compared to uncross-linked collagen. In general, the position of IR spectra for the cross-linked collagen could be same with the uncross-linked collagen because the secondary structure of collagen is not destroyed [30]. Also, amide A and OH band (3700–3100 cm⁻¹) can be much smaller for cross-linked collagen because the cross-linked collagen loses water bonded to the collagen [30]. From these IR results, we can convince that the porous collagen was well cross-linked.

3.3. Biodegradability of the 3D collagen scaffold

The biodegradability is one of important parameters of the biomedical scaffolds to regenerate various tissues. From the reason, we measured the weight change of the 3D collagen scaffold was measured in PBS solution for 2 and 5 days at 37 °C and the biodegradability was assessed by determining the weight loss. The weight loss was about 1.8 ± 1% on day 2 and 2.9 ± 2% on day 5. The result indicates that the collagen scaffold can degrade slowly. However, since the degradation of collagen scaffold is entirely dependent on the cross-linking degree, we will try to determine the effects on the biodegradability of the collagen scaffold in near future.

3.4. Mechanical properties of 3D collagen scaffold

Appropriate mechanical properties are highly important for biomedical scaffolds because the mechanical sustainability in the regenerative area can affect its biological function and/or the activities of the injected cells. Young’s modulus of the 3D collagen scaffolds after cross-linking process was 0.43 ± 0.12 MPa. Ultimate tensile strength and strain at break were 0.02 ± 0.01 MPa and 9 ± 3%, respectively. Although this measured value can be applicable only in the regeneration of chronic tympanic membrane
perforation, since the mechanical properties of the collagen scaffold is still low for various soft tissue regenerations, further studies are still required to enhance the mechanical properties of the collagen scaffolds to expand their practical applications by accommodating various synthetic polymers and bioactive ceramics as mechanical supporting structures and/or reinforcing materials.

### 3.5. Fluorescence images and cell-viability

Fig. 4(a–c) shows fluorescence images of the lyophilized 3D spongy and 3D collagen scaffolds cultured for 1 and 3 days. Attached cells (fibroblasts) were stained with calcein AM and ethidium homodimer–1 to identify live (green) and dead (red) cells. As shown in the fluorescence images, the cells proliferated well on both collagen scaffolds as increasing the culture period and the cell-viability, which was calculated through the Image J program (NIH, Bethesda, MD, USA), showed high value (93 ± 5% for spongy scaffold and 96.4 ± 2% for 3D scaffold) for both scaffolds. These results represent the 3D collagen scaffold can provide good cell viability and proliferation.

### 3.6. Otoendoscopic observation of the TM regeneration

As shown in Fig. 5(a and b), remained pores of the control and experimental groups were still existed at 7 days. There were no complete TM regeneration in both the control and experimental
groups. However, after 3 weeks, 82% of the experimental group showed complete regeneration of TM in the experimental group compared to 39% in the control group. Based on this simple observation, TM regeneration of the experimental group was statistically better than the control group at 3 weeks ($p = 0.025$) and at 4 weeks ($p = 0.016$). Fig. 6(a–c) showed a normal TM, regenerated TM using the control and 3D collagen scaffold, respectively, after 3 weeks. In the experimental group, all TM perforations were closed between 3 and 4 weeks post myringotomy, as judged by otoscopy. As shown in Fig. 7, the closure of the TM perforation was achieved in 100% of the experimental group vs. 43% of the control group, and this difference was statistically significant ($p = 0.034$).

3.7. Hearing recovery

Fig. 8 shows the ABR results. The ABR threshold at all frequencies of the experimental group was significantly recovered to the normal level compared to the control group. There was statistical significance between the control and normal groups ($p = 0.042$).

3.8. Tympanic membrane nanovibration using LDV

Change of tympanic membrane vibration in the control group significantly reduced the velocity in the low frequencies below 0.6 kHz compared to the normal group ($p = 0.034$). However, TM vibration velocity in the experimental group recovered similar to the normal control level. The differences are very small and they are not statistically significant below 1 kHz ($p = 0.074$) (Fig. 9).

3.9. OCT findings

Images were obtained from the normal, experimental, and control groups. The thickness of the experimental group revealed higher than that of the control group. The regenerated TM of the experimental group showed thickened fibrous and mucosal layer compared to the control group, which showed absence of fibrous layer like a dimeric TM (Fig. 10).

3.10. Light microscopic findings

Comparison of regenerated TM thickness between the two groups is shown in Fig. 10. Increased thickness in perforation site in the experimental group (22 μm) compared to the control group (4 μm, $p = 0.023$) (Fig. 11).

4. Discussion

Chronic TM perforation, due to otitis media or trauma, has long been regarded as the most common complication of ontological diseases. In order to accelerate the closure of TM perforation, research has targeted two different mechanisms. One is to provide additional stromal support for the guiding regenerating tissue. The other is to induce cellular replication and migration. The current concern is which biomaterials (or scaffolds) are the best choice regarding high susceptibility to support cell adhesion, proliferation, and differentiation, as well as their ability to promote tissue repair in vivo and consequently, repair the TM perforation [31,32]. Most TM perforations in animals, including chinchillas or rats, normally heal within 7–14 days [33]. The defined time period of chronic TM perforation in animals is controversial by researchers. There is a shortage of papers described definition. Weber et al. [6] defined the time periods as 6 weeks following TM perforation using chinchilla. Santa Maria et al. insisted that the perforation should be maintained for at least 8 weeks following intervention to be able to be defined as chronic and recommended rat as an ideal animal model [34]. Choi et al. followed Santa Maria’s recommendation for creation of
rat chronic TM perforation. In the present study, we used guinea pig instead of rat. The reason why guinea pigs were selected in the present study was obvious that external auditory canal (EAC) of rat is smaller than guinea pig. In the present study, we measured the nanovibration of the healed TM using laser Doppler vibrometer, and TM morphology using OCT. For measurement these devices’, guinea pig or rabbit is better than rat because of its small EAC. Of course rat is inexpensive and more tolerant to procedures under anesthesia. We followed the Weber's defined period because EAC and TM of guinea pig are more close to chinchilla than rat.

According to Babu et al. [35], using a high concentration of mitomycin C (>0.25 mg/ml) resulted in ototoxicity, with an increase in the auditory brainstem response threshold at 4 weeks and at 8 weeks. However, at low concentrations (<0.20 mg/ml), no change in auditory brainstem response threshold was noted. In the present study, we followed Babu et al.’s recommendation. Our dose of mitomycin C was 0.2 mg/mL and the topical steroid application for the potency of perforation is some different. In the present study, topical hydrocortisone was dropped every-other-day basis for 10 days instead of gelfoam impregnated steroid packing at the perforation. Because the EAC is opened to air environment, impregnated gelfoam is easy to dry up. And drug impregnated gelfoam is easy to deliver within several hours [36].

To date, biomaterials as artificial TM patches showed a very high closure ratio in cases of the acute TM perforation. However, regeneration efficacy is being challenged in the cases for chronic TM perforations [10].

In tissue engineering, one of the critical factors has been scaffold design. To create an ideal scaffold for inducing skin regeneration, it should possess excellent biocompatibility, suitable micro-pore structure (e.g., mean pore size of 100–300 μm with >90% porosity), controllable biodegradability, and proper mechanical properties [37,38]. Kim et al. reported the efficacy of 3D porous chitosan scaffolds for TM regeneration. In particular, number of studies demonstrated that the 3D porous scaffolds using chitosan were effective for tissue-engineering applications such as bone, skin, cartilage, liver, nerve, and blood vessel in vitro and in vivo [39].

From the perspectives, collagen has been one of the typical tissue engineering materials, particularly for TM regeneration [12,40]. Collagen scaffolds have been widely used to promote cell and tissue attachment and growth [41–43]. However, like the most natural biomaterials (e.g., alginate and silk fibroin), it is difficult to obtain...
a 3D collagen scaffold having a precisely controlled pore structure due to the low process-ability at room temperature and high hydrophilic nature of the collagen. In general, porous 3D collagen scaffolds have been fabricated using freeze-drying and critical-point-drying methods. However, the fabricated collagen scaffold has a broad pore-size distribution; moreover, the controllability of pore structure within the scaffold was impossible. To overcome these fabrication challenges, an indirect solid free form fabrication and low temperature direct plotting method was used [44,45]. In recent, we fabricated pore-size controlled collagen scaffolds and we used it in skin tissue regeneration. In this work, the in vitro keratinocyte/fibroblast co-culture results revealed that fibroblasts were well dispersed within the scaffold, and keratinocytes had completely migrated through the well-designed pore structure and differentiated on top of the scaffold surface [46].

In this study, we fabricated collagen scaffolds having precisely controlled pore structures (pore size, 382±67 μm; >96% porosity) and successive layers of perpendicular collagen struts (236±51 μm in diameter), by using a low temperature plate system. Recent advances in developmental biology and tissue engineering provide the opportunity to repair damage or lost tissues with cells supplied from exogenous sources. Combined biomolecules such as growth factors and an artificial bioscaffold as a TM patch to repair chronic TM perforations have been tried clinically [12,13,18,40].

Recently, the use of UCS in the form of eyepatches has been reported as a new treatment for severe ocular surface disorders [20,21]. UCS contains many growth factors, such as EGF, TGF-β, IGF-1, and NGF [22]. Besides these growth factors, UCS contains vitamin A and substance P [23]. Substance P also can accelerate tissue regeneration [47]. In addition, UCS derived mesenchymal stem cells modulate the immune systems, in part through mediator secretion and it lacks antigenicity [48].

Here, we present the first trial of UCS with the 3D collagen scaffold for TM regeneration. The healing process of the TM is not similar to that of other cutaneous structures. The acoustic-mechanical competence of the TM also needs to be evaluated for proper functioning of hearing. From the reason, hearing threshold by ABR and mechanical properties by measuring nanovibration should be performed.

In the experimental group using the 3D collagen scaffold, acoustic-mechanical properties restored to the normal compared to the control group. The umbo is used as the best site for transcanal measurement of the manubrium vibration. In addition, measurement of the umbo vibration also provides an indirect measure of stapes vibration, if the ossicular chain is intact [49]. Conductive hearing loss caused by TM perforation was frequency-dependent; the largest hearing loss at low frequencies [50,51]. In the control group, dimeric healed perforation did not recover the vibration velocity losses under 1 kHz. We evaluated the healed TM by OCT. OCT is an excellent tomographic imaging modality that is used to provide non-destructive, non-invasive real time imaging with micro-scale high resolution. OCT relies on differences in tissue optical properties to generate contrast, with axial resolution on the order of 10 μm and of a depth of penetration, of approximately 1–2 mm, depending on turbidity. Most OCT applications in the middle and inner ear have focused on either animal investigations or human temporal bone studies [52–54].

In our result, reformation of the fibrous component of the TM was incomplete in the control group. Paper patching has a fairly low success rate. In contrast, increased thickness in the experimental group was suggested due to ingrowth of fibroblasts and extracellular matrix. It may be that over a longer time period, the fibrous layer will become reorganized in a more efficient way, similar to that of the normal lamina propria, and thereby, the thickness of the TM may be restored. Our results demonstrated that 3D collagen scaffold with UCS provides rapid and effective mean to repair moderate- sized chronic TM perforations.

5. Conclusion
In summary, this study showed that 3D porous collagen scaffold with topically applied UCS significantly enhanced the regeneration of chronic TM perforation and achieved acoustic-mechanical recovery from an early stage compared to the paper patch. The healed TM showed comparable thickness to a normal TM. From these results, 3D collagen scaffold with topically applied UCS can be used in the treatment for chronic TM perforation in the future instead of time-consuming and expensive surgery.

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