The Ability of Human Nasal Inferior Turbinate–Derived Mesenchymal Stem Cells to Repair Vocal Fold Injuries

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. This study investigated the ability of implanted human nasal inferior turbinate–derived mesenchymal stem cells (hTMSCs) to repair injured vocal folds. To this end, we used quantitative real-time polymerase chain reaction (PCR) to analyze the early phase of wound healing and histopathological analysis to explore the late phase of wound healing in xenograft animal models.

Study Design. Prospective animal study.

Setting. Research laboratory.

Subjects and Methods. The right-side lamina propria of the vocal fold was injured in 20 rabbits and 30 rats. Next, hTMSCs were implanted into half of the injured vocal folds (hTMSC groups). As a control, phosphate-buffered saline (PBS) was injected into the other half of the injured vocal folds (PBS groups). Rat vocal folds were harvested for polymerase chain reaction (PCR) at 1 week after injury. Rabbit vocal folds were evaluated endoscopically and the larynges harvested for histological and immunohistochemical examination at 2 and 8 weeks after injury.

Results. In the hTMSC group, PCR showed that hyaluronan synthase (HAS)1, HAS2, and transforming growth factor (TGF)–β1 were significantly upregulated compared with the PBS group. Procollagen type III (COL III) messenger RNA expression was significantly upregulated in the PBS group compared with the normal group. Histological analyses showed that hTMSC administration afforded more favorable collagen and hyaluronic acid deposition than was evident in the controls. Implanted hTMSCs were observed in injured vocal folds 2 weeks after implantation.

Conclusions. Our results show that hTMSCs implantation into injured vocal folds facilitated vocal fold regeneration, with presenting antifibrotic effects.

Keywords

mesenchymal stem cell transplantation, turbinate, vocal fold wound healing

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The extracellular matrix (ECM) of the vocal folds and the associated epithelium cannot be replaced after severe injury.¹ Tissue defects in the vocal folds trigger scar formation. Histologically, the ECM of a scarred vocal fold differs from that of a normal vocal fold, and the changes cause voice problems. No well-established treatment exists for vocal fold scarring. Many treatments have been attempted, ranging from voice therapy to the injection of ECM substances such as hyaluronic acid (HA).²,³ However, treatment effectiveness is limited because the ECM composition does not become normalized. Therefore, the prevention of scar formation is important.⁴

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells of adult tissues. Several studies have reported that various types of MSCs exhibited favorable effects on the healing of injured vocal folds in animal models.⁵⁻⁸

Human nasal inferior turbinate–derived mesenchymal stem cells (hTMSCs) are an alternative source of stem cells. We earlier reported that hTMSCs exhibited MSC-like features.⁹,¹⁰ hTMSCs are relatively easy to harvest and are minimally affected by cell passage number and donor age.¹¹ Thus, hTMSCs may be ideal stem cells for clinical applications.

In this study, we investigated whether hTMSC aided the recovery of injured vocal folds. To this end, we measured messenger RNA (mRNA) expression levels in hTMSC-treated ECM in the early phase of wound healing and

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histopathologically examined tissue in the late phase of wound healing, using xenograft animal models.

**Materials and Methods**

**Isolation and Culture of hTMSCs**

The isolation and culture of hTMSCs were performed as described previously. The procedure was approved by the Institutional Review Board of Daejeon St Mary’s Hospital, The Catholic University of Korea (DC15TISI0022). With the informed consent of each patient, inferior turbinate tissues were obtained from tissues discarded after septoplasty and partial turbinectomy. Tissue was obtained from a position 1 cm behind the anterior end of the inferior turbinate under an endoscopic view using cutting forceps, washed with phosphate-buffered saline (PBS), and passed through a cell strainer (pore size: 100 mm) to remove tissue debris. The filtered cell suspensions were centrifuged and the cell pellets resuspended in α–Minimal Essential Medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin G, and 100 mg/mL streptomycin (all from Gibco, Grand Island, New York). The cells were seeded in culture plates and cultured at 37°C under 5% (v/v) CO₂. The cells were detached using 0.05% (w/v) trypsin-EDTA and replated at 5 × 10⁵ cells/dish (diameter: 100 mm). Fifth-passage hTMSCs were used for injection.

**Animal experiments.** This study was approved by the Animal Ethics Committee of The Catholic University of Korea (permit no. CMCDJ-AP-2014-005). The animals were cared for in accordance with our established institutional guidelines.

**Rats.** A total of 30 Sprague-Dawley rats were anesthetized via an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg). Each animal was placed in a semivertical position on a custom-made platform. The larynx was visualized with a pediatric endoscope. The right-side vocal folds were injured with microscissors until the thyroarytenoid muscle was exposed. This surgical procedure was described previously.

A total of 5 × 10⁵ hTMSCs in 0.05 mL PBS (the hTMSC group) or 0.05 mL PBS alone (the scar control group) was injected into the vocal folds (thyroarytenoid muscle) immediately after injury. Injections were performed using a syringe equipped with a 25-gauge long needle under direct vision employing a pediatric laryngoscope.

**Rabbits.** Twenty New Zealand white rabbits each weighing 3.5 kg were used. After premedication with xylazine (Rompun 10 mg/kg; Bayer, Berlin, Germany), all rabbits were anesthetized via the intramuscular administration of 50 mg/kg ketamine. Each rabbit was then placed in dorsal recumbency and the mouth opened. Right-side vocal fold injury was inflicted using a standard method featuring excision of the epithelium and lamina propria with the aid of microsurgical instruments. The contralateral vocal folds were not injured. Next, 1 × 10⁶ hTMSCs in 0.1 mL PBS (the hTMSC group) or 0.1 mL PBS alone (the scar control group) was immediately injected into the injured vocal folds. Injections were performed using a syringe equipped with a 25-gauge long needle under direct vision employing a pediatric laryngoscope. The correct injection site was confirmed by observing bulging of the vocal fold, reflecting the injected volume.

**Real-time reverse transcription polymerase chain reaction on rat materials.** The vocal folds of the rats were harvested 1 week after injury. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, California) according to the manufacturer’s instructions. Then, complementary DNA (cDNA) was synthesized from 1-μg amounts of RNA using a Reverse Transcriptase Premix Kit (Elpis Biotech, Daejeon, Korea). After reverse transcription of the RNA, the cDNA was used as a template in polymerase chain reactions (PCRs) employing gene-specific primer pairs (Table 1). The cDNA was amplified with the aid of Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Quantitative real-time PCR was performed on an ABI 7500 FAST platform (Applied Biosystems, Foster City, California). The relative levels of mRNA were normalized to that encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Macroscopic evaluation for rabbit vocal fold.** At 2 weeks and 2 months postinjury, photographs of each of the 10 rabbit vocal folds were taken with a rigid endoscope. The macroscopic evaluation of vocal folds was performed by a blinded method with rigid endoscopic photographs of experimental animals by 4 otolaryngologists who were not related to this experiment. Granulation tissue formation and fibrotic band formation were evaluated.

**Histological analysis of rabbit tissue.** Each of the 10 rabbit larynges was harvested for histological analysis after euthanasia at 2 weeks or 2 months postinjury with reference to previous studies.

Specimens were embedded in paraffin blocks and sectioned at a 4-μm thickness along the coronal axis using a microtome. The specimens were stained with hematoxylin and eosin, Masson’s trichrome (for collagen), or Verhoeff–Van Giesen stain (for elastic fibers) using standard pathology department protocols. The stained samples were observed under a light microscope (Eclipse TE300; Nikon, Tokyo, Japan). Densitometric analysis was performed by measuring the area of extracellular matrix stained with immunostaining. Stained areas were measured using ImageJ software (National Institutes of Health, Bethesda, Maryland).

**Histological Sample Preparation and Immunohistochemical Detection of hTMSCs**

Prior to immunohistochemistry, sections were rinsed in PBS and blocked with 4% (v/v) normal goat serum and 1% (w/v) bovine serum albumin (BSA) in PBS for 1 hour at room temperature. The sections were incubated overnight at 4°C with a 1:100 dilution of anti-human nuclear antibody...
using ImageJ in a blinded fashion.

**Statistics**

All statistical analyses were performed with the aid of SPSS software (version 18.0 for Windows; SPSS, Inc, an IBM Company, Chicago, Illinois). The Mann-Whitney test was used to explore the significances of differences between 2 groups, and the Kruskal-Wallis test was employed to compare 3 groups. Pearson χ² tests were used for analysis of the incidence rates of macroscopic findings. A P value less than .05 was considered statistically significant. All results are expressed as means ± standard deviations (SDs).

**Results**

**Gene Expression Analysis**

Real-time PCR showed that the expression levels of mRNAs encoding hyaluronan synthase (HAS) 1, HAS 2, and transforming growth factor (TGF)–β1 were significantly upregulated in the hTMSC group compared with the normal and PBS groups. Although the genes encoding HAS 3 and fibroblast growth factor (FGF) 2 were upregulated to some extent in the hTMSC group, there was no significant difference among the groups. Procollagen type III (COL III) mRNA expression was significantly upregulated in the PBS group compared with the normal group. In the hTMSC group, COL III mRNA expression was somewhat downregulated compared to the PBS group; however, statistical significance was not attained (Figure 1).

**Macroscopic Examination**

Endoscopic examinations were conducted at 2 weeks and 2 months after injury. In the hTMSC group, the morphology of the vocal folds was better than in the PBS group. At 2 weeks after injury, less granulation tissue was observed in the hTMSC group compared with the PBS group (P = .000, Table 2 (Figure 2 endoscopic finding)), and less scarring and fibrotic banding were observed in the hTMSC group at 2 months after injury (P = .001, Table 2 (Figure 3 endoscopic finding)).

**Histological Examination**

Histology performed at 2 and 8 weeks after injury showed that the hTMSC group exhibited less antifibrotic effects than the PBS group. These histological differences were pronounced at 8 weeks. Masson’s trichrome staining showed that collagen deposition was reduced in the hTMSC group compared with the PBS group. Verhoeff’s staining showed that the elastic fibers in the hTMSC group tended to be less disorganized and less tangled than in the PBS group. HA was detected by Alcian blue staining. The PBS group exhibited the least HA, but HA was retained to some extent in the hTMSC group. These histological findings were also confirmed by objective densitometric analysis. The ECM collagen density was significantly lower in the hTMSC group compared with the PBS group (17.18 ± 2.85 in PBS; P = .000 vs 24.52 ± 1.37 in hTMSC) and did not differ significantly between the hTMSC and the normal group (17.18 ± 1.37 in hTMSC vs 16.89 ± 1.11 in normal; P = .778). The elastic fiber density was significantly lower in the hTMSC group compared with the PBS group (5.34 ± 0.83 in hTMSC vs 7.49 ± 1.87 in PBS; P = .006) but was higher than in the normal group (5.34 ± 0.83 in hTMSC vs 4.7 ± 0.26 in PBS; P = .000). The density of HA was

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**Table 1. Sequences of the Primers Used for Quantitative Real-Time Polymerase Chain Reaction.**

<table>
<thead>
<tr>
<th>Rat Genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’-ACCACAGTCCATGCACTAC-3’</td>
<td>5’-TCCACACACCTGCTGTGTA-3’</td>
</tr>
<tr>
<td>COL III</td>
<td>5’-ATGGTCTTCAGTTCCAGC-3’</td>
<td>5’-CTGGCTGCTCCATTCAACCA-3’</td>
</tr>
<tr>
<td>FGF2</td>
<td>5’-AGGCCTCCTACTGCAAGAAC-3’</td>
<td>5’-GCCACCTCTTCCCTTCTA-3’</td>
</tr>
<tr>
<td>FN</td>
<td>5’-CGAGGTGACAGAGACCACAA-3’</td>
<td>5’-CTGGAGTCAGGAGACAGAC-3’</td>
</tr>
<tr>
<td>HAS 1</td>
<td>5’-TGGTCTGGATTTGAGGAGAT-3’</td>
<td>5’-AACGTGCTCCACATTTGAAGGCTA-3’</td>
</tr>
<tr>
<td>HAS 2</td>
<td>5’-CCAACTCGAGTTGGATCTG-3’</td>
<td>5’-ACTGGAGACGGCCCTGAT-3’</td>
</tr>
<tr>
<td>HAS 3</td>
<td>5’-CCCTATCGCCAGCTCATACA-3’</td>
<td>5’-CCACAGCTGACGTTTCTAG-3’</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5’-TGGGTTAATGTTGGTAAC-3’</td>
<td>5’-GGTGTAGCCCTTTCCAG-3’</td>
</tr>
</tbody>
</table>

Abbreviations: COL III, procollagen type III; FGF2, fibroblast growth factor 2; FN, fibronectin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HAS, hyaluronan synthase; TGF-β1, transforming growth factor–β1.
decreased significantly in both the PBS and hTMSC groups compared with the normal group (3.86 ± 1.18 in hTMSC vs 17.23 ± 3.41 in normal, \( P = .010 \); 1.30 ± 0.87 in PBS vs 17.23 ± 3.41 in normal, \( P = .004 \)), but the hTMSC group exhibited a significantly higher HA density compared with the PBS group (3.86 ± 1.18 in hTMSC vs 1.30 ± 0.87 in PBS, \( P = .003 \)) (Supplemental Figure S1, available in the online version of the article).

### Discussion

Studies of treatments for vocal fold fibrosis are ongoing, including efforts to replace ECM materials, to implant several types of growth factors with or without scaffolds, and to improve cell therapies such as stem cell injection (which has been reported to be effective in the laboratory). Stem cell implantation has been widely used in the field of regenerative medicine.

Kanemaru et al\(^7\) were the first to inject autologous bone marrow–derived stem cells (BMSCs) into the injured vocal folds of dogs. de Bonnecaze et al\(^{16}\) reported that the injection of autologous adipose tissue–derived stem cells (ASCs) improved vocal fold wound healing in a rabbit model. Hiwatashi et al\(^{17}\) compared the regenerative outcomes of ASC and BMSC transplantation and suggested that ASCs were more effective.

Most laboratory studies have used autologous or xenograft stem cells. In terms of clinical application, xenograft stem cells are associated with limitations such as rejection, and the use of autograft stem cells is sometimes problematic if host condition did not allow procedures such as bone marrow biopsy and liposuction that required for the harvest.

### Table 2. Macroscopic Evaluation of Vocal Folds at 2 Weeks and 8 Weeks.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2 Weeks</th>
<th>8 Weeks</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>hTMSC Group (n = 10)</td>
<td>PBS Group (n = 10)</td>
</tr>
<tr>
<td>Granulation tissue formation</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td>Fibrotic band</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: hTMSC, human nasal inferior turbinate–derived mesenchymal stem cell; PBS, phosphate-buffered saline.
\(^a\) \( P < .05 \).
of MSCs. In this study, we used hTMSCs, which can of course be collected from the patient. hTMSCs can be easily obtained during turbinate surgery, which is a common oto-laryngological procedure, being effective and safe in contrast to bone marrow biopsy and liposuction. We earlier verified that hTMSCs exhibited the properties of MSCs. hTMSCs were obtained at approximately a 30-fold higher yield than ASCs and proliferated approximately 5-fold better than did BMSCs.10 We previously published our regenerative medicine findings using hTMSCs in various contexts.9,10,18,19

Some studies used rabbits for both histological examination and PCR. However, the number of vocal fold tissues subjected to PCR was small.20,21 Therefore, it was difficult to attain statistical significance, even though a tendency may have been evident. In the present study, we subjected

![Figure 2](image2.png)

**Figure 2.** Endoscopic findings and histopathology of the vocal folds at 2 weeks (2 W) after injury. Histopathology: magnification ×100. hTMSC, human nasal inferior turbinate–derived mesenchymal stem cell; PBS, phosphate-buffered saline.

![Figure 3](image3.png)

**Figure 3.** Endoscopic findings and histopathology of the vocal folds at 8 weeks (8 W) after injury. Histopathology: magnification ×100. hTMSC, human nasal inferior turbinate–derived mesenchymal stem cell; PBS, phosphate-buffered saline.
tissues from 30 rats to PCR alone and found that, at the mRNA level, hTMSCs aided in the regeneration of damaged vocal folds, with statistical significance.

HA significantly influences the viscoelasticity of the ECM during the regeneration of vocal fold tissue. In addition, collagen fibers are necessary for wound healing, but excessive collagen triggers functional problems after healing. Therefore, the preferred course of vocal fold regeneration is the reduction of collagen deposition with enhancement of HA synthesis. The levels of HAS 1– and HAS 2–encoding mRNAs were significantly increased in the hTMSC group compared with the PBS group, and the HAS 3 mRNA level was increased compared with the PBS group, although statistical significance was not attained. The expression level of COL III mRNA was decreased in the hTMSC group compared with the PBS group.

Interestingly, TGF-β1– and FN-encoding mRNAs also increased in the hTMSC group. TGF-β1 and FN have been reported to be upregulated in the chronic phase of wound healing and are associated with increased collagen synthesis. Thus, TGF-β1 and FN reductions during the chronic phase of wound healing would aid vocal fold regeneration. However, in the early phase of wound healing, different results have been reported. Duflo et al reported that TGF-β1– and FN-encoding mRNA expression levels increased at this time and may improve vocal function in the early phase of wound healing. Lim et al observed TGF-β mRNA expression in the early phase of wound healing, and the downregulation of TGF-β aided scarless wound healing.

In the present study, the increases in TGF-β1– and FN-encoding mRNA levels in the hTMSC group did not trigger collagen deposition after 2 months, perhaps because of the expression of other factors such as hepatocyte growth factor (HGF), which suppressed TGF-β1 production. Ohno et al reported that TGF-β1 mRNA expression peaked 7 days postinjury, and HGF mRNA expression suppressing profibrotic activity peaked 14 days postinjury. Further studies that identify TGF-β1 and HGF mRNA expression at multiple timelines are needed.

Previous studies reported that elastic fiber was reduced in scarred voice folds. However, the present study showed that the PBS-injected vocal folds (PBS group) exhibited more elastic fiber deposition compared to the hTMSC group and normal group in densitometry analysis. This result can be explained by the fact that the tissue slides are made by coronal section. In normal vocal folds, elastic fibers run parallel to the free edge of the vocal, so these fibers are observed as small black dots in the Verhoeff–Van Giesen stain. In the scarred vocal fold, compact and disorganized elastic fibers are detected as big points or bundles in the Verhoeff–Van Giesen stain.

We sought to confirm how the expression of these mRNAs changed histological features. However, the rat vocal fold is so small that it is relatively difficult to identify histological changes. Rabbit vocal folds can be subjected to endoscopic and histological morphological examinations. Histological examination of the rabbit vocal fold reflected the changes in mRNA levels, and the rat results were thus confirmed.

In the present study, injected hTMSCs persisted for more than 2 weeks, but we found no surviving cells after 4 weeks. The survival period of injected stem cells remains controversial. Some studies reported that the survival time was more than 1 month, and cell differentiation was observed. It has been suggested that the observed improvements in vocal fold healing were attributable to stem cell differentiation. However, other studies have
reported survival times of less than 1 month and the absence of any differentiation.\textsuperscript{17}

It is difficult to determine whether the observed regeneration of injured vocal folds was attributable to the transdifferentiation of hTMSCs to vocal fibroblasts or a paracrine effect of hTMSCs on such fibroblasts. However, differentiated hTMSCs would have been easily detected using anti–human antibodies. Therefore, the observed improvements in vocal fold healing reflect a paracrine effect of hTMSCs rather than cell differentiation and engraftment.

The paracrine effects of stem cells have been studied in various contexts.\textsuperscript{36-39} However, the paracrine effects of stem cells in terms of conditioned medium have not been studied in the sphere of vocal fold regeneration. Further study is needed.

A xenograft animal model that did not control host immunity could be the limitation of this study. The ultimate goal of our study is to use hTMSCs to heal human vocal folds. However, prior to clinical use, we chose the xenograft model to demonstrate the effectiveness of hTMSCs. If hTMSCs are applied clinically, an autograft model is used. Some studies used an immunosuppressant agent to control host immunity in a xenograft animal model.\textsuperscript{6,40,41} However, in this study, there were no signs of immune reaction and T lymphocyte infiltration around the injected hTMSCs. This result shows the hypoinmunogenic characters of hTMSC. The immune evasion of MSC is not fully understood, but several biological properties of MSCs have been identified and accepted, such as the immune-modulatory property and suppression of innate immune cells or adaptive immune cells.\textsuperscript{42} Recently, many studies have been done without the use of immunosuppressant in a xenograft model that transplants human stem cells.\textsuperscript{34,43-45}

Conclusion

Implantation of hTMSCs into injured vocal folds was associated with the expression of useful genes 7 days after injury in a rat model. Also, endoscopic and histopathological examinations showed that scar formation was reduced in a rabbit model. These results suggest that hTMSCs could present anti-fibrotic effects and facilitate vocal fold regeneration.

Author Contributions

Choung-Soo Kim, study design, animal experiment, data collection and analysis, wrote the manuscript, checked the reference, final approval of the version to be published; Hyunsu Choi, helped with animal experiment, data collection and analysis, drafting the work or revising it critically for important intellectual content, final approval of the version to be published; Ki Cheol Park, helped with animal experiment, data collection and analysis, drafting the work or revising it critically for important intellectual content, final approval of the version to be published; Sung Won Kim, data collection and analysis, compiled the literature sources, interpreted data, drafting the work or revising it critically for important intellectual content, final approval of the version to be published; Dong-II Sun, study design, animal experiment, compiled the literature sources, interpreted data, drafting the work or revising it critically for important intellectual content, final approval of the version to be published.

Disclosures

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Supplemental Material

Additional supporting information is available in the online version of the article.

References


