Decellularization of Human Nasal Septal Cartilage for the Novel Filler Material of Vocal Fold Augmentation

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Summary: Objectives. The clinical application of allogenic and/or xenogenic cartilage for vocal fold augmentation requires to remove the antigenic cellular component. The objective of this study was to assess the effect of cartilage decellularization and determine the change in immunogenicity after detergent treatment in human nasal septal cartilage flakes made by the freezing and grinding method.

Methods. Human nasal septal cartilages were obtained from surgical cases. The harvested cartilages were treated by the freezing and grinding technique. The obtained cartilage flakes were treated with 1% Triton X-100 or 2% sodium dodecyl sulfate (SDS) for decellularization of the cartilage flakes. Hematoxylin and eosin stain (H&E stain), surface electric microscopy, immunohistochemical stain for major histocompatibility complex I and II, and ELISA for DNA contents were performed to assess the effect of cartilage decellularization after detergent treatment.

Results. A total of 10 nasal septal cartilages were obtained from surgical cases. After detergent treatment, the average size of the cartilage flakes was significantly decreased. With H&E staining, the cell nuclei of decellularized cartilage flakes were not observed. The expression of major histocompatibility complex (MHC)-I and II antigens was not identified in the decellularized cartilage flakes after treatment with detergent. DNA content was removed almost entirely from the decellularized cartilage flakes.

Conclusion. Treatment with 2% SDS or 1% Triton X-100 for 1 hour appears to be a promising method for decellularization of human nasal septal cartilage for vocal fold augmentation.

Key Words: Decellularization–Cartilage–Filler–SDS–Triton X-100.

INTRODUCTION

Unilateral vocal palsy causes the voice to change and brings about aspiration, leading to a decrease in the quality of life. There are several methods, such as injection laryngoplasty, thyroplasty, and arytenoids adduction, which are used to treat unilateral vocal palsy. Although thyroplasty and arytenoid adduction are effective, these results in neck scar.[1] Therefore, recently, because vocal fold augmentation with injectable materials is an easy and simple operation, it has become a widely popular method for medialization of unilateral paralyzed vocal fold.

There are several materials used in injection laryngoplasty, such as fat,[2] collagen,[3] hyaluronic acid,[4] cartilage,[5] and calcium hydroxylapatite.[6] Although calcium hydroxylapatite was approved by the U.S. Food and Drug Administration (FDA) for vocal fold augmentation, several complications (migration, granulation formation, and impaired vocal fold vibration) were reported.[7] The ideal materials for vocal fold augmentation need to involve little or no resorption, high tissue biocompatibility, no tissue reaction at the injection site, reversibility (or potential for later modification), ease of handling, and no migration.[8] As of yet, no ideal material has been found.

Lee et al[9,10] previously reported that the volume and histological presence of intracordal autologous cartilage were preserved after 3 years. Based upon these results, it was suggested that autologous cartilage might be an excellent augmentative material for long-term treatment of glottic incompetence. Recently, Lim et al[5] described the clinical outcome of intracordal autologous auricular cartilage injection that provided effectiveness for up to 1 year. Lee et al[11] also reported that allogenic cartilage treated with Triton X-100 for decellularization is well tolerated for 2 years and may be an effective material for volumetric augmentation in the paralyzed vocal fold. So, they suggested that a filler made of allogenic cartilage treated with detergent could have the possibility of being commercialized for the augmentation and the cosmetic fields.

Although autologous and allogenic cartilages are effective materials for volumetric augmentation, it was not easy to make harvested cartilage into small flakes enough to be passed through a finer needle than a 19-gauge needle using a knife and scissors. In addition, it is difficult to perform a clinical application to the commercialized cartilage flake injection using a 19-gauge needle under local anesthesia. Small size of cartilage flake passing through a 24-gauge needle is an advantage for use in the clinical application of commercialized cartilage. Consequently, Park et al[12] detailed a new preparation technique for intracordal cartilage injection, specifically the freezing and grinding method. Essentially, one uses liquid nitrogen and a mortar to produce smaller-sized cartilage flakes easily and rapidly. The cartilage flakes created with the freezing and grinding method can then be passed through a 24-gauge needle. Therefore, they suggested that this new technique would also be applicable to the production of commercialized cartilage material for widespread use.

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Although autologous cartilage might be the ideal material for vocal fold augmentation, allogenic and xenogenic cartilages would be more advantageous as these are commercially available and straightforward to obtain without the need for general anesthesia. The acellularized xenogenic cartilage preserving relatively nonimmunogenic extracellular matrix may be a useful option to find material for vocal fold augmentation because xenogenic cartilage is a fairly limitless and cost-effective source.

The clinical application of allogenic and/or xenogenic cartilage for vocal fold augmentation has been required to remove the antigenic cellular component in these tissues. For instance, an acellular dermal matrix has seen successful use clinically as the FDA-approved Alloderm product.[13] However, studies demonstrating the effect of the decellularization on cartilage are limited, as there are no studies demonstrating the effect of the decellularization of cartilage for vocal fold augmentation in the case of allogenic and/or xenogenic cartilage. The objective of this study was to therefore evaluate the effect of cartilage decellularization and to establish the change in immunogenicity after detergent treatment in human nasal septal cartilage flake made by the freezing and grinding method. We also measured the change in size of these cartilage flakes after detergent treatment to estimate their potential to pass through a 24-gauge needle for vocal fold augmentation.

MATERIALS AND METHODS

Preparation of harvested cartilage

After the Pusan National University School of Medicine Review Board approved this study, human nasal septal cartilages were obtained from surgical cases. The harvested cartilages were treated with the freezing and grinding technique described previously.[12] The harvested cartilages were placed into mortar and were frozen using liquid nitrogen, at which point the frozen cartilages were ground manually with a mortar and pestle. After that, the yielded cartilage flakes were passed through a strainer (150 μm), their sizes were measured, and they were decellularized by being exposed to 1% Triton X-100 or 2% sodium dodecyl sulfate (SDS).

Tissue decellularization

The human nasal septal cartilages were decellularized using modified detergent-enzyme treatment as previously described.[14,15] Cartilage flakes immersed in 0.02% Tris/EDTA with protease inhibitor and incubated for 48 hours at 4 °C. The tissues were then transferred into 1.0% Triton X-100 solution or 2% SDS solution and stirred for 1 hour at 4 °C. After washing with PBS, tissues were incubated with PBS containing DNAse/RNAse (15 μg/mℓ: Sigma, St. Louis, MO, USA) for 24 hours. Tissues were then retreated with 0.02% Tris/EDTA solution for 48 hours. After washing again with PBS, tissues were stored in physiologic saline at 4 °C until they were utilized. The cartilage flakes were then assessed for their gross appearance using hematoxylin and eosin stain (H&E stain) and surface electric microscopy (SEM) analysis. In addition, the tissues were evaluated based on the expression of major histocompatibility complex MHC I and II antigens by immunohistochemistry and the total DNA content gauged by quantitative biochemistry.

Size measurement and scanning electric microscopy (SEM) of cartilage flakes

The size of the cartilage flakes was measured using 200-fold magnification before and after detergent treatment. Following H&E staining, the cartilage flakes were examined cytologically. The tissue samples were then treated with 2.5% glutar-aldehyde plus 0.1 M sodium cacodylate buffer. Subsequently, the samples were dehydrated through a series of ethanol and critical-point dryings before being coated in gold. The samples were then examined and photographed using a Cambridge 360 scanning electron microscope (Cambridge Instruments, Cambridge, United Kingdom).

MHC immunohistochemistry

Paraffin sections were prepared from each of the tissue samples. The sections were washed three times in PBS and transferred to blocking buffer (PBS containing 2% bovine serum albumin) for 1 hour at room temperate prior to application of the primary antibody. The sections were incubated with biotin-conjugated mouse antihuman MHC I or II antibodies (MCA 1044; Serotec, Oxford, United Kingdom) overnight at 4 °C. The sections were then washed three times with phosphate-buffered saline (PBS) again. Phycoerythrin (PE)-conjugated streptavidin was diluted 1:200 with blocking buffer, and incubated for 1 hour at room temperature. After another round of washing three times with PBS, the slides were cover slipped with a Glycerol vinyl alcohol aqueous (GVA) mount (Zymed, S. San Francisco, CA, USA). The slides were then viewed with a confocal laser-scanning microscope.

DNA quantification by elisa

The total DNA was extracted from tissue samples treated with 1% Triton X-100, 2% SDS, or no treatment at all using a DNA Extraction kit (QIAGEN GmbH, Germany) reagent. DNA amounts were determined by optical density at 450 nm.

Statistical analysis

Statistical analysis was performed using Wilcoxon signed-rank test. Significance was defined at $P < 0.05$.

RESULTS

Size and morphology of cartilage flakes

A total of 10 nasal septal cartilages were obtained from surgical cases for these experiments. After making cartilage pieces employing the freezing and grinding method, cartilage flake sizes were measured at the magnified view of 200-fold H&E stain before detergent treatment and after 1% Triton X-100 or 2% SDS treatment (Figure 1). The average size of cartilage flakes before detergent treatment was 36.7 μm, whereas the average sizes of decellularized cartilage flakes after 1% Triton X-100 and 2% SDS treatment were 29.9 μm and 21.3 μm, respectively (Table 1). Apparently, detergent treatment significantly reduced the average size of the cartilage flakes. As well, the average size of the decellularized cartilage flakes after 2% SDS was significantly less than after 1% Triton X-100 treatment. Furthermore, after detergent treatment, the largest size of the cartilage flakes was significantly diminished. However, there was no significant dif-