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Mesenchymal stem cell-laden hybrid scaffold for regenerating subacute tympanic membrane perforation

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ABSTRACT

Tympanic membrane (TM) perforation is one of the most common otology complications. To date, there has not been reported TM regeneration using bioprinted scaffold. The purpose of this study was to evaluate the efficacy and feasibility of bioprinted polycaprolactone/collagen/alginate-mesenchymal stem cell (PCAMSC) scaffolds for the regeneration of subacute TM perforation. Sprague-Dawley rats were used in an animal model of subacute TM perforation. In the experimental group (n = 7), bioprinted 3D PCAMSC scaffold was placed on the perforation. The control group (n = 7) were treated with polycaprolactone/collagen/alginate (PCA) scaffold. 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. 72% of the control group, and this difference was statistically significant ($p < 0.05$). The ABR threshold at all frequencies of the experimental group was 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 are 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 well regenerated but less thickened than experimental group. From these results, the cell-laden PCAMSC scaffold offers a significant advantage in the TM regeneration in a rat subacute TM perforation model. It may offer attractive opportunities in the conservative clinical treatment.

1. Introduction

Chronic tympanic membrane (TM) perforation due to otitis media or trauma has long been regarded as the most common complication of ontological diseases. Closing a chronic tympanic membrane (TM) perforation restores the vibratory area of the membrane and affords round window protection, thus improving hearing and prevents contamination by exposure to pathogens introduced via the external auditory canal. Various materials have been used as a scaffold, including paper patch, gelfoam, and alloderm in acute TM perforation [1–3]. However, it is difficult to treat a chronic TM perforation due to decreased regenerative activity of the TM at the margin of the perforation. Autologous tissue tympanoplasty (fascia or perichondrium) increases successful rates but requires more invasive technique and greater operative time despite using endoscopic tympanoplasty.

In an effort to overcome this drawback, the use of the novel adjuvant substances and tissue engineering techniques have been applied to enhance the healing of chronic TM perforations and to potentially replace autologous grafts in human patients [4,5]. In our previous work [6], a 3D porous collagen scaffold combined with human umbilical cord serum (UCS) was used in the chronic TM perforation model, and the collagen/UCS scaffold considerably accelerated the regeneration.

To date, stem cells have been rarely used for TM regeneration. There were only three papers reported by same group in the Medline search [7–9]. They used stem cells impregnating gelatin sponge over the perforation. In acute TM perforation, stem cell treated ears did not show any enhanced healing of the perforation, but stem cells have shown great promise in enhancing TM regeneration of chronic TM perforation model. Previous local transplantation of stem cells using a scaffold has some advantages over direct transplantation, such as local injection or intravenous infusion [10]. Processed biological scaffold facilitates host cell infiltration and revascularization and results in integration of the implant into the autologous tissue at the repair site [11]. However, TM

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perforation is different environment from local injection or intravenous infusion into the body. The external ear and middle ear contains normal air. The wetted stem cell impregnated gelatin sponge can be easy to dry and it cannot make a microenvironment which enhances mesenchymal stem cells to proliferate and differentiate. Therefore the stable bioscaffold including mesenchymal stem cell is necessary to overcome microenvironment in TM regeneration. Recently, several research groups have embarked on engineering three-dimensional (3D) living structures using bioprinting. Bioprinting has emerged in recent years as an attractive method for creating 3D tissues and organs in the laboratory, and therefore is a promising technology in a number of regenerative medicine applications [12–14]. However, there has not been reported TM regeneration using a bioprinted scaffold. As far as we know, this is the world's first trial of the bioprinted scaffold laden with mesenchymal stem cells (MSCs) for TM regeneration.

In this study, we present the first trial of bioprinted 3D polycaprolactone/collagen/alginate-MSC (PCAMSC) scaffold for closing subacute TM perforation in an animal model. As a control, a cell-free scaffold, polycaprolactone/collagen/alginate (PCA), was used. The PCAMSC scaffold was consisted of polycaprolactone (PCL) fibers, which were attained using centrifugal melt spinning, type-I collagen, and a MSCs-laden alginate. In the cell-laden structure, the melt-spun fibrous PCL was accommodated as a mechanical supporter. By using the cell-laden scaffold, the closing efficiency for subacute TM perforations in rats was evaluated.

2. Materials and methods

2.1. Scaffold fabrication

To obtain the cell-laden structure consisting of PCL micro/nanofibers supplemented with collagen and MSCs-laden alginate, PCL (M_w = 45.000 Da, T_m = 60 °C, Sigma–Aldrich, USA), type-I collagen (density = 1.3 g cm^{-3}, Matrixen-PSP; Bioland, South Korea) from porcine tendon, and low-viscosity, high-G-content LF10/60 alginate (FMC BioPolymer, Drammen, Norway) were used.

As shown in Fig. 1A, to attain the fibrous PCL structure, we used a centrifugal spinning process with the processing conditions (melting temperature of PCL = 210 °C, 30 gauge spinning nozzle, and rotating rpm = 1300 ± 50). To increase the low bioactivity of the PCL fibers, the melt-spun PCL fibers were dipped in 2% (*w*/*v*) collagen solution (Fig. 1B). After coating the collagen in the fibrous structure, it was immediately dried in a freeze-dryer (FD, SFDSM06, Samwon, South Korea) at −75 °C for 10 h and then the coated collagen was cross-linked with a 50-mM 1-ethyl-(3-3-dimethylaminopropyl) hydrochloride (EDC) solution in 95% ethanol for 2 h at room temperature. After the process, the PCL/collagen fibrous structure was washed four times in 0.1-M Na_2HPO_4 for 2 h each and rinsed four times in demineralised water for 30 min each.

The bioink, MSCs-laden alginate, was attained by the mixture of alginate (3.5% *w*/*v*) with PBS and 0.5% (*w*/*v*) $CaCl_2$. In this study, the MSCs were provided by Prof. Eun-Ju Lee (Seoul National University Hospital, Seoul, South Korea). To obtain the bioink, the mixture ratio was 7:3 of alginate:calcium chloride ($CaCl_2$) solution. Finally, MSCs (density: 1×10^7 mL^{-1}) were incorporated into the mixture of alginate and $CaCl_2$ solution. The MSCs-laden bioink was plotted on the fibrous surface with the processing conditions (processing temperature = 32 °C, pneumatic pressure of the nozzle = 170 ± 15 kPa; moving speed of nozzle = 10 ± 2 mm s^{-1}) (Fig. 1B). After plotting the MSCs-laden bioink on the fibrous structure, the bioink was finally cross-linked with the 2% $CaCl_2$ solution prepared in PBS. Through the fabrication processes, the hybrid structure, which is consisted of three main components (PCL fibers, collagen, and MSCs-laden alginate), was obtained (Fig. 1B).

2.2. Mechanical test

To measure the tensile properties of the cell-laden fibrous structure, it was cut into small strips (10 × 40 mm). The tensile test was performed using a tensile testing machine (Top-tech 2000, Chemilab, South Korea). The stress–strain curves for the scaffolds were recorded at a stretching speed of 0.5 mm s^{-1}. All values are expressed as means ± standard deviation (SD) (n = 5).

2.3. Live/dead analysis

After printing the MSCs-laden bioink on the fibrous structure, to observe the initial cell-viability the structure was immediately exposed to 0.15 mM calcein AM and 2 mM ethidium homodimer-1 for 45 min and stained specimens were analyzed with fluorescence microscopy (CKX41; Olympus, Japan). In the images, green and red mean live and dead cells, respectively. To calculate the initial cell-viability, the ratio of the number of live cells to the total number of cells was counted using Image-J software. Finally, the value was normalised relative to the cell-viability (the value before cell printing), which was determined using trypan blue (Mediatech, Herndon, VA, USA).

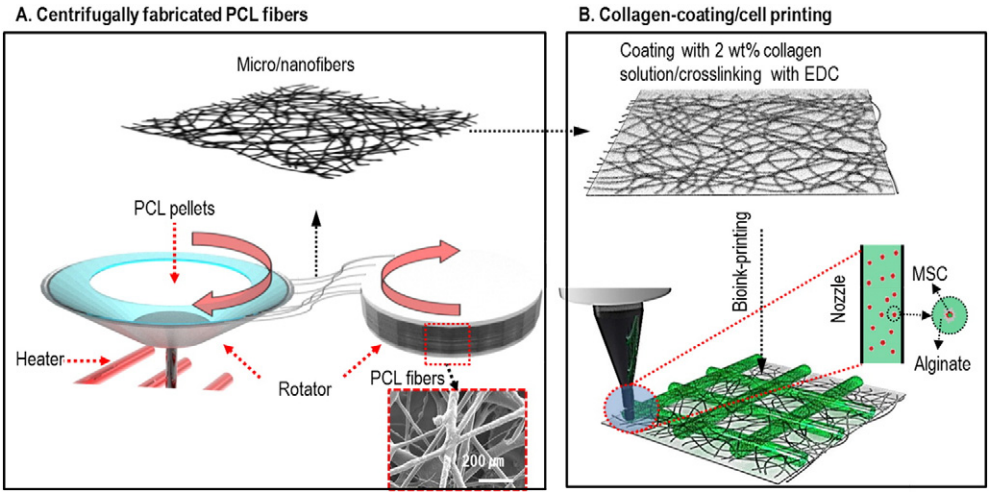

Fig. 1. Schematic diagram of the fabrication procedure for the MSCs-laden fibrous structure. (A) Centrifugal melt-spinning for PCL fibers. (B) Collagen-coating/cell-printing process for MSCs-laden fibrous structure (PCAMSC).