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의학박사 학위논문

Injection Laryngoplasty with Human Adipose Tissue Derived Extracellular Matrix and Methylcellulose Hydrogels

인간 지방 조직 추출 세포외기질을 이용한 성대주입술

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김 동 욱

Injection Laryngoplasty with Human Adipose Tissue Derived Extracellular Matrix and Methylcellulose Hydrogels

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A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Medicine
(Otorhinolaryngology)

In the Seoul National University, Seoul, Korea
July 2017

Doctoral committee:

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Professor Seong Keun Kwon

Professor Sang Hyuk Lee

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Abstract

Injection Laryngoplasty with Human Adipose Tissue Derived Extracellular Matrix and Methylcellulose Hydrogels

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Objective: To introduce a mixture soluble extracellular matrix (sECM) and methylcellulose (MC) hydrogels as an injection laryngoplasty material and present the preliminary animal study focusing on a bio-suitability of sECM/MC hydrogels

Subjects and Methods: sECM was fabricated from human adipose tissue which was obtained by a liposuction technique. After removing blood and oil components from adipose tissue, the tissue was homogenized, centrifuged and lyophilized. sECM/MC solution was prepared by a mixture of lyophilized sECM and MC solution that was obtained by dispersion technique. Unilateral vocal fold palsy was made to twenty New Zealand white rabbits by recurrent laryngeal nerve section and five rabbits without procedure were used as a normal group. Twenty animals of vocal fold palsy were randomly divided into 4 groups. Group 1-3 received sECM/MC injection at paralyzed vocal fold and sacrificed 1, 4 and 8 weeks after injection, respectively.

Group 4 was not injected and used as a control. Laryngoscopic exams were performed at 1, 4 and 8 weeks after injection laryngopalsty; then vocal fold vibration was evaluated for functional analysis by high speed video recording and histologic study was performed in each groups.

Results: All animals except one rabbit survived until the scheduled period. Laryngoscopic analysis showed that sECM/MC maintained its volume through 8 weeks after injection. Histologic studies also revealed the augmentation effect in paralyzed vocal fold without significant inflammatory response. There were increased number of collagen fibers and fatty granules at the injection site without significant inflammation or fibrosis. On functional analysis, high speed camera examination revealed regular and symmetric contact of vocal fold mucosa without a distorted movement by injected sECM/MC in experimental group. Also asymmetric vocal fold movement was improved compared with the control group.

Conclusion: In the paralyzed vocal fold, sECM/MC hydrogels showed a good bio-compatibility and effective augmentation results histologically and functionally. These results indicate that the sECM/MC hydrogel can enhance vocal function in paralyzed vocal folds without early resorption and has potential as a promising material for injection laryngoplasty for stable vocal fold augmentation which can overcome the shortcomings of autologous fat such as unpredictable duration and morbidity associated with the fat harvest.

Keywords: vocal fold paralysis, adipose tissue, extracellular matrix, injection laryngoplasty, adipogenesis

Student Number: 2013-31136

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Introduction

Glottic insufficiency resulting from vocal fold paralysis causes numerous voice complaints, including vocal fatigue, hoarseness, loss of projection, and breathiness. Dysphagia often occurs with vocal fold paralysis and paresis. The most common etiology of vocal fold paralysis is iatrogenic nerve injury. Surgical procedures commonly associated with iatrogenic vocal fold paralysis include thyroidectomy/parathyroidectomy, anterior cervical disc surgery, esophagectomy, thymectomy, neck dissection, carotid endarterectomy, mediastinoscopy, and cardiothoracic surgery, including aortic surgery, coronary artery bypass grafting, and pulmonary lobar resection. Endotracheal intubation, prolonged nasogastric tube placement, and even esophageal stethoscope placement have all been implicated as occasional causes of VFP. [1-5] Various techniques, including laryngeal framework surgery and injection laryngoplasty, have been applied in the management of VFP and resultant glottal insufficiency. In recent years, injection laryngoplasty has regained popularity because of its initial low cost, minimal invasiveness, and low morbidity as compared to surgical repair methods such as thyroplasty type I and arytenoid adduction.[6]

Based on clinical experience and studies related to glottal insufficiency, an ideal material for injection laryngoplasty should have the following characteristics. It should be easily injectable into the vocal folds in an outpatient setting. It should sufficiently restore the volume of the atrophied glottis. It should medialize the vocal

fold permanently, be highly biocompatible and not induce an inflammatory response. However, none of the currently used materials for injection laryngoplasty possess all of these characteristics. A number of injection materials have been proposed and tested in the clinical setting of glottic insufficiency,[7] including paraffin, cartilage particles, bovine bone dust, polytetrafluoroethylene (PFTE/Teflon®), gelatin (Gelfoam®), bovine collagen, autologous collagen, acellular dermis (Alloderm®), micronized formulation of acellular dermis (Cymetra®), autologous tissues (i.e., fascia and fat), calcium hydroxylapatite (CaHA), and hyaluronic acid (HA). [6, 8, 9]

Calcium hydroxyapatite (CaHA) is the only injection material approved by the FDA for laryngoplasty, and it has been commonly used because of its lower cost, morbidity, and invasiveness compared with those of conventional open thyroid framework surgery.[9] However, CaHA cannot maintain its augmentation effect long term, and it has been reported to induce various complications such as inflammation, granulation, migration, and inhibition of vocal fold vibration.[10]

Autologous fat injection is considered to be durable and long-lasting, with excellent biocompatibility. Several studies have reported that autologous fat injection has long-lasting effects that are comparable to the functional outcomes seen in framework surgery such as medialization thyroplasty.[11-15] However, unlike injection laryngoplasty using CaHA, which can be easily performed in an outpatient clinic, autologous fat injection laryngoplasty requires a liposuction procedure that needs to be performed under general anesthesia in an operating room. Injection laryngoplasty with fat requires large gauge needles, such as 18 gauge, which makes it difficult to

localize the injected material precisely into the muscle layer of vocal fold. Another concern with fat injection is the possibility of unpredictable early resorption. The efficacy of fat injection is often doubted due to variable resorption rates and unpredictability in postoperative outcomes. The resorption rate of grafted fat tissue ranges from 20% to 90%.[16-18] It was reported that injected fat was absorbed within 1 month and was completely resorbed within 5 months.[19, 20] To prevent early resorption, lipoinjection into vocal folds is performed such that there is substantial over injection of the volume. However, this excessive fatty tissue can induce poor voice quality and respiratory distress.

To overcome these limitations, we developed an easily injectable, soluble extracellular matrix (sECM) and methylcellulose (MC) hydrogel for use in injection laryngoplasty. The sECM/MC solution exhibited thermosensitive sol-to-gel transition, remaining a viscous liquid at 4 ℃ and forming a translucent hydrogel at 37 °C. Human adipose tissue, the most prevalent and expendable tissue in the body, can be harvested in large quantities with minimal morbidity and has received much attention as a rich source of ECM. Numerous studies have reported on the biological effects of ECM-based three-dimensional (3-D) tissue engineering scaffolds for use in regenerative medicine.[21-25] Human adipose-derived ECM grafts have greater biostability than autologous fat graft.[23, 24] ECM scaffolds derived from human adipose tissue have been reported to increase graft volume and form new adipose tissue with numerous blood vessels.[21-25] Here, we introduce soluble ECM hydrogel as a novel

material for injection laryngoplasty and present preliminary animal study data focusing on the effects of stable augmentation and its biocompatibility.

Materials and Methods

All experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (approval number: 14-0005-S1A0) and performed in accordance with the ethical guidelines of the committee. All efforts were made to minimize suffering.

Preparation of sECM/MC hydrogel

The sECM was prepared from human adipose tissue obtained by liposuction technique.[21-25] The adipose tissue obtained by liposuction (~20 ml) was washed several times with distilled water to remove blood components. Distilled water (10 ml) was added to the adipose tissue and the tissue/water mixture was homogenized at 12,000 rpm for 5min. The tissue suspension was centrifuged at 3000 rpm for 5 min and the upper layer containing oil components was discarded. This process was repeated several times. The final gel-like, thick tissue suspension (~5 ml) was washed three times by addition of ~25 ml of distilled water to the gel-like tissue suspension, gentle mixing by pipetting, and centrifugation at 3000 rpm for 5min. The final gel-like tissue suspension was frozen at −70 °C, freeze-dried, and crushed using a manual mill. Finally, the sECM solution (30% w/v) was prepared by dissolving 3 g of lyophilized sECM in 10 mL of phosphate buffered saline (PBS) for 24 h at 4°C. MC solution (7.5% w/v) was prepared using dispersion. Briefly, 0.75 g of MC (viscosity of 15 cP) was thoroughly wetted in 10 mL of PBS and incubated at 90°C for 60 min. Then, the solution was equilibrated overnight at 4°C. Subsequently, sECM and MC

solutions were combined at a volume ratio of 1:4 to a final concentration of 6% ECM and 6% MC, and the mixture was stirred to homogeneity at 4°C. The sECM/MC solution was stored at 4°C until use.

Recurrent laryngeal nerve (RLN) section, sECM/MC hydrogel injection laryngoplasty, and group classification

Twenty five male New Zealand white rabbits (Koatech Laboratory Animal Company, Korea) weighing 3.0 to 3.5 kg were obtained. Five animals were used as normal control. Unilateral recurrent laryngeal nerve section to induce vocal fold palsy was performed in twenty animal as previously described.[26] Zoletil (50 mg/kg) was administered intramuscularly for anesthesia and subcutaneous fat and strap muscles were dissected at the midline through a 2.5 cm vertical incision made at the level of the cricoid. After thyroid isthmectomy, the left thyroid lobe was laterally reflected, and the inferior thyroid vessel was identified at the medial surface of the thyroid gland. The left RLN was identified parallel to the inferior thyroid vessels. The RLN was severed for approximately 2 cm to prevent spontaneous re-anastomosis. Subcutaneous tissue and skin were closed with a surgical stapler. Immediately after the procedure, laryngeal endoscopic exam was performed to confirm unilateral vocal fold palsy using a 4.0 mm 30 degree rigid endoscope (Richards, Knittlingen, Germany). Prophylactic antibiotics (cephazolin) were administrated intramuscularly for 3 days.

One week after RLN sectioning, rabbits were randomly divided into 4 equal groups of 5 rabbits using a blocked randomization method. Groups 1, 2, and 3 received sECM/MC hydrogel injection laryngoplasty and group 4 acted as the control group with no injection procedure. The sECM/MC hydrogel injection was conducted under anesthesia with intramuscular injection of Zoletil (50 mg/kg). Each injection (0.1 cc/rabbit) was administered using a syringe with a 23 G spinal needle under the guidance of a 4.0 mm 30 degree rigid endoscope. The injection needle was placed laterally at the tip of the vocal process such that the vocal process could rotate medially. For the following 3 days, rabbits were given prophylactic antibiotics. All animals were monitored daily for weight, coughing, sputum production, wheezing, and dyspnea.

For histological studies and functional analyses, groups 1, 2, and 3 were sacrificed at 1, 4, and 8 weeks after sECM/MC hydrogel injection laryngoplasty, respectively. From group 4, two rabbits were randomly selected for sacrifice at 4 weeks after sECM/MC hydrogel injection laryngoplasty and the remaining rabbits were sacrificed at 8 weeks. All groups received standard laryngoscopic examination using a 4.0 mm 30degree rigid endoscope immediately post injection laryngoplasty and at 1, 4, and 8 weeks after sECM/MC hydrogel injection laryngoplasty. Images were taken of vocal folds with a digital camera (E4500, Nikon, Tokyo, Japan) that was attached to the endoscope.

Assessment of vocal fold vibration for functional analysis

Group 3 and control group animals were humanely sacrificed, and the larynges were removed through total laryngectomy. Also, the larynges of animals in normal group were obtained by the same manners. Vocal fold vibrations were examined using a high-speed camera.[1, 26] To better visualize the vocal fold, the superior portion of the thyroid cartilage and supraglottic structures were removed. The arytenoid cartilage was sutured using a Prolene 6-0 suture to close both vocal folds (Figure 1). The trimmed larynx was mounted, and humidified air was injected into the larynx to generate vocal fold vibration. Vocal fold vibration during induced phonation was imaged using MotionXtra NR4S2 high-speed video camera (DEL Imaging Systems, Cheshire, Connecticut, USA). High-speed video data were recorded at 8,000 images per sec with a spatial resolution of 256 horizontal × 512 vertical pixels. Illumination was provided by a 300-W xenon light source.

The maximum amplitude of the vocal wave was measured to evaluate the symmetricity of the vocal mucosal vibration using videokymograms that were obtained from the high speed images using Metamorph® (Molecular Device, Sunnyvale, CA) and *Image J* imaging software (National Institute of Mental Health, Bethesda, MD).[27] The maximum distance from the midline of the glottis to the free edge of the vocal fold was measured at the mid antero-posterior portion of the vocal fold using the maximum open phase of a videokymogram (Figure 2A). The maximum distance of the left denervated vocal fold (*a*) was divided by the maximum distance

of the right vocal fold (*b*) to create a ratio that was referred to as the asymmetry index. The asymmetry index is calculated as follows (Figure 2B):

$$\text{Asymmetry index} = a / b$$

The asymmety index shows a degree of the symmetricity in bilateral larynx. A physiologic phonation of normal mammals (dog, rabbit and human, etc.) is achieved by the symmetrical vibration of bilateral larynxes. Therefore, the asymmetry index of healthily vibratory larynx should be expected to be near the value of 1.0. In diseased conditions, including vocal paralysis or severe scar formation at vocal mucosa, the index deviates from the value of 1.0.[28]

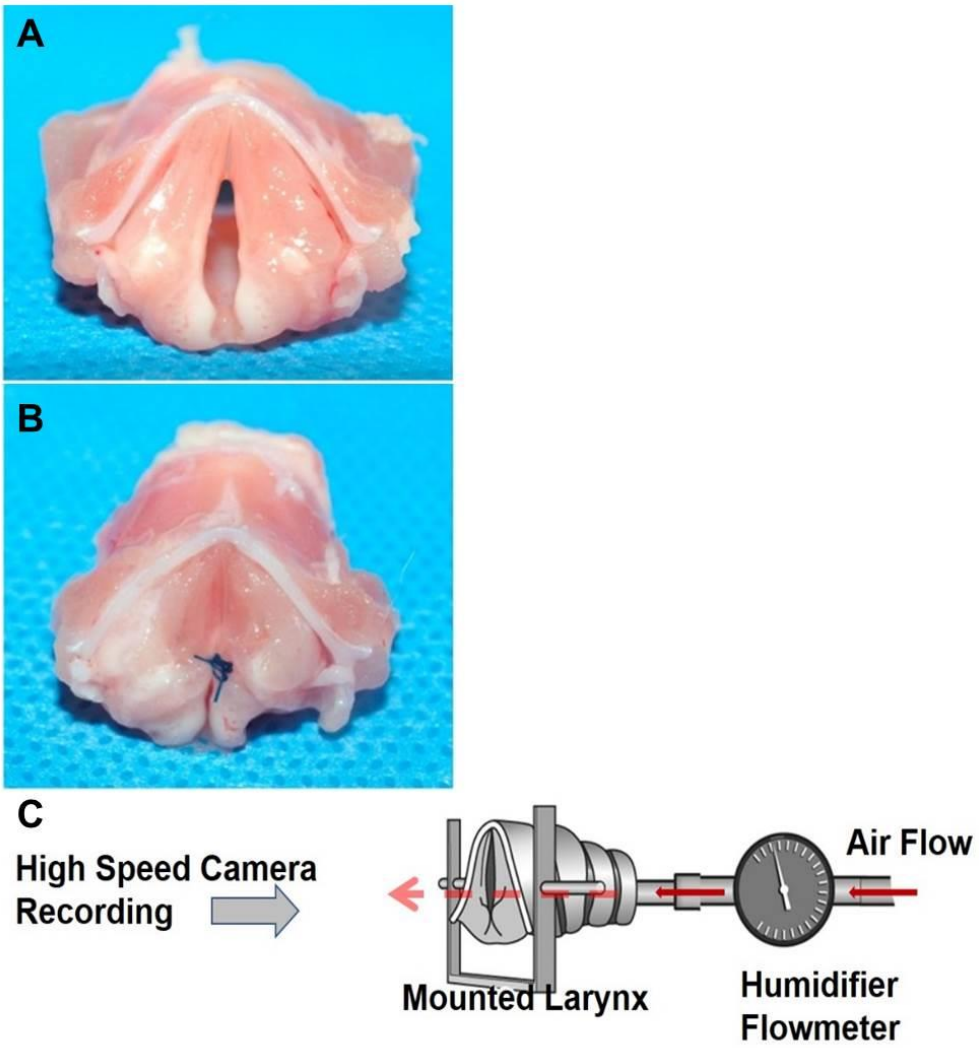
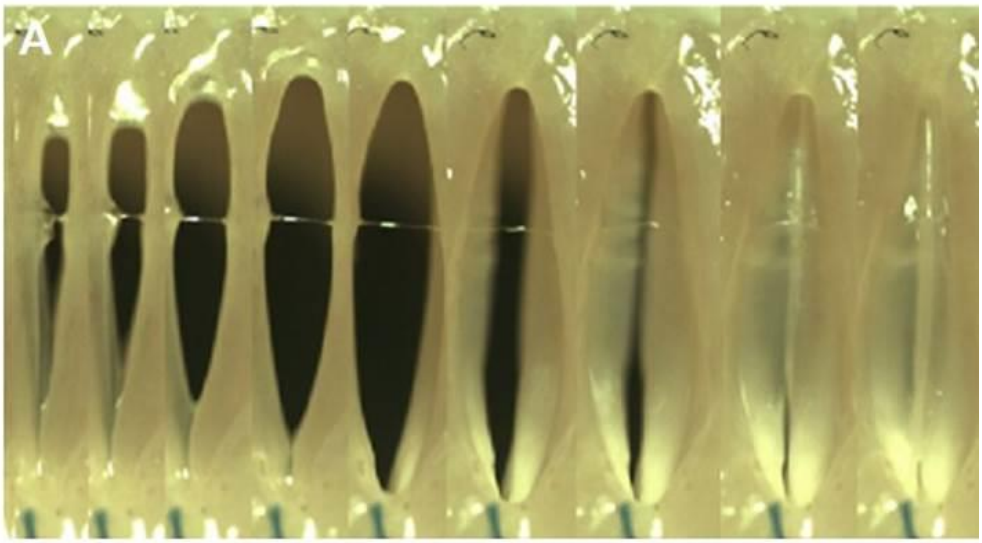

A
B
C
High Speed Camera
Recording
Air Flow
Mounted Larynx
Humidifier
Flowmeter

Figure 1. Functional assessment of vocal fold mucosa vibration using high speed camera recording

The left denervated vocal fold was lateralized after total laryngectomy (A). The arytenoid cartilage sutured to close both vocal folds and trimmed before mounting at air flow outlet of high speed recording (B). The trimmed larynx was mounted, and humidified air was injected into the larynx to generate vocal fold vibration during high speed camera recording (C).


A

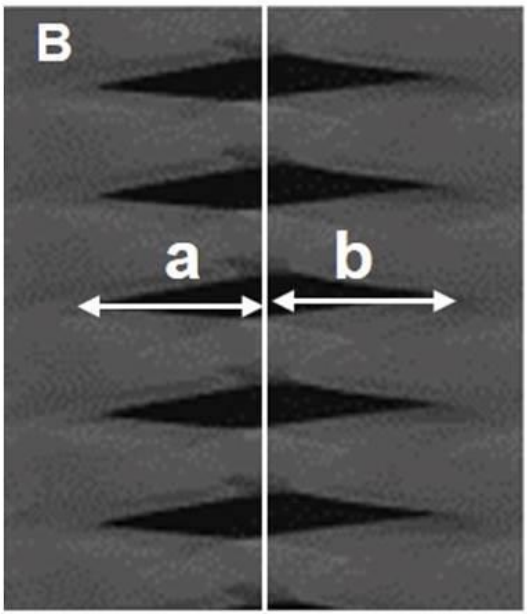

B
a
b

Figure 2. The asymmetric index using videokymograms for vocal functional analysis

(A) The maximum distance from the midline of the glottis to the free edge of the vocal fold was measured at the mid antero-posterior portion of the vocal fold using the maximum open phase of a videokymogram. (B) The maximum distance in the left denervated vocal fold (*a*) was compared to the right vocal fold (*b*) using a videokymogram to generate an asymmetry index. The asymmetry index was calculated as follows: asymmetry index = a / b.

Histological analysis and volume calculations of the injected ECM/MC hydrogel

Specimens from groups 1, 2, 3 and 4 were fixed for 24 h in 10% formalin, and were serially sliced at 4 μm thick using a microtome in the axial plane from the false vocal fold to the level of the subglottis. Standard hematoxylin and eosin (H&E) staining was performed to assess the amount of remaining injection material and determine the presence of any inflammatory tissue reactions. For collagen, hyaluronic acid, and elastin fiber staining, sections were stained with Masson's trichrome (MT, Scytek, Logan, Utah 84323, USA. TRM-1), Alcian blue (AB, Scytek, Logan, Utah 84323, USA. AFR-1), and Verhoeff-Van Gieson (VVG), respectively. In the key section of MT histology, quantitative analysis of collagen fiber deposition within the area of sECM/MC hydrogels injection was performed using Metamorph® software to calculate the total area of staining. The mean area percentage (%) of collagen fiber in the cross-sectional area of the injection material was analyzed according to the experimental period. In addition, an immunofluorescence assay for collagen type I was performed to evaluate fibrosis. Tissue sections were deparaffinized, incubated with a hot stirrer for 10 min in citrate buffer (Invitrogen, Frederick, USA, 005000). Samples were then incubated at room temperature for 30 min followed by 0.5% Triton X-100 solution for 15 min. Samples were blocked using 3% BSA in PBS for 1 h at room temperature, and primary antibody (Collagen type I Human/Rabbit : Abcam, Bristol, UK, ab6308) was incubated at 4°C overnight. After washing with

PBS, secondary antibody (Invitrogen, Frederick, USA, Alexa Goat-594/Alexa mouse-488) was incubated at room temperature for 1 h, and nuclei were stained with DAPI (Molecular Probes, Oregon, USA, H3570). Imaging was performed on a fluorescent microscope. Immunohistochemistry (IHC) using monoclonal mouse anti-rabbit macrophage clone RAM11 (Dako, Ely, UK, M0633) was also performed for confirmation of foamy histiocyte. An experimentally blinded pathologist reviewed the samples.

The volume of remaining material was calculated using a modified procedure of a previously reported method.[29] For each time point, the area of remaining injected material was measured in pixels from 10 consecutive H&E-stained slides (each 100 μm interval) using computerized image analysis software (Leica Q win V3, Konvision Corporation, Seoul, South Korea).[26] The volume of the remaining ECM/MC hydrogel was estimated using the following formula:

Total volume = the sum of cross-sectional material areas from 10 consecutive slides × 100μm.

Statistical analysis

Experimental data were expressed as median with interquartile ranges. Non-parametric statistical test using SPSS 21.0 statistical software (SPSS) was performed, and statistical significance was recognized as $p < 0.05$.

Results

The sECM/MC hydrogel was readily injected into the paralyzed vocal folds of all rabbits for groups 1, 2, and 3, without complications. Endoscopic evaluation suggested that the injected ECM/MC hydrogel remained in the paralyzed vocal folds for up to 8 weeks and augmented volume in the paralyzed left vocal fold in all rabbits of group 3 ($n = 4$). Moreover, group 3 showed straighter vocal folds on the paralyzed side than those of the control group, which were curved and shortened (Figure 3).

High-speed imaging of vocal fold vibration that was induced by intra-tracheal airflow showed regular and symmetrical vocal contact in the sECM/MC hydrogel groups. In addition, the left vocal fold, which was injected with the sECM/MC hydrogel, had a vibration similar to the normal side (Figure 4A). The mean asymmetry index in the sECM/MC hydrogel groups was closer to 1.0 than that of the control group (1.020 ± 0.069, 0.787 ± 0.102, respectively). There was significant difference between two groups ($p = 0.047$ using a Mann-Whitney U test, Figure 4B).

Histological analysis of H&E-stained sections revealed no significant resorption of the injected sECM/MC hydrogel until 8 weeks post injection. The control group showed smaller area of the larynx on the denervated side than on the normal contralateral side at 8 weeks post procedure (Figure 5A, green dotted line). This is likely due to the obvious atrophy of the intrinsic laryngeal muscles. However, in the sECM/MC group, the laryngeal volume was compensated for by the injected sECM/MC hydrogel (Figure 5A, brown dotted line). Quantitative analysis using

H&E staining of sequential tissue sections indicated that the median volume of the sECM/MC hydrogel was stably sustained up to 8 weeks post procedure, and there was no volume difference between 1, 4, and 8 weeks ($p > 0.05$ by Kruskal-Wallis test, Figure 5B).

Focal capillary ingrowth into the injection site (Figure 6, arrowheads) was observed without any significant inflammatory response, including neutrophil and lymphocytes aggregation in the surrounding muscle, lamina propria, or epithelium (Figure 5A). Interestingly, cell aggregates with intracellular fatty lobules increased as function of times and mature adipose tissue developed in the area of the sECM/MC hydrogel injection (Figure 6, white asterisk). Also, mature adipose tissue appeared at 8 weeks post procedure (Figure 6, arrow). Additional IHC analysis using RAM 11 suggested that these cells were foamy histiocytes that had phagocytized fatty granules in the injected sECM/MC hydrogel (Figure 7).

Collagen fibers were weakly stained with MT in the area of sECM/MC hydrogel injection in group 1 (1 week). However, MT staining in group 2 (4 weeks) and group 3 (8 weeks) indicated increased collagen deposition (Figure 8A). Similar results were observed using quantitative analysis of collagen deposition (Figure 8C). The mean cross-sectional area (%) of collagen fiber at the injection site in group 1 was 33.72 ± 11.48%; in group 2, 46.9 ± 10.36%; and in group 3, 58.23 ± 10.55%. The total collagen deposition at 4 and 8 weeks was significantly higher than that at 1 week ($p = 0.0218$ by Kruskal-Wallis test). The deposition of human/rabbit (green) collagen type I was slightly increased up to 4 weeks post procedure and remained at 8 weeks

procedure in the injection area as determined using IF (Figure 8B). These results of collagen study indicate that the collagen type I increased in the injected sECM/MC. However, the formation of elastin fibers or hyaluronic acid was not detected in the area of injection (assessed by VVG and AB staining, Figure 9).

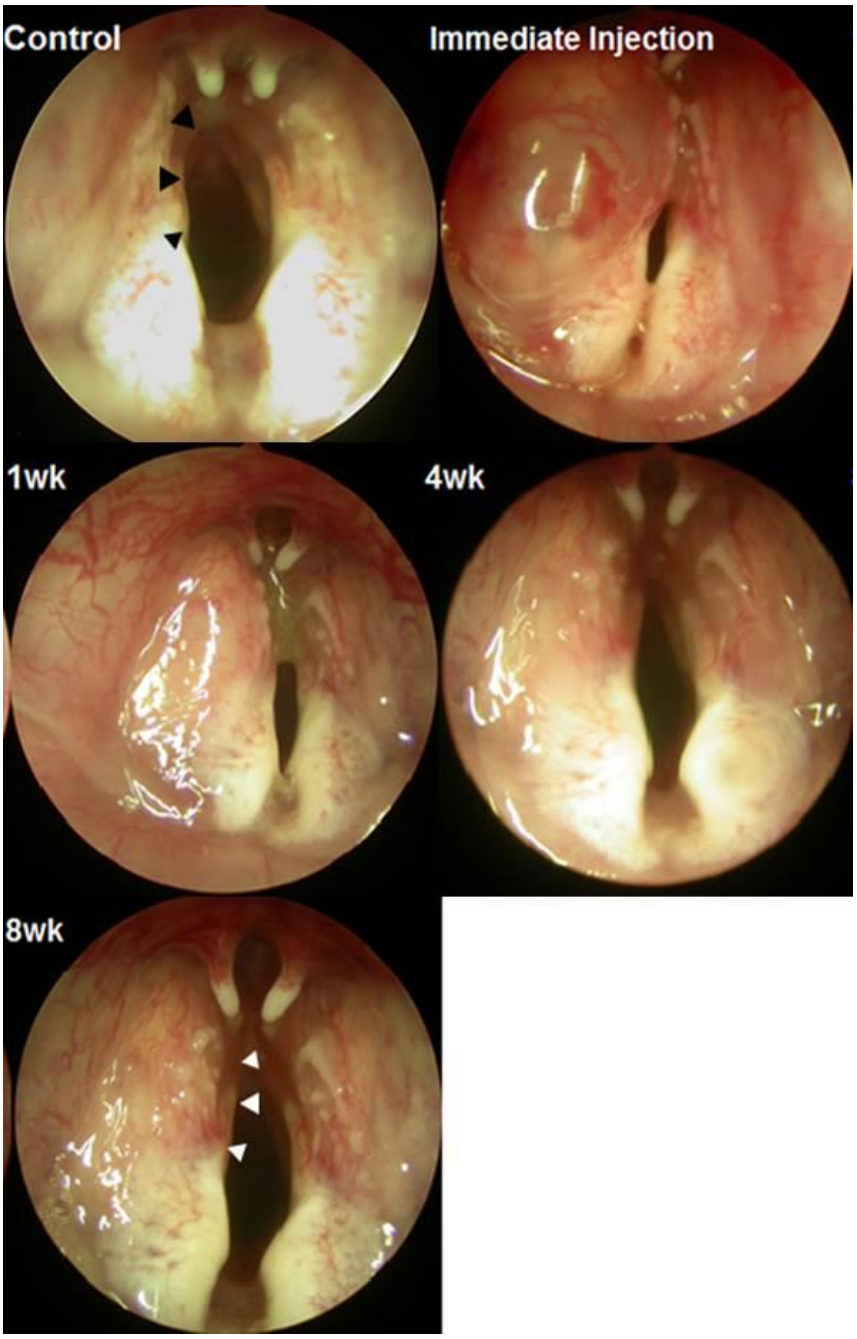
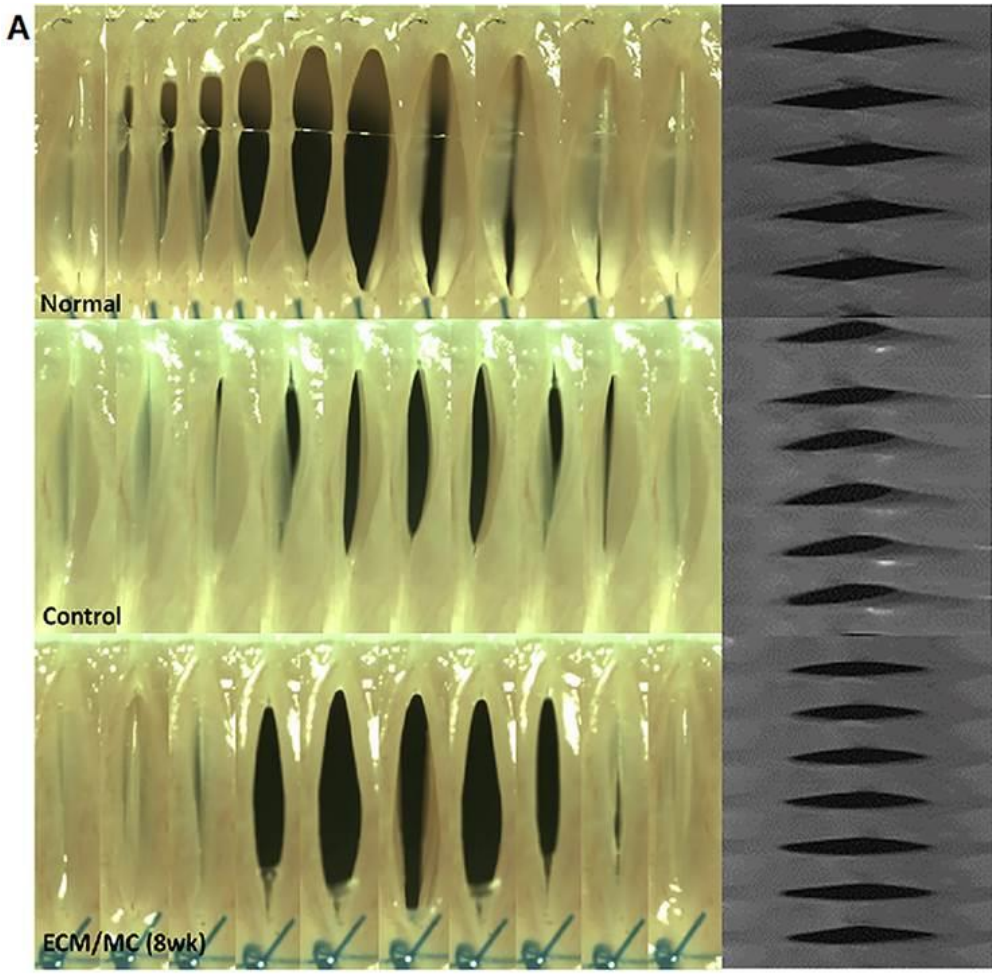

Control
Immediate Injection
1wk
4wk
8wk

Figure 3. Laryngoscopic images after injection laryngoplasty of sECM/MC hydrogels into the left paralyzed vocal fold.

The sECM/MC hydrogel injection group exhibited a straight and medialized vocal fold (white arrowhead) while the control group had a curved and lateralized vocal fold (black arrowhead), which is likely due to denervation of the recurrent laryngeal nerve.


A
Normal
Control
ECM/MC (8wk)

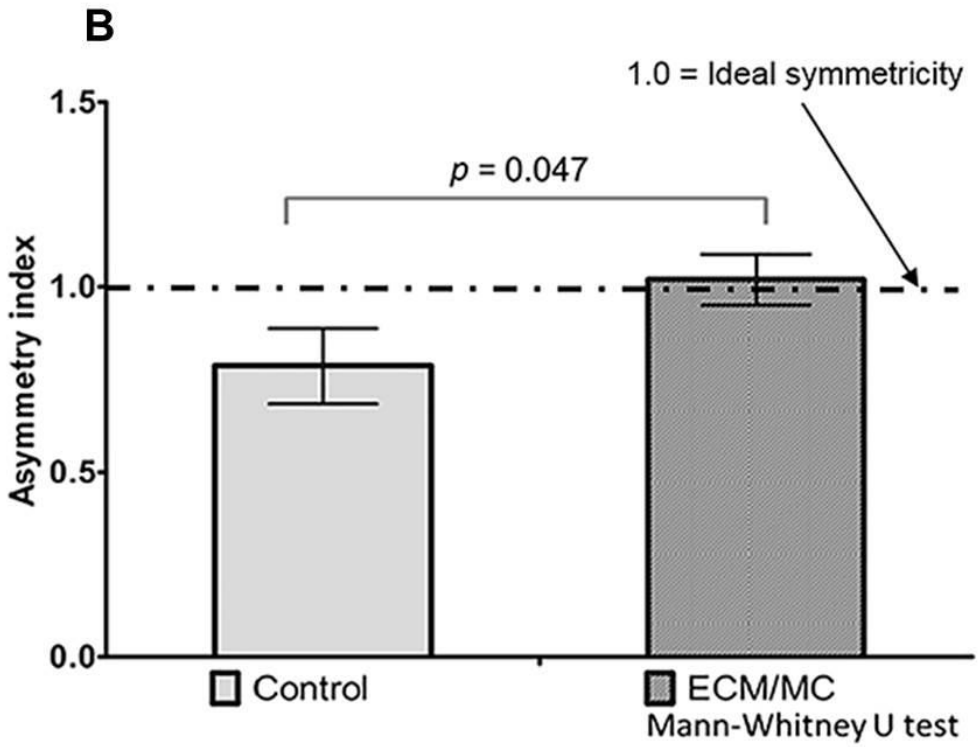
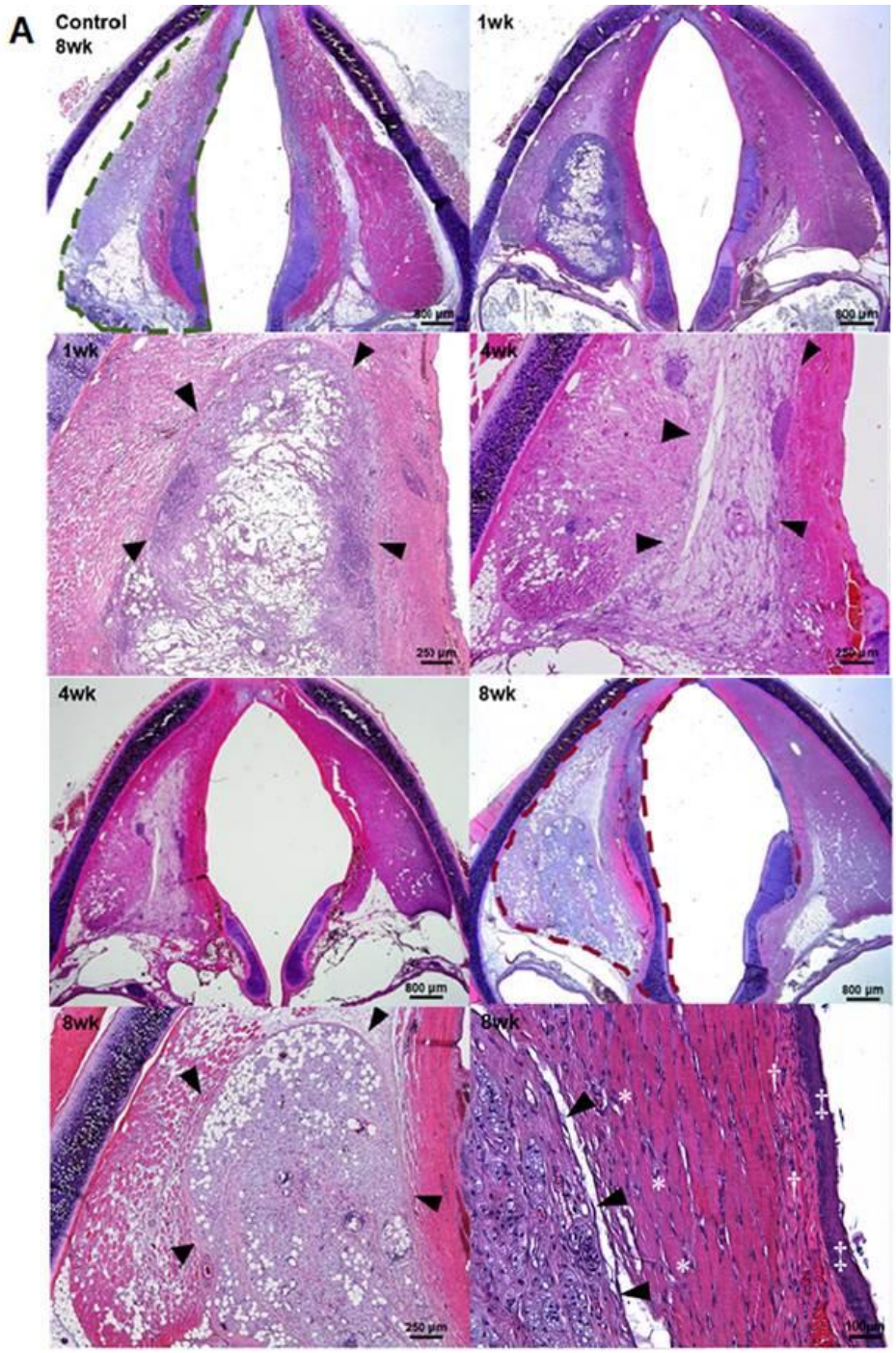

B
1.0 = Ideal symmetricity
1.5
p = 0.047
Asymmetry index
1.0
0.5
0.0
Control
ECM/MC
Mann-Whitney U test

Figure 4. Representative serial images of high-speed camera recording at 8 weeks (A), the asymmetric index using videokymograms and the results of asymmetry index for vocal functional analysis (B).

(A) Normal and symmetrical vocal contacts showed no change in the vibration of vocal mucosa in sECM/MC groups relative to the control group. (B) The mean asymmetry index of the sECM/MC hydrogel group (1.020 ± 0.069) and the control group (0.787 ± 0.102) are shown (p = 0.047 using a Mann-Whitney U test). In diseased conditions, the index deviates from the value of 1.0. The asymmetry index was calculated as follows: asymmetry index = a / b.


A
Control
8wk
1wk
800 μm
1wk
4wk
250 μm
250 μm
4wk
8wk
800 μm
800 μm
8wk
8wk
250 μm
100 μm

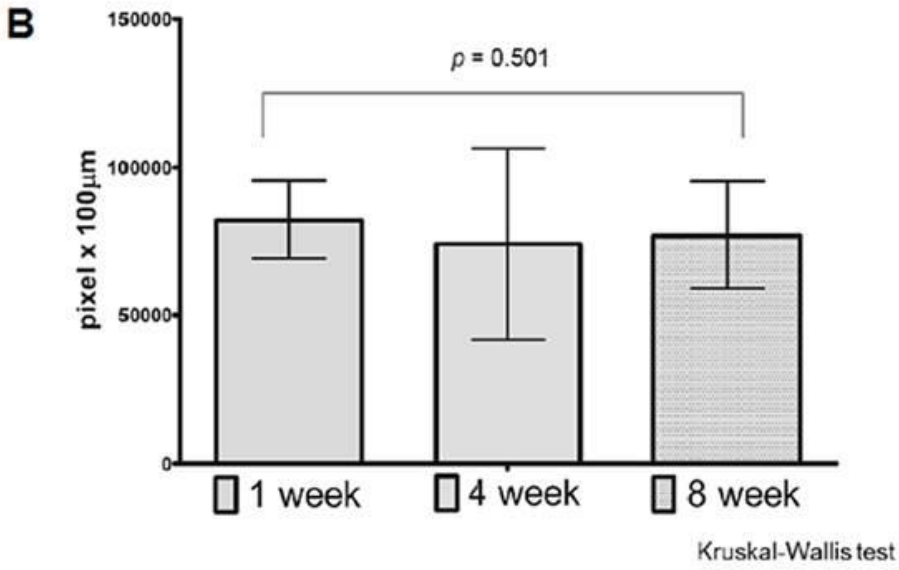

B
150000
100000
50000
0
pixel x 100µm
p = 0.501
1 week
4 week
8 week
Kruskal-Wallis test

Figure 5. Standard hematoxylin and eosin (H&E) staining of rabbit larynx after injection laryngoplasty into the left paralyzed vocal fold (A) and the quantitative analysis of remaining volume of sECM/MC hydrogels (B).

(A) Histological examination of the injected biomaterials at 8 weeks post procedure. Area of the laryngeal intrinsic muscle was smaller on the denervated side in the control group (green dotted line) than on the contralateral normal side. In the sECM/MC group, the laryngeal muscle area was compensated for by the injected sECM/MC hydrogel (brown dotted line). The injected ECM/MC hydrogel (arrowhead) induced no significant inflammatory response including neutrophils or lymphocytes aggregation in the surrounding muscle (*), lamina propria (†), or epithelium (‡). (B) Quantitative analysis of remaining sECM/MC hydrogel volume (p = 0.501 using Kruskal-Wallis test).

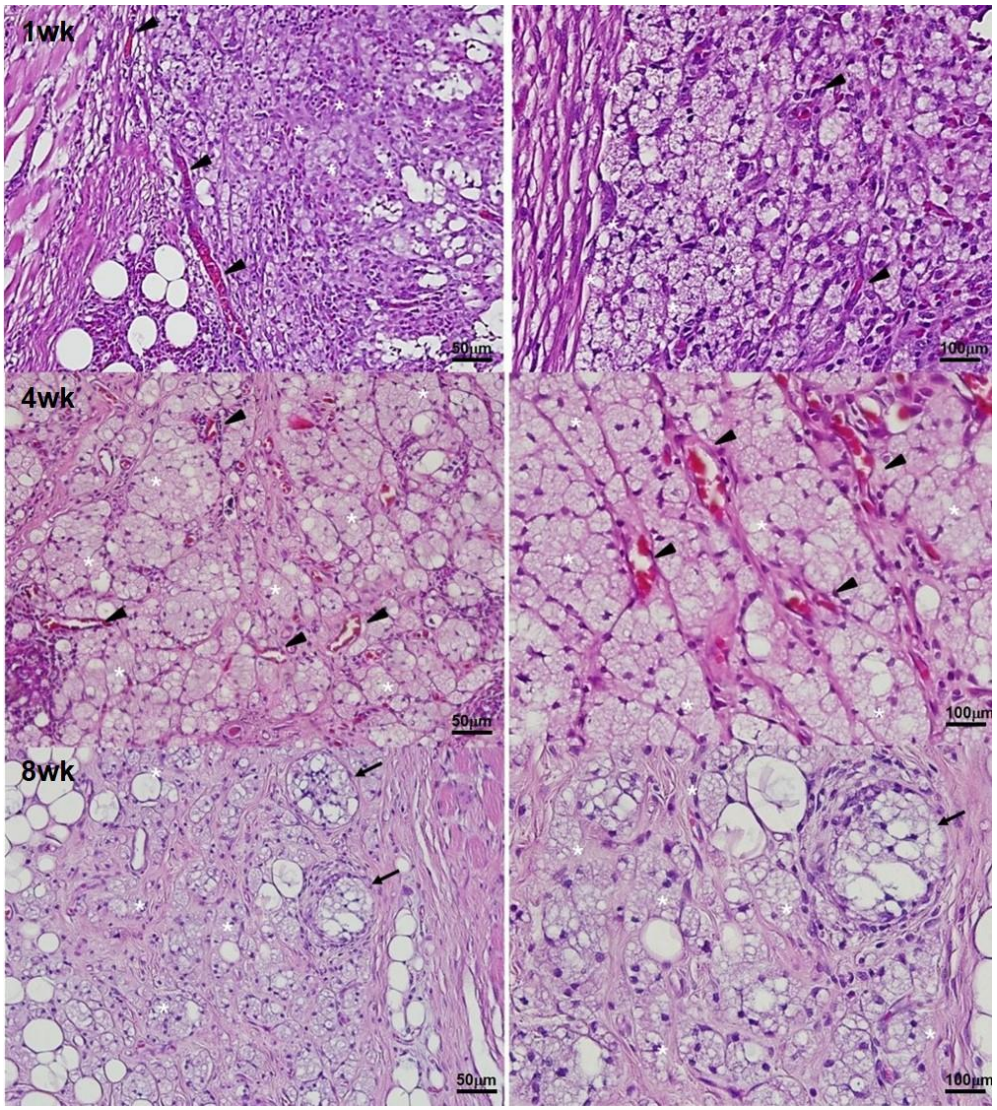

1wk
50μm
100μm
4wk
50μm
100μm
8wk
50μm
100μm

Figure 6. Foamy histiocyte with intracellular fatty lobules and neo-vascularization in the injected sECM/MC hydrogels.

Focal capillary ingrowths (arrow head) into the injection site. Cell aggregations with intracellular fatty lobules in the injection area (white asterisk). Mature adipose tissue appeared at 8 weeks post procedure (arrow).

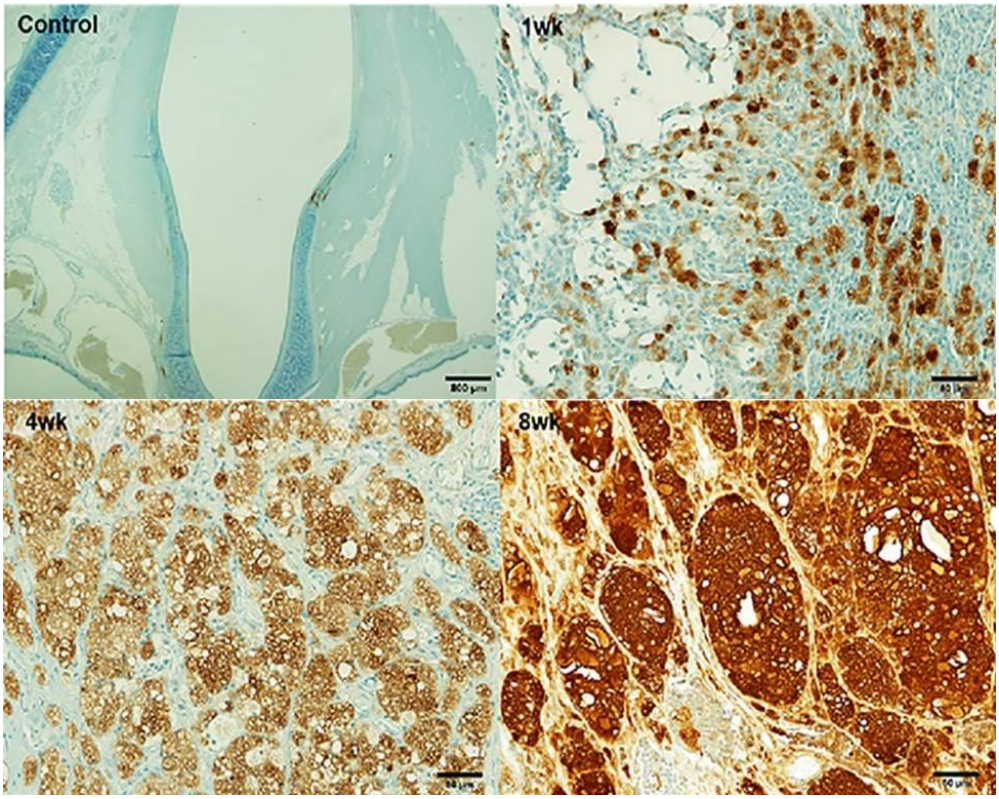
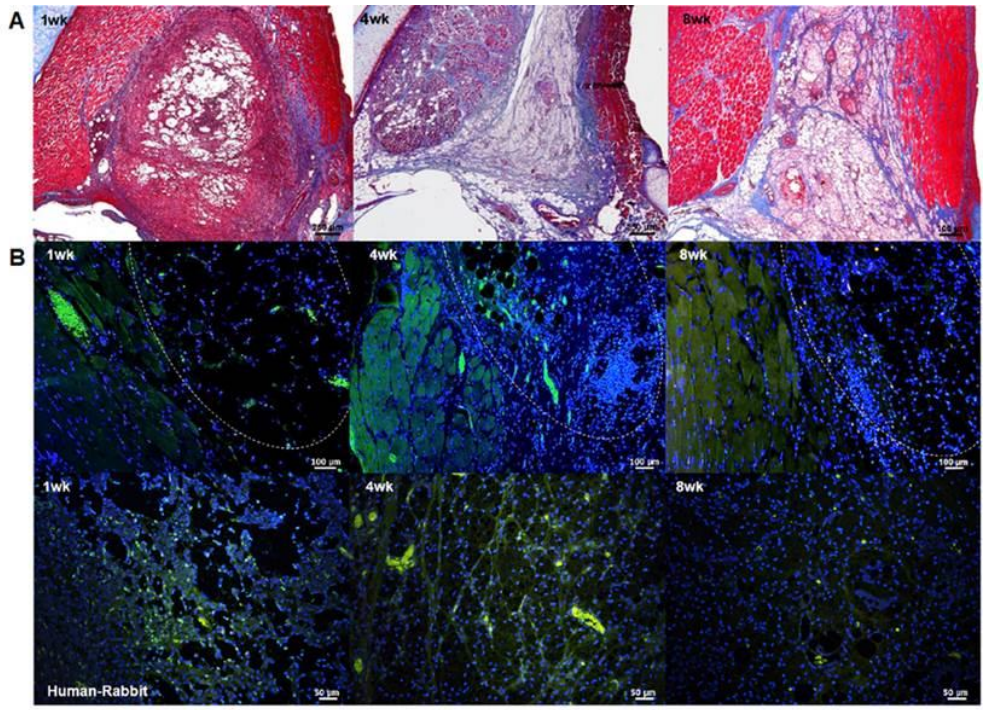

Control
1wk
4wk
8wk

Figure 7. Immunohistochemistry (IHC) using monoclonal mouse anti-rabbit macrophage clone RAM11 for foamy histiocytes in the injected sECM/MC hydrogels

RAM 11 positive cells (dark brown) are foamy histiocytes that had phagocytized fatty granules in the injection site.


A
1wk
4wk
8wk
B
1wk
4wk
8wk
1wk
4wk
8wk
Human-Rabbit

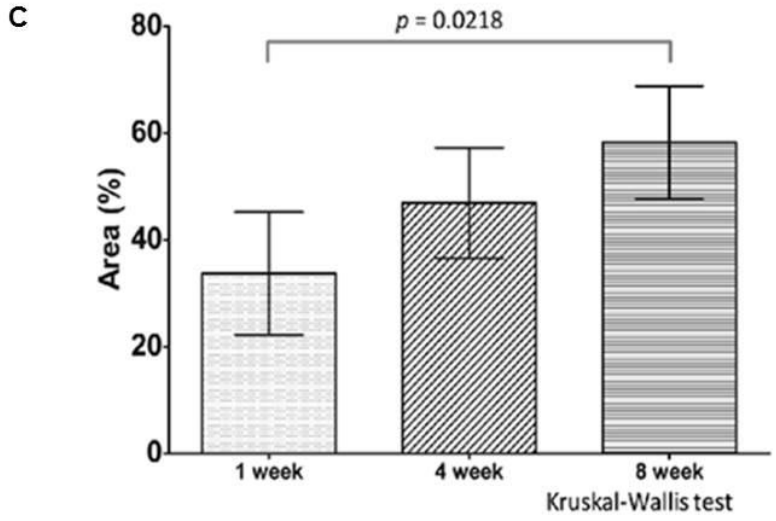

C
p = 0.0218
Area (%)
80
60
40
20
0
1 week
4 week
8 week
Kruskal-Wallis test

Figure 8. Collagen fiber stained by MT staining (A), IF for Type I collagen of Human/Rabbits (B) and the quantitative analysis of collagen fiber deposition (area %) in the injected sECM/MC hydrogel (C).

(A) MT staining of collagen (blue) in the injected sECM/MC hydrogel at 1, 4 and 8 weeks post procedure. (B) Type I collagen fibers of the intrinsic vocal muscle and the injected sECM/MC hydrogel (white dotted line) are shown (blue: DAPI, green: collagen type I). (C) The mean percent areas (%) of collagen fibers in the injection area are shown ($p = 0.0218$ by Kruskal-Wallis test).

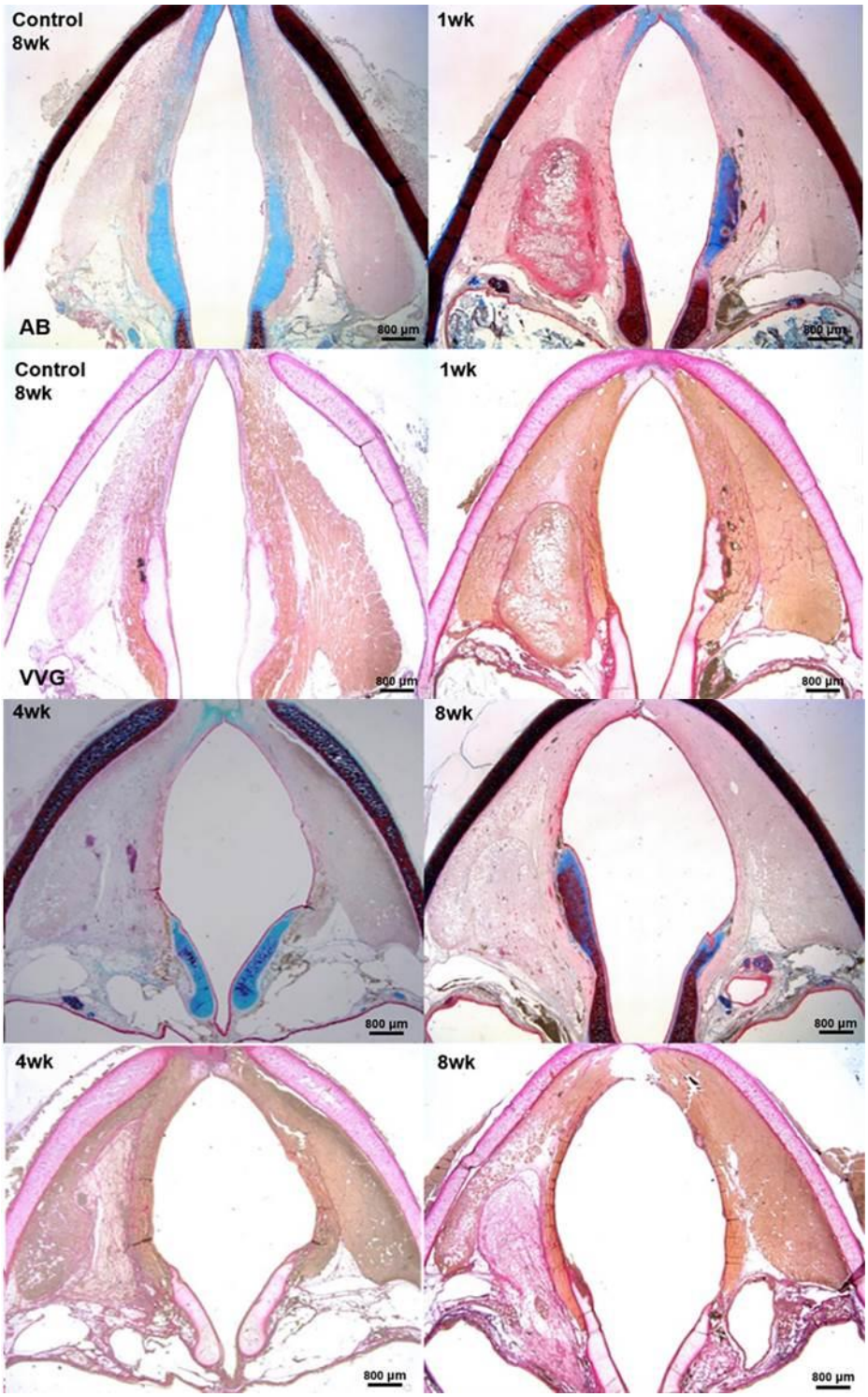

Control
8wk
AB
800 µm
1wk
800 µm
Control
8wk
VVG
800 µm
1wk
800 µm
4wk
800 µm
8wk
800 µm
4wk
800 µm
8wk
800 µm

Figure 9. Alcian blue (AB) and Verhoeff-Van Gieson (VVG) staining in the injected sECM/MC hydrogel. .

Discussion

Injected sECM/MC was stable up to 8 weeks after injection without early resorption, and augmented volume in the paralyzed vocal fold lasted for at least 8 weeks as indicated by endoscopy and histology. Symmetricity of vocal fold vibration was recovered to normal levels in the sECM/MC-injected groups, whereas vibration in the control group exhibited asymmetricity. These results suggest that injection of sECM/MC hydrogel in paralyzed vocal folds augmented volume and resulted in recovery of vocal fold function.

ECM plays a central role in tissue engineering by preserving tissue volume, providing temporary mechanical functions, and guiding the complex multicellular processes of tissue formation and regeneration.[24, 30, 31] ECM influences biological responses such as cell survival, development, cell shape, migration, and cell polarity by interacting with cellular adhesion molecules, growth regulators, binding proteins, proteolytic enzymes, and enzyme inhibitors.[23, 32] Our hypothesis that injecting sECM/MC would induce vocal tissue regeneration was supported by histological findings. The total collagen fiber content was increased up to 8 weeks post procedure as determined using MT staining and collagen type I specific IF. Furthermore, we detected foamy histiocytes that phagocytized fatty granules at the injection site, which suggests that adipose tissue-derived ECM may induce regeneration of fatty components.

Many studies of the human adipose tissue derived ECM has supported and indicated a clear role for adipogenesis during transplantation of adipose tissue-

derived ECM.[21-25] Human adipose tissues contains a variety of cells, and therefore is rich in ECM components and releases a wide variety of cytokines. [33, 34] The autologous adipose tissue transplantation has yielded poor results, with a 40–60% reduction in graft volume. [19] Human adipose tissue derived ECM exhibited not only an increased graft volume, but also the formation of new adipose tissue with numerous blood vessels in the grafts without any signs indicating adverse immune responses. Human adipose tissue derived ECM induce ingrowth and differentiation of preadipocytes from the neighboring host tissues. [35, 36] Other works including adipogenic-specific genes study revealed that PPARγ, aP2, leptin, and adiponectin play a key role in regulating adipogenesis. [23, 34, 35] They are involved in various functions of adipose tissue such as the transport or storage of lipids, glucose metabolism, and homeostasis. Adipocyte-specific mRNAs were expressed in the grafts of human adipose tissue derived ECM and adipogenic differentiation of host preadipocytes. Although the exact mechanisms of how host cells migrated into grafts and influenced adipogenesis are not clear, many studies have documented the ability of human adipose tissue derived ECM to secrete various paracrine factors, which may positively influence differentiation of preadipocytes. [22-24, 36, 37]

Phagocytosis of human fatty materials is a predictable result in the immune-competent rabbit rather than in immune-compromised animals.[38-41] These results are more favorable for vocal fold tissue recovery than our previously published reports using CaHA injection, which induced the formation of abundant giant cells. However, they are based only on a preliminary animal study with a relatively short time course

(8 weeks). Further studies on sECM/MC injections will be needed to corroborate these results for long-term tissue regeneration in paralyzed vocal folds.

The novel sECM/MC hydrogel has several advantages as a material for injection laryngoplasty. First, the burden of repeated injections would be alleviated by the permanent augmentation effects of sECM/MC hydrogels that is stimulated through vocal tissue regeneration. For conventional injection laryngoplasty, patients with vocal fold paralysis typically receive periodic procedures at 3 to 6 month intervals in order to maintain vocal fold volume. Second, the biostability of sECM/MC hydrogels could enable injections to be more accurate in quantity and localization than that achieved with conventional fat or CaHA injections. Conventional injection materials for augmentation must be over-injected to compensate for early resorption of the material. Avoiding over-injection would directly improve post injection voice quality. Finally, the straightforward ECM solubilization process and ability to store the sECM/MC hydrogels using refrigeration could permit more frequent use of autologous adipose tissue-derived ECM injections. Considering that liposuction has become one of the most commonly performed aesthetic surgery procedures,[42] autologous fat tissue is easily obtained. If aseptic facilities capable of harvesting and handling adipose tissue are improved, injection of autologous soluble ECM extracts may become more readily available.

We believe there are direct clinical applications for injection laryngoplasty using sECM/MC hydrogels. The material is effective for volume augmentation of paralyzed vocal folds, and the long-term effects are likely due to adipogenesis. These injections

use small bore needles and can be administered at outpatient clinics. Finally, the material has excellent biocompatibility and restores vocal fold vibration.

Conclusions

We propose sECM/MC hydrogels as a highly suitable material for augmentation injection laryngoplasty. Further studies are needed to elucidate the mechanisms by which sECM/MC injections induce tissue regeneration and their long-term effects.

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국문 초록

목적: 지방 및 결체조직 재생을 유도하는 인간지방조직 추출 세포외기질 (extra cellular matrix, ECM)을 이용하여, 성대 마비 및 성대부전증 치료를 위한 성대주입술 (injection laryngoplasty)에 적용시켜, 부작용이 없고 주사하기 편리하며 성대의 물리적 특성을 복원 시키면서 오래 유지되는 이상적인 성대주입술 제재를 개발하고자 하였다.

방법: 지방흡입술 방법을 통해 채취한 인체 지방조직으로부터 원심분리 및 세척 후 동결건조하여 세포외기질을 분말화 한다(soluble ECM, sECM, 7.5% w/v). 이후 주입이 용이하게끔 Methyl cellulose (MC) 분말과 1:4 로 혼합하여 sECM/MC 혼합 용액을 형성한다. 20 마리의 토끼 (male, 3.0-3.5kg)의 좌측 후두되돌이신경을 절제하여 성대부전 동물 모델을 만든 후, 무작위 추출을 통해 대조군 (5 마리)와 3 개의 실험군으로 나눈뒤 3 개의 실험군의 편측 마비된 성대에 각각 0.1cc 의 sECM-MC 혼합 용액을 4.0mm, 30° 내시경 유도하 23 게이지 spinal needle 사용하여 성대주입술을 시행한다. 제 1 실험군은 성대주입술 시행 1 주일 뒤 내시경 평가 및 기능적, 면역조직학적 분석을 시행하고, 제 2 실험군은 성대주입술 4 주 뒤, 제 3 실험군은 성대주입술 시행 8 주 뒤 내시경 평가 및 기능적, 면역조직학적 분석을 시행한다. 성대주입술의 평가는 4.0mm, 30° 내시경을 이용한 후두내시경 촬영하였으며 후두적출술

시행 후 high-speed video camera 을 이용하여 성대 점막 진동의 기능적 평가를 시행하였다. 그리고 적출된 성대 조직을 이용 조직학적 검사 및 collagen, macrophage 에 대한 면역학 검사를 시행 하였다.

결과: 한마리를 제외한 모든 실험동물들이 계획된 실험을 완료하였다. 후두내시경 평가에서 sECM/MC 혼합 용액은 마비된 성대 내에서 8 주차까지 지속되어 마비된 성대의 부피를 유지하였다. 조직 면역학적 평가에서도 또한 마비된 성대 내에서 주입 후 8 주까지 유지되는 것을 확인하였고 sECM/MC 주입 주변 조직의 염증 소견도 보이지 않아 우수한 생체적합성을 갖고 있음을 알 수 있었다. 초고속 비디오 촬영을 이용한 sECM/MC 의 성대 점막 운동의 기능적 평가에서는 sECM/MC 성대주입술이 시행이 성대의 점막운동을 제한하지 않고 성대의 대칭적인 운동을 향상시킴을 확인하였다.

결론: 인간지방조직에서 추출 sECM/MC 을 이용한 성대주입술은 마비된 성대를 염증반응 없이 유지되면서 마비된 성대 운동을 효과적으로 복원할 것으로 기대된다.

주요어: 성대마비, 지방조직, 세포외기질, 성대주입술, 지방분화

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