Application of a Collagen Patch Derived from Duck Feet in Acute Tympanic Membrane Perforation

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Abstract We investigated the utility of the duck-feet collagen extraction patching procedure in the traumatic tympanic membrane (TM) perforation via a comparison with spontaneous healing or paper patch. Fifty-six ears of adult male Sprague-Dawley rats, each weighing in the range of 250 to 300 g, were used for the animal studies. Sixteen rats had one-side ear in the control group and the opposite-side ear in the treated groups. The remaining twelve rats had a one-side ear with the duck-feet collagen patch and the opposite-side ear with a paper patch. The proliferating cell nuclear antigen (PCNA) expression cells were calculated among the 200 basal cells, and the expression percentage was identified as the labeling index. The healing of the perforation in the duck-feet collagen patch group was confirmed to be more rapid compared to the healing of the other groups. PCNA staining was observed in the migrating portion of PCNA enhanced cell to collagen scaffold in Post operative day (POD) 7 of collagen patch group. Thus, the adhesive effect of the duck-feet collagen patch to perforated margin was better than that of the paper patch. After completing the healing process, the collagen patch shrank and detached from the tympanic membrane (POD 14). In this study, we confirmed that the use of a duck-feet collagen patch had the advantage of early healing, inducing natural TM contour, and disappearing ability after the patch effect is complete.

Keywords Middle ear · Tympanic membrane perforation · Duck's feet · Fibroin

1 Introduction

Numerous studies have been reported regarding proper management of traumatic ear tympanic membrane (TM) perforation. Although high rates of spontaneous recovery following conservative management of traumatic TM

perforation are reported in many studies [1–3]. But, specific TM conditions, such as large perforation, flap shape development of the perforation margin, suspicious ossicular problem, or annulus involvement, require immediate patch application to facilitate the healing process. Currently, paper patches are being used worldwide. The advantages of a paper patch are easily obtainable properties and cost effectiveness. However, the paper patching approach also has considerable issues. First, the paper is occasionally displaced after the procedure. TM is well-known for its movement in reaction to noise and middle ear pressure. To prevent such an accident, antibiotic ointment is applied to the paper patch because of the effects of the ointment viscosity [4]. Second, a paper patch might induce an inflammatory reaction and/or infection. The material properties of paper are not considered as biocompatible; as a result, a foreign body reaction might be induced through the

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trimmed area of the TM. In addition, the middle ear environment, in which mucosal secretion is consistently maintained, and normal floras exist as potential sources of post-patch infection, is not an appropriate condition for a non-bio-compatible material. Third, a paper patch must be removed practically after termination of their function for TM healing. However, it is difficult to remove the patch material for children or poor accessibility patients during the follow-up periods. For these reasons, our institute investigated the development of an appropriate patch material for use in TM regeneration. As an initial trial, a silk paper patch was made for acute and chronic tympanic membrane perforation [5, 6]. Compared with paper patches, silk material (which is a natural polymer produced by *Bombyxmori* silk worms) was more effective in facilitating the healing process of drums in patients with acute TM perforation. We confirmed these differences in animal and human studies [4, 5]. Although good properties were obtained in using a silk patch to facilitate the healing process while preserving the natural TM contour, the limitation that the applied patch must be removed from the external auditory canal or TM remains an issue because silk displays long-term degradation behavior [7]. In addition, the use of a replaceable material for the fibrous layer of tympanic membrane (temporalis fascia is globally used) was required. This material requires special characteristics to survive in the air-filled space. Thus, we considered another material property: the disappearance of the material from the external auditory canal. The patch extraction based on duck-foot collagen has a sponge-type property. Collagen from duck feet is a naturally derived polymer, making it beneficial for cell seeding and cell attachment and rapid biodegradation [8, 9]. The method of fabrication and bio-compatibility was evaluated in our institute previously [8]. These characteristics of a collagen patch have several advantages for application on TM. Easy application and self-removal are possible, thereby enabling a minimal invasive procedure to be performed in children or infant patients with traumatic TM perforation under local anesthesia or no anesthesia.

In the present study, we investigated the utility of duck-foot collagen extraction patching in the traumatic TM perforation via comparison with spontaneous healing or a paper patch.

2 Materials and methods

2.1 Materials and duck-feet collagen extraction

Joowonori (Jincheon-gun, Chungcheongbuk-do, Korea) provided Duck feet. Collagen with freeze-dried sponge type was harvested through extraction and separation from the duck feet. Paper patches were handmade from cigarette

paper in the outpatient clinic and later sterilized with ethylene oxide gas [8].

Blood was subsequently removed over a period of 24 hours at room temperature. 0.5 M NaOH at a ratio of 3:1 of NaOH were used to remove of fat. The fat-removed samples were washed with methanol:chloroform (ratio of 3:1), acetone, and 70% Et-OH serially. 5% citric acid was mixed in at a ratio of 3:1 of acid to the sample and then stirred at 4 °C during 72 hours. Next, the solution was centrifuged at 12,000 rpm, 4 °C during 15 minutes. The supernatant was collected and then precipitated with 100% EtOH. The collagen was harvested by centrifuging the sample at 3,500 rpm, 4°C for 5 minutes. The collagen pellet was collected and later lyophilized in a freeze-dryer (Genesis 25-LE, Virtis) at 20 mmHg and -40°C, eventually obtaining a collagen sponge (Fig. 1) [8].

2.2 Fabrication of collagen patches

Collagen patches were prepared via the lyophilization process. 0.5 M acetic acid solution to prepare a 4% (w/v) solution was used to dissolve of the collagen. The collagen solution was poured into a Petri dish. The solutions were maintained in a deep freezer at -80 °C during 24 hours to lyophilize. Thickness of patch was 60 µm. A patch was punched into 6-mm size. The patches were immersed gradually in 100, 80, 50, 30, and 0% ethanol solution during 3 hours in each. Next, refrigerating and lyophilizing were performed again (Fig. 1) [8].

2.3 The study design for *in vivo* test

Fifty-six ears of adult male Sprague-Dawley rats, each weighing in the range of 250 to 300 g, were used for the animal studies. This study was approved by the institutional review board of Hallym University, Chuncheon, Korea (IRB no. Hallym 2014-103). All the surgical procedures were performed under general anesthesia using 3% isoflurane in a 75:25 mixture of nitrous oxide and oxygen (Air Liquid America, Houston, TX). After anesthesia, the rats were ventilated with a small rodent respirator (Harvard Apparatus, South Natick, MA). Bilateral myringotomies 1.8 mm in diameter were performed with a hemisphere-shaped red-hot needle in the pars tensa under otomicroscope examination (Opmi 1, Zeiss, Germany). Sixteen perforated ears were randomly selected to act as the control group. Twenty perforated ears were managed with duct-feet collagen patches, and the other twenty perforated ears were treated with a paper patch. Sixteen rats had one-side ear in the control group and the opposite-side ear in the treated groups. The remaining twelve rats had one-side with the duck-feet collagen patch and the opposite-side with the paper patch. All animals were observed in separate cages with free access to

Fig. 1 Schematic process of the extraction of the duck feet collagen using acid and the preparation for the duck-foot collagen using a freeze drying method

food and water, and two drops of antibiotic otic solution (Tarivid otic solution[®], ofloxacin 3 mg/cm³; Daiichi Sankyo, Tokyo, Japan) were dropped into the external auditory ear canals during the first 3 days for infection prophylaxis. Each tympanic membrane was photographed each daily through an endoscope before and immediately after the perforation procedure until the perforations were closed. To examine the healing processes at different time points, three rats were killed at 1, 3, 7, and 14 days after making tympanic membrane perforations [5]. The animals were decapitated under general anesthesia, and both external ears were subsequently separated at the osteo-cartilaginous junctions. The cranial vault and brain were removed, and the temporal bone was identified. The tympanic bulla was isolated and stored in 10% formaldehyde solution and reserved for histopathological evaluation. After fixation, the specimens were decalcified for 3 days in an EDTA solution containing formaldehyde and formic acid. The specimens were then dehydrated, embedded in paraffin (Shandon Citadel 1000, Thermo Electron Corp., Waltham, MA), and cut through a line bisecting the perforation and running perpendicular to the manubrium of the malleus [5]. The resulting tissue blocks were embedded in paraffin, cut into 5- μ m-thick sections, stained with hematoxylin-eosin (H&E) and subjected to

immunohistochemistry for proliferating cell nuclear antigen (PCNA) under a light microscope.

2.4 Morphology observation

A low vacuum scanning electron microscope (S-3500N, Hitachi, Tokyo, Japan) was used for the cross-sectional morphology of the scaffolds. A thin 10-nm layer of silver/palladium for 120 seconds at a discharge current of 15 mA using an Ion Sputter coater (1010, Hitachi, Tokyo, Japan) was used to coat the samples. A variable pressure field emission scanning electron microscope (FE-SEM) EVOVR LS10 equipped with energy dispersive X-ray spectroscopy, manufactured by Carl Zeiss SMT was used for energy dispersive X-ray spectroscopy (EDX) analysis at Korea Basic Science Institute (KBSI), Chuncheon [8].

2.5 Quantification: analysis of proliferation characters

PCNA expression cells were calculated among the 200 basal cells, and the expression percentage was identified as the labeling index (LI). A mean value of the LI counts were

subdivided and estimated to the epithelial and endothelial portions. The pars tensa of the TM is composed of five layers: 1) epidermal layer of keratinizing squamous epithelium (epithelial layer), 2) thin connective tissue layer, 3) dense connective tissue layer (lamina propria), 4) thin layer of connective tissue, and 5) single layer of flattened epithelial cells (mucous layer) [10]. For evaluation of the effect of each portion, we counted the PCNA expression cell at the epithelial and mucous portion separately. At $\times 40$ magnification, the numbers of positive cells per visible field were counted for PCNA from the perforation margin.

2.6 Statistical methods

Statistical analyses were performed using the SPSS version 21 (SPSS IBM, New York, USA). The tympanic membrane perforation healed area and the LI of PCNA enhanced cells according to each groups were compared using the analysis of Kruskal-Wallis Test. The level of significance was set at $p < 0.05$.

3 Results

3.1 Morphology of duck-feet collagen patch

The external appearance of the collagen patch is shown in Fig. 2A, and the cross-sectional LV-SEM (Low Vacuum Scanning Electron Microscope) image of the collagen patch is shown in Fig. 2B. Each collagen patch has a three-dimensional interconnected porous structure. The distribution of the pore shapes was homogeneous. The pore size was 150 (0.05 of standard deviation), the porosity was 65 (10.05 of standard deviation). Such a structure of a collagen patch facilitates the tympanic membrane regeneration via intercellular metabolism and gas exchange to form a good environment for cell growth.

Fig. 2 Duck-feet fibroin patch. **A** the external appearance of the duck-feet fibroin patch with a size of 6 mm. **B** The cross-sectional LV-SEM images of the duck feet fibroin patch

3.2 Gross inspection and healing rate

Figure 3 shows the endoscope findings of the tympanic membranes. Specific abnormal findings, such as inflammation in all groups, were not observed. The duck-feet derived collagen patch was observed to be well attached to the tympanic membrane perforation site compared to the paper patch. The perforation of the tympanic membrane still remained until day 7 in the control group. However, no perforation was observed in the duck-feet derived collagen and paper patch group on day 7. These results indicated that the duck-feet derived collagen and the paper patch induced a more rapid healing process compared to the control group. After 7 days, a portion of the paper patch became detached from TM spontaneously; however, it must be manually removed from the external auditory canal. In contrast to the paper patch, all the duck-feet derived collagen patches were absorbed and disappeared gradually. The period of absorption was short: the duck-feet derived collagen patch on day 3 was much smaller than the collagen patch on day 1 (Fig. 3, middle columns).

The TM perforation healed area was measured to compare the effects of each patch regarding the acceleration of the healing processes (Fig. 4). After 3 days, the percentages of healed areas of the duck-feet derived collagen patch, paper, and control groups are 44%, 28%, and 16%, respectively ($P = 0.044$). On day 7, in contrast to the 100% healing rate of the duck-feet derived collagen patch group, the percentages of the healed area of the control and paper patch groups are 97% and 93%, respectively. In other words, some of the TM perforation remained for the control and paper patch groups. Thus, the healing of the perforation in the collagen patch group can be confirmed to be more rapid than the healing of the other groups.

3.3 Histological investigations

Figure 5 shows the H&E stain result for histological observation. Perforation of the tympanic membrane was still

Fig. 3 The endoscopic findings of the TM. Perforation of the tympanic membrane remained until day 7 in the control group (control, 7 days). The duck-feet derived collagen and paper patch groups on day 7 did not exhibit perforation (collagen, 7 days and paper, 7 days). The duck-feet derived collagen patch was observed to be better attached to the tympanic membrane perforation site (collagen, 3 days) compared to the paper patch (paper, 3 days)

Fig. 4 The measurement results of the healed area of the tympanic membrane perforation. The calculated area of the healed portion was compared with the pre-treatment TM perforation size using the InnerViewTM program. The healing process of the collagen patch group was faster than that of the paper patch and control groups. X control, C duck-feet collagen patch, P paper patch (Kruskal-Wallis Test, $P = 0.044$, 3 day)

observed on day 3 in all groups. In both the duck-feet derived collagen group and the paper patch group, the patches were well attached to the perforation site. 7 days after performing the patching procedure, the proliferation and hyperplasia of the all five layers close to the edge of the perforation site was identified in the control and paper

groups. However, the collagen group did not confirm this finding. Proliferation of the epithelial layer of the perforation margin may cause thickening or tympanosclerosis of the healed tympanic membrane. The post-operative 14 days photos show completely healed tympanic membranes in each of the groups. We observed that the newly healed

Fig. 5 H&E staining. Some portion of perforated tympanic membrane was directed in the wrong direction in the control group (control, 7 days). In addition, the margin thickness of the perforated tympanic membrane was maintained until post-perforated 7 days (control, 7 days). Tympanic membrane attaching to the collagen scaffold was maintained in the collagen patch group (collagen, 7 days). In contrast to the collagen patch group, some portion of the paper patch was detached, and the thickened portions of the perforated margin were still maintained (*, paper, 7 days). *Arrow* collagen patch, *arrowhead* paper patch. *ME* middle ear, *EAC* external auditory canal

Scale bar=300μm

tympanic membrane is relatively thicker in the control group compared to that of the collagen and paper groups (Fig. 5, 14 days in control, collagen and paper).

3.4 Immunohistochemistic findings of PCNA

Figure 6 shows the Immunohistochemistic result for histological observation. To examine the proliferation after attaching the patch that induced the tympanic membrane healing process, we evaluated the expression cells by PCNA. As determined by immunohistochemistry, PCNA staining was observed in the attaching portion of the PCNA enhanced cell to collagen scaffold in 7 days for the collagen patch group. The proliferation activity was observed to remain until 7 days in the collagen patch group, and the duck-foot derived collagen patch was found to shrink and detach after complete healing followed by thinning of the TM (collagen 14 days). In contrast, the thickened TM and continued proliferation activity were observed for some of the paper patch group (paper, 14 days). To compare this phenomenon using quantitative analysis, the LI of PCNA enhanced cells was calculated. The LI of PCNA enhanced cells was different between the keratinized epithelial layer and the mucous layer in all three groups. In contrast to the control and paper patch groups, the healing process of the keratinized epithelial

layer was accelerated at the immediate post-treatment stage. In addition, the proliferation of the mucous layer peak followed. After the maximum proliferation activity of the keratinized epithelial layer on day 3 ($P=0.039$), the activity of the keratinized epithelial layer decreased dramatically; otherwise, the peak of mucous layer's activity was developed on day 7 in the duck-foot collagen patch group ($P = 0.027$, day 7). All activities of PCNA for the three groups were normalized at their respective values on day 14 (Fig. 7, $P=0.148$).

4 Discussion

To address the perforation of the tympanic membrane developed by trauma, patch application is globally performed to induce healing process. However, the currently used procedure has some problems. For example, the paper patch used is not demonstrated to be a biologically compatible material and has limitations for use in infants or children. To address these issues, clinical and experimental trials have been performed regarding the use of other materials to improve the healing success and reduce the healing time [10]. Recently, some authors reported a surgical trial using a variety of materials, such as silk, collagen, chitosan, and calcium alginate [11, 12].

Scale bar=300µm

Fig. 6 The immunohistochemistic results of PCNA expression. The PCNA enhanced cells were observed in the collagen scaffold (arrowhead collagen, 7 days). PCNA enhancing was observed on the epithelial layer in 3 days (arrow collagen, 3 day). And, following enhancement was observed on the mucous layer in 7 days (arrow

collagen, 7 day). The thickened margin inside the mucosal plug was observed at the paper patch at 7 days after treatment. The hyper-proliferated portion of the healed TM was observed in the paper patch group (paper, 7 and 14 days)

Fig. 7 Labeling index of PCNA enhanced cells. *C* marked in dark blue denotes the activity of the mucous layer's proliferation cell around the perforated TM. *C* marked in light blue denotes the activity of the keratinized epithelial layer's proliferation cell around the perforated TM. The activities of both *C* were higher than those of the control group (*X* marked in dark black: mucous layer's proliferation activity, *X* marked in light black: keratinized epithelial layer's proliferation activity) and the paper group (*P* marked in dark red:

mucous layer's proliferation activity, *P* marked in light red: keratinized epithelial layer's proliferation activity) at day 3. After the maximum proliferation activity of the keratinized epithelial layer at day 3, the activity decreased dramatically; the peak of the mucous layer's activity occurred at day 7 in the duck-foot collagen patch group. All activities of PCNA in the three groups were normalized to the levels at day 14 (Kruskal-Wallis Test, $P = 0.039$, day 3; $P = 0.027$, day 7; $P = 0.148$, day 14). (Color figure online)

Many bio-materials have been developed based on collagens from other species, for example, bovine collagen, sea urchin, horse serum and deer antler collagen [13–17]. To replace human collagen material and to commercialize

an easily obtainable product, many researchers have performed experimental and clinical tests on many materials to achieve the appropriate properties. However, several limitations exist when applying experimentally proven bio-

compatible materials to humans because of social and cultural situations. For example, Arab societies following the Islamic religion are banned from eating pigs, and people in India following the Hindu religion are banned from eating cows. Issues with prion disease that induce bovine spongiform encephalopathy can negatively effect the application of bovine collagen during a pandemic period. As a result, applicable collagens from a variety of species must be studied and developed to satisfy all the demands of a wide range of applications. Considering these demands, we developed and experimentally studied duck feet collagen.

As the first trial of this new collagen material to human application, we made duck-feet collagen in patch shape for TM perforation. To evaluate the applicability of duck-feet collagen, we previously confirmed the characteristics, structure and basic material properties of duck-feet collagen. The cell proliferation and attachment properties of duck-feet collagen in the tissues was confirmed via animal studies [8].

In the present study, patch application to a perforated drum with trimming accelerates proliferating activities of keratinized epithelium and the mucous layer of the TM [18]. If the TM was positioned in the middle ear space without a supporting structure, then some portion of the perforated margin will grow in an unexpected direction. To prevent this problem, patch application is usually performed. However, the typically used material, paper, is not verified as a proper material for use in such a process, and a portion of patients suffer delayed healing process and failure results when using a paper patch.

The wound healing process of TM perforation starts with proliferation and migration of epithelial and fibroblast cells from the region of the handle of malleus and annulus to the region of the perforation [19]. Accelerated inflammatory cells are thought to be involved in the tissue formation and remodeling phases [19]. In the present study, PCNA expression of the mucous layer peaked at day 7, while keratinized epithelium peaked at day 3. After the maximum expression of PCNA at the keratinized epithelium and the mucous layer, the proliferation activity was normalized to the value at day 14 when compared with the activity of the control group. Naturally, the migration activity of the keratinized epithelium layer is higher than that of the mucous layer regarding the bridging of the perforated gap after trimming or perforation [18]. In the results of our present study, the healing process of the duck-feet collagen patch might induce and accelerate the natural healing process better than the paper patch via epithelial proliferation to the collagen scaffold (adhesion effect). Some in-growing epithelial cells to the sponge micro-cavities maintained firm attachment between the

patch and the trimmed area. For this reason, the collagen patch has a good benefit as a patch material compared to a paper patch in the wet environment of the middle ear cavity caused by secretion from the middle ear mucosa cells. From our results, the duck-feet collagen patch was observed to accelerate the healing process and reduce the healing time. An SEM image analysis revealed that the micro-structure of the collagen patch has good three-dimensional structure for cell proliferation; this structure accelerates the metabolic and gas exchanges between in-growing cells. Figures 2B and 5 (7day collagen) was proved this characteristic. A number of epithelial cells from trimmed margins grow into the margin portion of sponge typed duck feet collagen patch. These attaching cells with supporting tissues might be enhanced and maintained firm adhesion between trimmed margin of TM perforation and collagen material; therefore, the role of patching during the immediate healing process was better when we used the duck feet collagen patch.

We confirmed that the rapid healing process of TM perforation was induced by the proliferation process of the collagen patch through the immunohistochemistry of PCNA. Cell proliferation was accelerated over the entire portion of TM when the TM was in the perforated condition [20]. In previous studies, enhancement of the healing process was induced by several markers, including epidermal growth factor, endothelial growth factor, and hepatocyte growth factor. However, the proliferation processes of the paper patch group were continued up to 14 post-operative days. We think that these processes might be induced by foreign body reaction caused by the presence of the paper material. In contrast to the paper patch, the duck-feet collagen patch induced a rapid healing process without thickening of the newly generated TM. The sponge type of patch induced the adhesion effect via migration of the newly generated cells to the empty spaces of the patch material. This effect was differentiated from that of the paper patch, which only provides guidance for the healing direction. In addition, the results of this study might be used as preliminary data for the next set of experimental and clinical trials of the application of duck feet collagen material to other human organs.

In this study, we confirmed that a duck-feet collagen patch had the advantages of rapid healing, induction of the natural TM contour, and disappearance ability after the patch effect is completed. Further studies involving the development of a more adhesive and biocompatible material are required for proper application of the material.

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Compliance with ethical standards

Conflict of interest The authors have no financial relationships or conflict of interest to disclosure.

Ethical statement This research protocol was approved by the Hallym University institutional Animal Care and Use Committee (IRB no. Hallym 2014-103), and the experiments were performed in compliance with the ARRIVE guidelines on animal research.

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