*The Laryngoscope*


# Chronic Vocal Fold Scar Restoration With Hepatocyte Growth Factor Hydrogel

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**Objectives/Hypothesis:** Therapeutic challenges exist in the management of vocal fold scarring. We have previously demonstrated the therapeutic potential of hepatocyte growth factor (HGF) in the management of acute phase vocal fold scarring using a novel hydrogel-based HGF drug delivery system (DDS). However, the effect of HGF on matured vocal fold scarring remains unclear. The current study aims to investigate the effect of HGF-DDS on chronic vocal fold scarring using a canine model.

**Study Design:** Animal model.

**Methods:** Vocal folds from eight beagles were unilaterally scarred by stripping the entire layer of the lamina propria; contralateral vocal folds were kept intact as normal controls. Six months after the procedures, hydrogels (0.5 mL) containing 1 $\mu$g of HGF were injected into the scarred vocal folds of four dogs (HGF-treated group). Hydrogels containing saline solution were injected into the other four dogs (sham group). Histological and vibratory examinations on excised larynges were completed for each group 9 months after the initial surgery.

**Results:** Experiments conducted on excised larynges demonstrated significantly better vibrations in the HGF-treated group in terms of mucosal wave amplitude. Although phonation threshold pressure was significantly lower in the HGF-treated group compared with the sham group, no significant differences were observed in the normalized glottal gap between HGF-treated and sham groups. Histological examinations of the HGF-treated vocal folds showed reduced collagen deposition and less tissue contraction with favorable restoration of hyaluronic acid.

**Conclusions:** Results suggest that administration of HGF may have therapeutic potential in the treatment of chronic vocal fold scarring.

**Key Words:** Chronic vocal fold scarring, drug delivery system, hepatocyte growth factor.

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## INTRODUCTION

There continue to be therapeutic challenges in the management of vocal fold scarring.[1] Vocal fold scarring occurs following injury, inflammation, or phonosurgery and disrupts the layered structure of the lamina propria altering the biomechanical properties of the vocal fold. Vocal fold scarring often causes glottal insufficiency and severe intractable dysphonia.

Previous histologic studies[2–5] on vocal fold scarring have revealed changes in the organization and distribution of extracellular matrix components (ECM), such as dense and/or disorganized type I collagen deposition, decreased elastin and decorin, increased fibronectin, and occasional decreases in hyaluronic acid (HA). These results confirmed the aberrant synthetic phenotype of vocal fold scar fibroblasts.[6] Given that these histological changes stiffen the properties of the vocal fold, phenotypic changes of vocal fold fibroblasts and a correction of the distribution of ECM components is needed to restore the vocal fold after scarring.

Hepatocyte growth factor (HGF) is a multifunctional polypeptide that plays a significant role in embryogenesis, angiogenesis, organ regeneration, and wound healing.[7] HGF has strong antifibrotic potency and has been shown to contribute to the prevention or complete resolution of fibrosis in the liver, kidney, and lung in animal models.[7] Another study has shown the therapeutic potential of HGF in the management of vocal fold scarring by demonstrating that HGF can increase HA production and decrease collagen production in vocal fold fibroblasts.[8]

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We have previously shown the therapeutic potential of HGF in the management of vocal fold injury at acute phase.[9–11] Although those studies have shown the therapeutic potential of HGF, incomplete restoration of scarred tissue and individual variability of these effects were also reported.[11,12] These effects were attributed to insufficient retention time of HGF in the injected site, as the biological activity of HGF may be limited due to rapid dispersal by diffusion. To overcome this limitation and enhance the effect of HGF, we have developed a novel drug delivery system (DDS) for HGF using a gelatin hydrogel.[12] The previous study, however, revealed only the inhibiting effect on scar formation at the acute phase, and the effect of HGF on matured, chronic vocal fold scarring remains unclear. Given the clinical use of HGF it is important to determine if it has a restorative remodeling effect on chronic vocal fold scarring. The current study aims to investigate the effect of HGF-DDS on chronic vocal fold scarring using a canine model.

## MATERIALS AND METHODS

### *Animals*

Eight beagles weighing 10 to 17 kg were used in this study. All experimental protocols were approved by the Animal Committee of the Graduate School of Medicine, Kyoto University. Animal care was provided under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University.

### *Preparation of HGF Hydrogel*

Biodegradable hydrogels were developed by the Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University.[13,14] The hydrogel was constituted by chemically cross-linking acidic gelatin with glutaraldehyde. A 50-mL quantity of acidic gelatin aqueous solution (5% w/w) was mixed with 50 $\mu$L of glutaraldehyde aqueous solution (25% w/w) to give a final concentration of 6.25 mM. The water content of the hydrogel was 94.8%. A solution of 1 $\mu$g of HGF (Human recombinant HGF; PeproTech Inc., Rocky Hill, NJ) in 20 $\mu$L of phosphate buffered saline (PBS) was dripped onto the gelatin hydrogels and left overnight at 4°C to create the HGF hydrogel.

### *Surgical Procedure*

The surgical procedures that were used for generating the vocal fold injury models had been established in previous studies.[11,12] All animals were sedated under general anesthesia with intramuscular injections of ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (6 mg/kg). The glottis was visualized using a direct laryngoscope, and the vocal folds were unilaterally scarred by stripping the entire layer of the lamina propria down to the muscle. The contralateral vocal folds were kept intact as normal controls. The sides for scarring were randomly selected.

After stripping the vocal fold lamina propria of test animals, vocal fold scars were allowed to mature for 6 months. This period of vocal fold scar maturation was based on data from Rousseau et al., who proposed that it takes 6 months for vocal fold scarring to mature in canine and rat models.[3,4] Six months after the procedure, 0.5 mL of hydrogel solution containing 1 $\mu$g of HGF was injected into the scarred vocal folds of four dogs (HGF-treated group) using a transoral intracordal injector, and 0.5 mL of hydrogel solution containing 1 $\mu$g of PBS was injected into the scarred vocal folds of the four dogs in the sham group. Because HGF was expected to act on the fibroblast in the lamina propria, hydrogel was carefully injected into the subepithelial layer of the vocal fold. To enhance the effect of injection, the injection was performed twice at an interval of 1 month. It is reported that HGF acts on some kinds of cells in an autocrine manner,[15–17] and vocal fold fibroblasts may be similar to those cells. Thus, the effect of HGF might continue for some time after the administration and release period. For this reason, we set the interval to 1 month, which is longer than the 2-week release period.

All animals were euthanized 9 months after the surgery by intracardiac injection of Nembutal. The larynges were harvested and used for vibratory examinations then subjected to histological examination.

### *Setup for Vibratory Examination of Excised Larynges*

Vocal fold vibration was examined with an excised larynx setup developed in previous studies.[10,12] For better visualization of the vocal folds supraglottic structures, including the epiglottis, false vocal folds and aryepiglottic folds were removed after resection of the superior portion of the thyroid cartilage. The arytenoid cartilages were sutured together, and an arytenoid adduction procedure was bilaterally performed using a 3-0 Prolene suture to close the glottis. The larynx was mounted on a table and an intubation tube was inserted into the trachea and tightly clamped. Air was pumped through the tube to generate vocal fold vibrations. During the vibratory examination, saline was dripped onto the vocal folds to prevent dehydration. A pressure sensor (PG-100; Nidec Copal Electronics Corp., Tokyo, Japan) was inserted into the tube to monitor subglottic pressure, and a high-speed digital imaging system (MEMRECAMci; NAC Image Technology, Osaka, Japan) was used to record vocal fold vibrations from the superior view. The camera was mounted 50 cm above the larynx, and the image was displayed on a monitor. The images were recorded at a frame rate of 1,000 frames per second, which is the maximum rate to give an acceptable resolution level in our equipment, and the images were then scanned into a computer.

As an indirect measurement, we used phonation threshold pressure (PTP) to evaluate the mucosal vibration. PTP, which is regulated by factors such as vocal fold thickness, property, and glottal width, is defined as the minimum pressure required to initiate phonation.[18,19] Further, the amplitude of the mucosal wave and glottal gap were measured using image analysis software (Scion Image beta4; Scion Corp., Frederick, MD). The distance (d1) from the midline of the glottis to the free edge of the vocal fold was measured at the anteroposterior middle portion of the vocal fold during the closed phase. Closed phase was recognized by the motion of the upper and lower lips of the vocal folds. The same distance (d2) was measured at the maximum open phase. The mucosal wave amplitude was defined by subtracting d1 from d2 and the amplitude ratio was derived by dividing the amplitude in the HGF-treated side by the amplitude in the normal side. The following formula was used: amplitude ratio (AR) = (d2−d1 in the HGF-treated side)/(d2−d1 in the normal side). The glottal gap was examined from the images during the closed phase. The length (L) from the anterior commissure to the vocal process and the glottal area (a) were measured, and the glottal area was normalized by dividing it by $L^2$. The following formula was used: normalized glottal gap (NGG) = $a/L^2 \times 100$ unit (u).

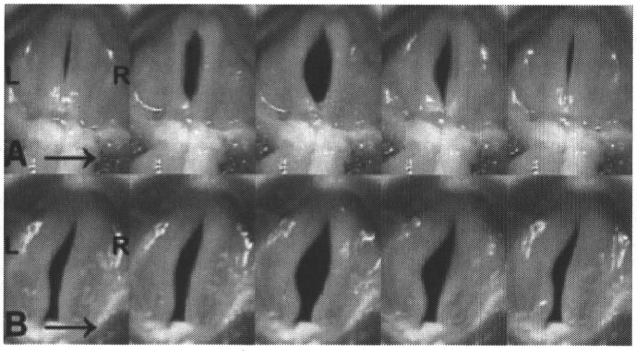


Fig. 1. Vibratory patterns experimentally generated from treated vocal folds of excised larynges. In both cases the left vocal fold was scarred. The hepatocyte growth factor-treated vocal fold showed almost normal mucosal vibration (A), however the sham-treated vocal fold was bowed and mucosal vibration was limited (B).

### *Histological Examination*

Collagen, elastin, and HA in the lamina propria of each vocal fold were examined using light microscopy. The thickness of the lamina propria was also assessed to determine the degree of scar contraction. The thickness of the lamina propria was determined by measuring the distance from the free edge of the vocal fold down to the thyroarytenoid muscle and normalized by dividing the distance on the treated side (t1) by that of the normal side (t2). The following formula was used: normalized thickness of lamina propria (NTLP) = t1/t2.

Immediately following the vibratory examinations, the larynges were fixed in 10% formaldehyde for later tissue examination. Larynges were subsequently embedded in paraffin, and 5-$\mu$m-thick serial sections were prepared in the coronal plane from the anteroposterior middle portion of the vocal folds.

Elastica van Gieson staining was performed to identify collagen and elastin. Alcian blue staining was used to identify HA. A hyaluronidase digestion technique was used to detect HA. Images were captured with a BIOREVO BZ-9000 microscope (Keyence Corp., Osaka, Japan).

These assessments were performed in a blinded fashion, in which the examiners were not informed which slide belonged to each group.

### *Statistical Analysis*

An unpaired $t$ test was used to ascertain differences in PTP, AR, NGG, and NTLP between treatment groups. A $P$ value $< .05$ was considered statistically significant.

## RESULTS

### *Vibratory Examinations*

The experiments on excised larynges showed better mucosal vibration in the HGF-treated group, as compared with the sham group. Figure 1 shows representative cases in the HGF-treated group (Fig. 1A) and in the sham group (Fig. 1B). Injured vocal folds were bowed and the mucosal vibration was limited in the sham group; however, their vibration was comparable to the uninjured side in the HGF-treated group.

An unpaired $t$ test revealed significantly lower PTP in the HGF-treated group, compared with the sham group (Fig. 2A, $P = .015$). Although no significant differences were observed for NGG between the two groups (Fig. 2B), AR was significantly higher in the HGF-treated group compared to the sham group (Fig. 2C, $P = .012$).

### *Histological Examinations*

Histological examinations revealed better restoration and less tissue contraction in the HGF-treated vocal fold compared with the sham-treated vocal fold.

Disorganized collagen deposition was found to be minimal in the HGF-treated vocal fold (Fig. 3A, 3B), whereas there was excessive collagen deposition in the sham-treated vocal fold (Fig. 4A, 4B). Elastin and HA in the HGF-treated vocal fold appeared to be well

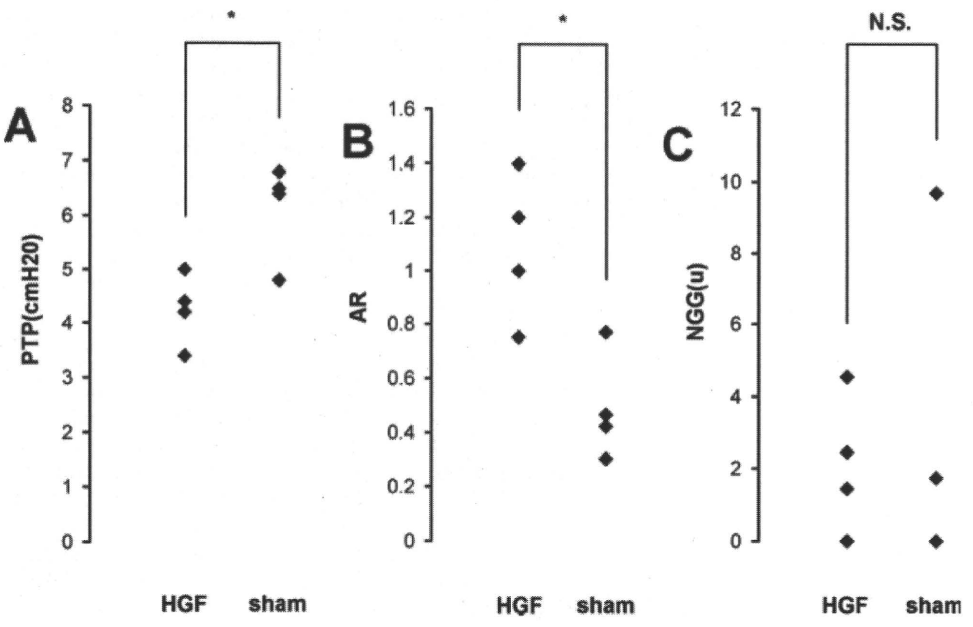


Fig. 2. Results of vibratory examinations. The hepatocyte growth factor (HGF)-treated group demonstrated significantly lower phonation threshold pressure (PTP) (A) and higher amplitude ratio (AR) (B) compared with the sham-treated group. No differences were observed for normalized glottal gap (NGG) between the two groups (C). * $P < .05$.

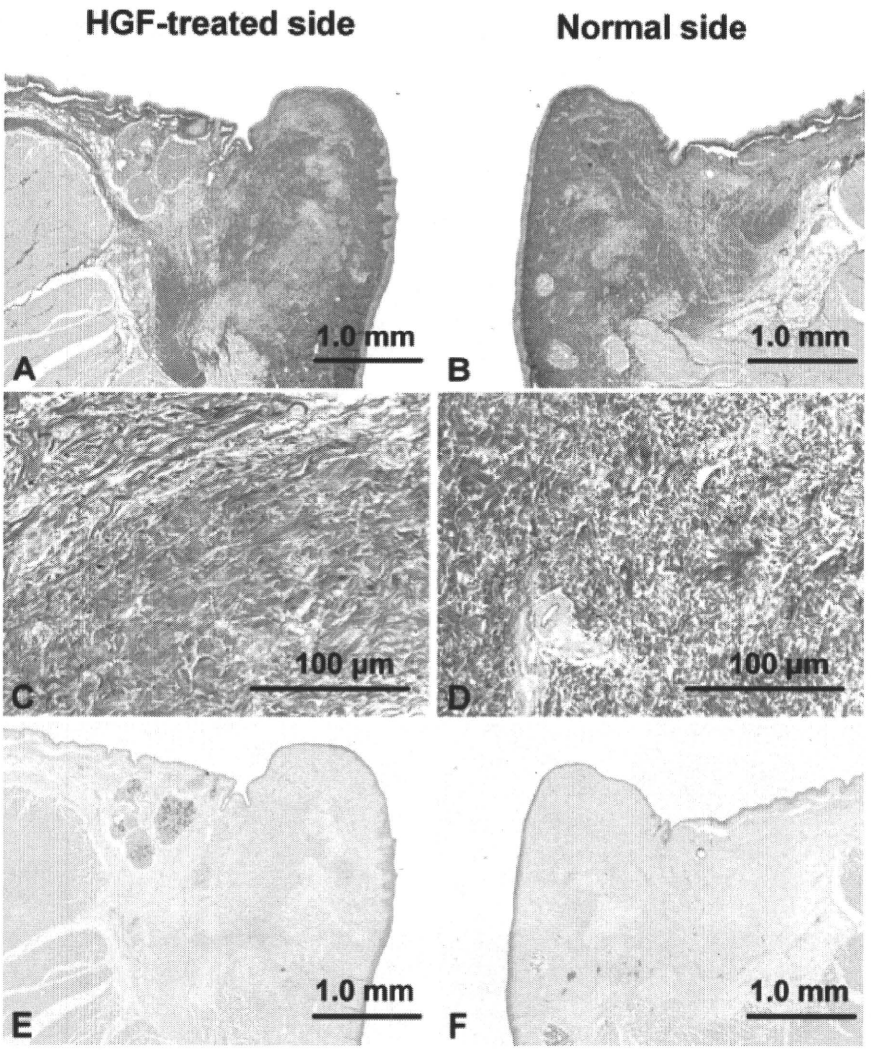


Fig. 3. Histologic findings in the hepatocyte growth factor (HGF)-treated group. (A–D) Elastica van Gieson stain. (E–F) Alcian blue stain. Tissue contraction and collagen deposition were found to be minimal (A, B), and elastin (C, D) and hyaluronic acid (E, F) were favorably restored.

organized, similar to that of the unscarred contralateral side (Fig. 3C–3F), whereas both were decreased in the sham-treated vocal fold (Fig. 4C–4F). There were no remarkable findings in terms of the underlying muscle.

NTLP was close to normal in the HGF-treated vocal folds, whereas NTLP was significantly thinner in the sham group as compared with the HGF-treated group (Fig. 5, $P = .03$).

## DISCUSSION

The restoration of normal vocal fold properties is essential to the treatment of vocal fold scarring. With the advancement of phonosurgery, most voice disorders have been overcome, and many therapeutic strategies, including medialization thyroplasty, fat/collagen injection, and scar dissection have been tried in an attempt to restore normal properties to scarred vocal folds.[20] Medialization thyroplasty and fat/collagen injection result in augmentation effects that improve glottal insufficiency and facilitate entrainment of vocal fold vibrations. However, the restoration of normal vocal fold properties is not achieved by these treatments. The effect of scar dissection depends on the individual's healing ability, and stable outcomes with this approach cannot be achieved. Thus, there is no optimal strategy for the treatment of vocal fold scarring to date, and development of a new regenerative pathway is needed.

In tissue engineering, regeneration of tissues or organs can be achieved by the combination of scaffold, cells, and regulatory factors under appropriate conditions. Applying this concept, we have focused on two kinds of therapeutic strategies—cell therapy[21] and growth factor therapy[8–12]—for the treatment of vocal fold scarring. As a cell source for cell therapy, we have shown the therapeutic potential of autologous mesenchymal stem cells (MSCs).[21] We have previously injected MSCs into injured vocal folds at an acute phase in a canine model, and histologic examinations revealed improved healing after 2 months. Furthermore, we have shown the efficacy of HGF as a candidate for growth factor therapy in the management of vocal fold scarring by demonstrating its ability to control ECM production in vocal fold fibroblasts. As described before, HGF has strong antifibrotic potency and has been shown to contribute to the prevention or complete resolution of

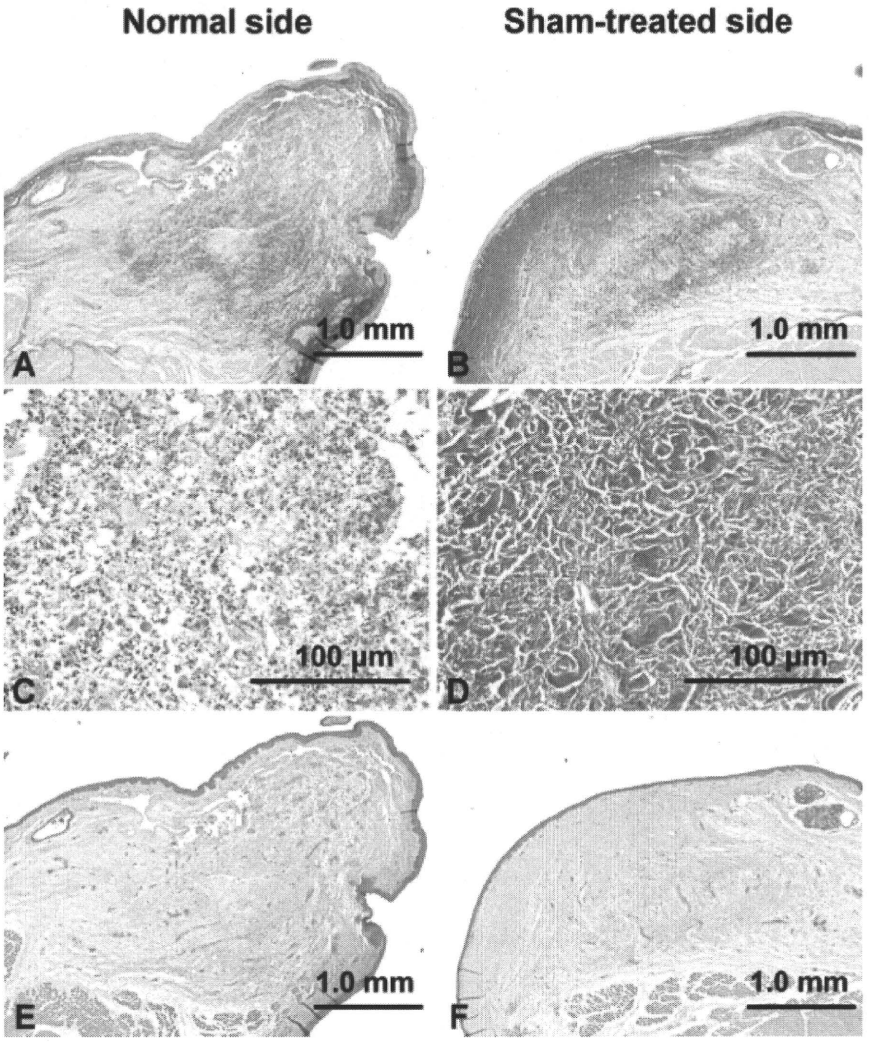


Fig. 4. Histologic findings in the sham-treated group. (A–D) Elastica van Gieson stain. (E–F) Alcian blue stain. Severe tissue contraction and excessive collagen deposition were observed in sham-treated vocal folds (A, B). Elastin (C, D) and hyaluronic acid (E, F) were decreased in the superior portion of the treated vocal fold.

fibrosis in some organs.[7] Hirano et al. reported the effects of HGF for the treatment of acute vocal fold injury using canine[11] and rabbit[10] models. In these studies, HGF was injected into injured vocal folds, and histological examination revealed reduced collagen deposition and decreased tissue contraction of the lamina propria in HGF-injected vocal folds as compared with saline-injected controls. However, these previous studies revealed only the inhibiting effect on scar formation at the acute phase of wound healing, and it is not clear whether these approaches have a restorative effect on the aberrant synthetic phenotype of vocal fold fibroblasts. In the current study we have investigated the effect of HGF administration on chronic matured vocal fold scarring.

A biodegradable hydrogel developed to enhance the in vivo regenerative effects of growth factors, such as HGF, basic fibroblast growth factor, platelet-derived growth factor, and epidermal growth factor, has been shown to be successful in the controlled release of biologically active growth factors in other parts of body.[13,14,22–24] In this system, HGF was embedded in gelatin hydrogel and gradually released in a continuous fashion over a 2-week period in vivo.

This study represents the first investigation of HGF for the treatment of matured, chronic vocal fold scarring in vivo. The vibratory experiments in the present study showed significant improvement of mucosal vibration in terms of PTP and AR in the HGF-treated group compared with the sham group. As mentioned above, PTP is regulated by the vocal fold property and glottal gap. Improvement in PTP without smaller NGG indicates that administration of HGF restored the scarred vocal fold in terms of stiffness and tissue contraction. Histological examination also showed positive restorative effects with the administration of HGF, including reduced collagen deposition, less tissue contraction, and improved restoration of elastin and HA. These results suggest that HGF-DDS has restorative remodeling effects on chronic vocal fold scarring; however, there was still individual variability and complete restoration could not be achieved. Particularly, a possible reason for incomplete improvement of glottal gap may be insufficient volume obtained in the treated vocal folds. Here may be some limitations in growth factor therapy, which warrant a combined use of cells and/or appropriate scaffoldings to obtain adequate tissue volume and function.

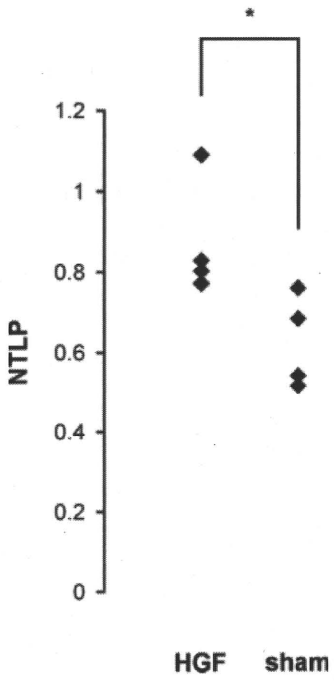


Fig. 5. Normalized thickness of lamina propria (NTLP) was significantly thinner in the sham-treated group than in the hepatocyte growth factor (HGF)-treated group. *$P < .05$.

## CONCLUSION

The present study demonstrated that the HGF-DDS significantly improved the vibratory properties of matured, chronic vocal fold scarring in a canine model. HGF-DDS reduced excessive collagen deposition and tissue contraction with favorable restoration of elastin and HA. Results suggest that administration of HGF may have therapeutic potential in the treatment of chronic vocal fold scarring.

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*The Laryngoscope*


# Temporal Changes in Vocal Functions of Human Scarred Vocal Folds After Cordectomy

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**Objectives/Hypothesis:** The maturation process of scarred vocal folds has previously been investigated using animal models. However, in human models the features of scarred vocal folds have rarely been described, and the process by which the scar changes with time is not well known. The present study aimed to investigate the maturation process of human vocal folds scarred by cordectomy in terms of vibratory and aerodynamic functions.

**Study Design:** Prospective case series.

**Methods:** Eight patients with early glottic carcinoma and two patients with leukoplakia of the vocal fold underwent endoscopic cordectomy at Kyoto University Hospital between 2006 and 2008. The temporal changes in their vocal functions were evaluated using acoustic and aerodynamic analyses and videostroboscopic examination.

**Results:** Normalized mucosal wave amplitude, mean flow rate, and the amplitude perturbation quotient appear to stabilize about 6 months after the procedure. Although there were individual variations in the changes in normalized glottal gap and maximum phonation time, it appears to take at least 6 months to reach plateau. The other parameters—pitch perturbation quotient and noise to harmonic ratio—varied by individual, and thus it was difficult to identify commonalities in the healing process.

**Conclusions:** Some individual variation was observed in the temporal changes of vocal function of scarred vocal folds after cordectomy. However, in terms of vibratory and aerodynamic functions, this study suggests that it takes at least 6 months for maturation of vocal fold scarring.

**Key Words:** vocal function, human, scarred vocal fold, maturation.

**Level of Evidence:** 4

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## INTRODUCTION

Vocal fold scarring occurs after injury or inflammation of the vocal fold mucosa. It impairs mucosal vibration and results in severe, intractable dysphonia. Given that voice is one of the most important tools for communication, dysphonia might lead to psychological and social distress. Although many voice disorders have been overcome by advances in voice therapy and/or phonosurgery, to date there is no optimized therapeutic strategy for vocal fold scarring.[1]

To develop a therapeutic strategy for the management of vocal fold scarring, it is necessary to understand the process of vocal fold scar maturation. Previously, animal models have been used to characterize the features of scarred vocal folds.[2–4] In these studies, histological analysis of scarred vocal folds has revealed changes in the organization and distribution of extracellular matrix components, including dense and/or disorganized type I collagen deposition, decreased elastin and decorin, increased fibronectin, and occasional decreases in hyaluronic acid.[2–4] Similar changes have also been reported for the scarred vocal folds of humans.[5]

Rousseau et al. have proposed that it takes 6 months for vocal fold scarring to mature in canine and rabbit models.[2,3] However, owing to the difficulty in performing histological evaluations on human vocal folds, the features of scarred human vocal folds have rarely been characterized. Similarly, how the scar changes over time is not well known. To properly evaluate and diagnose scarred lesions and to determine the proper

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TABLE I.
Clinical Information About the Patients and Surgical Procedures.

| Case number | Sex | Age (years) | Side | Cordectomy type | Diagnosis |
|---|---|---|---|---|---|
| 1 | M | 55 | Rt. | I | Carcinoma |
| 2 | M | 69 | Bil. | I | Carcinoma |
| 3 | M | 65 | Bil. | I | Leukoplakia |
| 4 | M | 70 | Rt. | II | Carcinoma |
| 5 | M | 64 | Rt. | I | Carcinoma |
| 6 | F | 47 | Bil. | I | Carcinoma |
| 7 | M | 74 | Lt. | I | Carcinoma |
| 8 | F | 53 | Lt. | I | Leukoplakia |
| 9 | M | 56 | Rt. | III | Carcinoma |
| 10 | M | 69 | Lt. | II | Carcinoma |

M: Male, F: female, Rt.: Right, Lt.: Left, Bil.: Bilateral.

therapeutic strategy, it is essential to know how the vocal fold scar changes and matures over time.

Because human vocal function can be followed temporally, we have focused on the functional rather than histological changes in the process of vocal fold scarring. To know the changes in vocal function during the scar maturation would help us to understand its condition. In the current study, we investigated this maturation process by examining the temporal changes in vibratory, acoustic, and aerodynamic properties of scarred vocal folds in postcordectomy patients.

## MATERIALS AND METHODS

### *Patients*

Clinical information about the patients and surgical procedures is summarized in Table I. Ten patients (eight men and two women), eight with early glottic carcinoma and two with leukoplakia of the vocal fold, underwent endoscopic cordectomy from 2006 to 2008 at Kyoto University Hospital. Their ages ranged from 47 to 74 years (average, 62 years). Seven patients underwent type I cordectomy, two patients underwent type II cordectomy, and one patient underwent type III cordectomy. Seven cases were treated with unilateral resections, whereas the others received bilateral resections.

### *Assessments*

Two trained laryngologists made blind measurements. Assessment consisted of stroboscopic, acoustic, and aerodynamic examinations. Voice and stroboscopic samples were recorded three times at normal pitch and loudness in each examination.

Stroboscopic examinations were performed with a Digital Video System Model 9295 (Kay PENTAX, Lincoln Park, NJ) to assess temporal changes in the mucosal wave and glottic closure. The amplitude of the mucosal wave and glottal gap was examined using image analysis software (Scion Image Beta3b; Scion Corp., Frederick, MA). The distance (d1) from the midline of the glottis to the free edge of the vocal fold was measured at the anteroposterior middle portion of the vocal fold during the closed phase, and then the same distance (d2) was measured at the maximum open phase. Mucosal wave amplitude was normalized by the distance (L) from the anterior commissure to the vocal process. The normalized mucosal wave amplitude (NMWA) was calculated from the formula NMWA = (d2 − d1)/L. This measurement was done on the treated side of the vocal fold. Glottal gap was examined in the images at closed phase. Glottal area (a) was measured, and the normalized glottal gap (NGG) was calculated as $NGG = a/L^2$.

Aerodynamic examinations included maximum phonation time (MPT) and mean flow rate (MFR). For acoustic examinations, Computerized Speech Lab software (Kay PENTAX) was used to evaluate pitch perturbation quotient (PPQ), amplitude perturbation quotient (APQ) and noise to harmonic ratio (NHR).

## RESULTS

### *Vibratory Examinations*

Figure 1 shows the temporal changes of NMWA and NGG. NMWA showed gradual improvement in all cases, and it appears to stabilize about 6 months after the procedure. Although there were individual variations in the changes of NGG, it appears to take at least 6 months to reach plateau.

### *Aerodynamic and Acoustic Examinations*

Although the changes in MPT differed by individual, the majority of cases had stabilized by 6 months after surgery (Fig. 2A). Similarly, MFR also appeared to become stable 6 months postprocedure (Fig. 2B).

In some cases (case 2 at 2 months postsurgery and case 10) the voice was too harsh and rough for acoustic analysis to be performed. Although APQ showed gradual improvement and stabilized after about 6 months (Fig. 2D), the individual variability within the other parameters—PPQ and NHR—made it difficult to draw any generalized conclusions about the healing process (Fig. 2C, 2E).

### *Representative Case*

***Post-bilateral type I cordectomy scar (case 2).*** A 69-year-old man with laryngeal carcinoma in both vocal folds underwent bilateral type I cordectomy. Two months after surgery, no mucosal waves were seen in either of the vocal folds, and incomplete glottal closure was observed (Fig. 3A). Four months after surgery, the

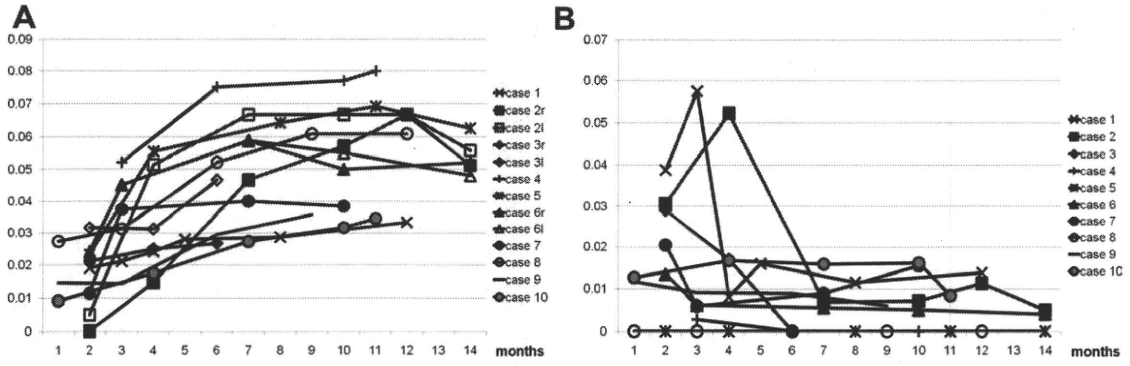


Fig. 1. Temporal changes in normalized mucosal wave amplitude (NMWA) and normalized glottal gap (NGG). Although there were individual variations in the changes of NGG (B), NMWA (A) and NGG appear to stabilize about 6 months after the procedure. r = right vocal fold; l = left vocal fold.

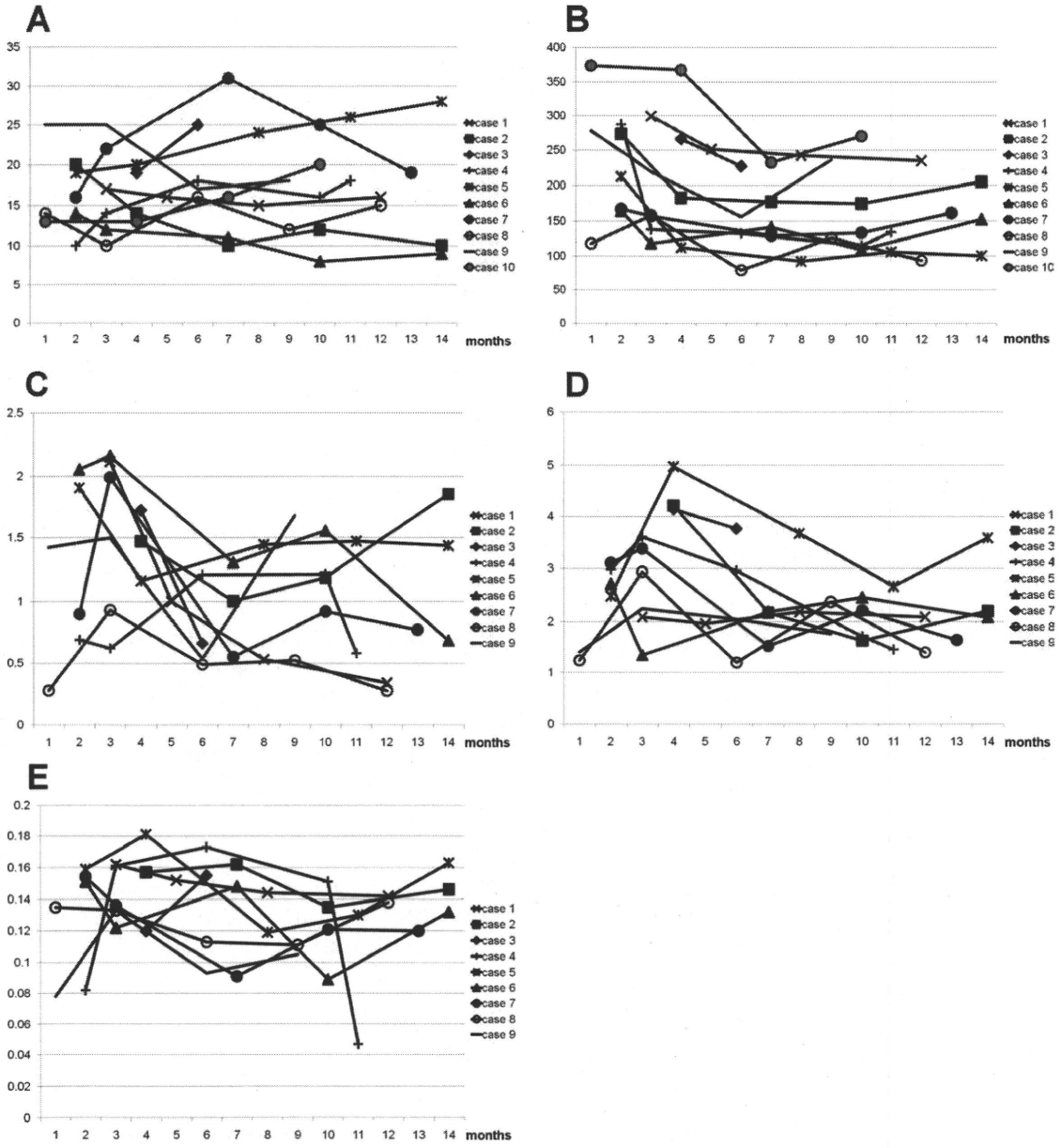


Fig. 2. Temporal changes in aerodynamic and acoustic measurements. (A) Maximum phonation time (MPT). (B) Mean flow rate (MFR). (C) Pitch perturbation quotient (PPQ). (D) Amplitude perturbation quotient (APQ). (E) Noise to harmonic ratio (NHR). The majority of cases had stabilized by 6 months after surgery in MPT and MFR. Though PPQ and NHR varied individually, APQ stabilized after about 6 months.

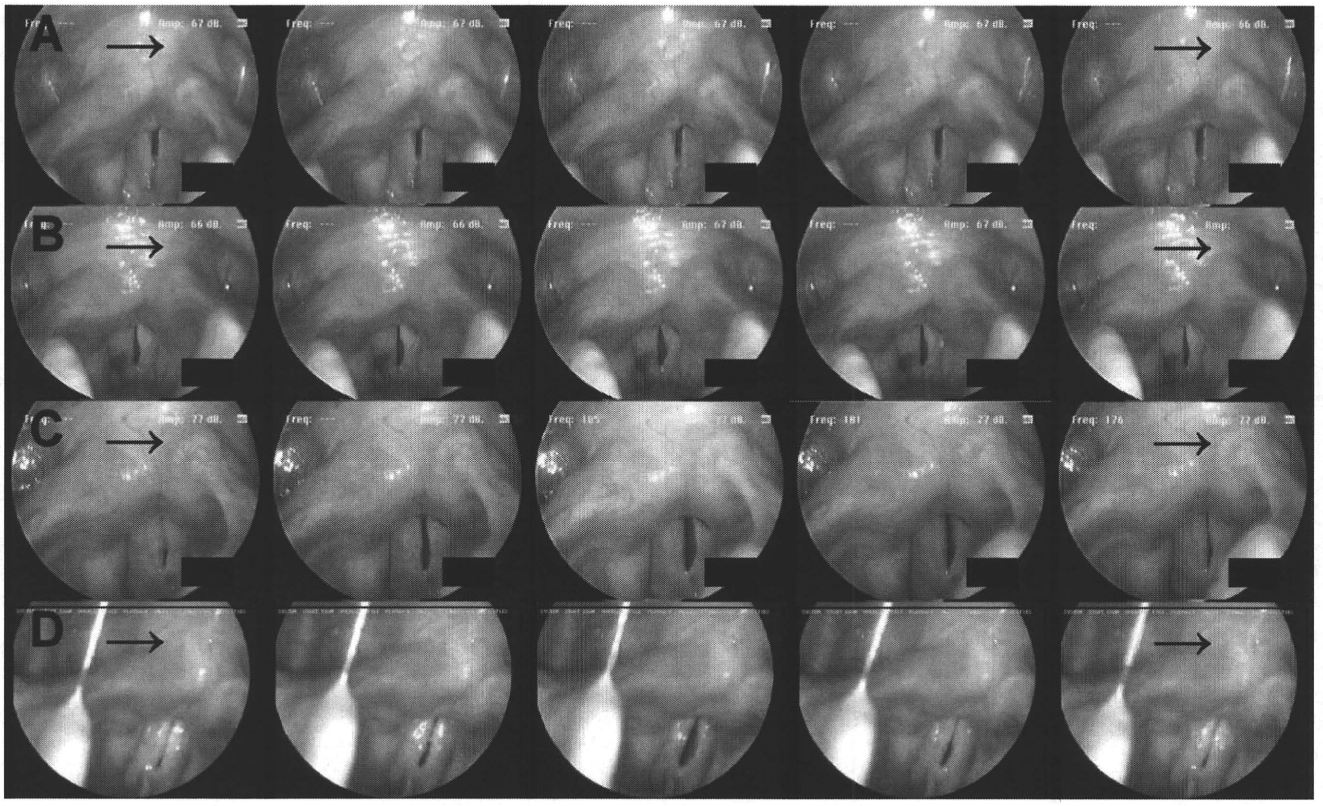


Fig. 3. Stroboscopic findings in case 2. No mucosal waves were seen in either vocal fold 2 months after surgery (A). Four months after surgery, the vocal folds showed some vibration; however, the vibration of the right vocal fold was still limited (B). Improved mucosal waves were observed 7 (C) and 14 (D) months after the procedure.

vocal folds showed some ability to vibrate; however, the vibration of the right vocal fold remained limited (Fig. 3B). Although an anterior glottic web was present, the glottal gap had become smaller and improved mucosal waves were observed 7 months after the procedure (Fig. 3C). These improvements continued 14 months after the procedure (Fig. 3D).

## DISCUSSION

With recent developments in regenerative medicine, new therapeutic strategies are being established for previously intractable disorders of organs systems.[6,7] In the treatment of vocal fold scarring, various restorative approaches based on the principles of tissue engineering—cell therapy,[8,9] growth factor therapy,[10–13] and scaffolding therapy[14–16]—have been utilized in attempts to restore normal function. Although these approaches are not fully established, some restorative effects have been reported and show promise as the basis for a new unified therapeutic strategy. To measure the clinical potential of such new approaches, it is important to have a comprehensive understanding, including the functional aspects, of the maturation process of human vocal fold scarring.

Cordectomy, which is one of the main treatments for early glottic carcinoma and laryngeal precancerous lesion, is a common cause of iatrogenic vocal fold scarring. Cordectomy has been shown to have high cure rates for these conditions while enabling laryngeal preservation.[17] It has also attracted much attention as a minimally invasive therapeutic strategy because it offers several advantages over radiation therapy, including the elimination of the adverse effects of irradiation, shorter hospitalization times, and high cost effectiveness.[17]

Postcordectomy patients were enlisted for this study as it is possible to precisely identify the onset of the scarring process following surgery. The wound healing response consists of three successive but overlapping phases—inflammation, proliferation, and tissue remodeling. Wound healing has been well documented on cutaneous wounds, and some valuable data pertaining to vocal fold scarring also exists.[18,19] A previous study on acute vocal fold injury reported that the vocal fold wound healing process is analogous to wound repair in the skin during the inflammatory and proliferative phases, but differs during the remodeling phase.[20] Additionally, Bond et al. have reported that cutaneous wound scar redness fades at 7 months, and scar maturation occurs over the course of 1 year.[21,22] Consistent with this, Xu et al. have reported that vocal quality becomes steady 6 months after surgery in type III–IV cordectomy patients.[17] In the current study, vibratory, aerodynamic, and acoustic parameters required at least 6 months to stabilize, and full scar maturation is thought to occur over a period of 1 year. These results suggest that the

maturation process in vocal fold scarring is temporally similar to that of cutaneous scarring.

The presence of individual variation in vibratory and functional changes of the scarred vocal folds made it difficult to identify commonalities for some parameters. These variations are thought to primarily depend on the individual healing mechanisms. Moreover, most patients in this case series underwent type I cordectomy, which is the shallowest resection. The variations might stand out because the scarring effect of this procedure is thought to be minimal and does not obscure the individual variations in healing ability. Furthermore, MPT and acoustic parameters are affected by other factors, such as pulmonary function, and might not accurately depict the condition of the vocal fold.

This study has identified the temporal changes that occur in vibratory function during maturation of human vocal fold scarring. Although these temporal changes do not reflect the maturation process of vocal fold scarring directly, they would help our understanding of the process and complement other basic science work in providing benchmarks in clinical use. This is, however, a preliminary study owing to the small number of enlisted patients. Further study is necessary to fully understand the wound healing process that occurs following vocal fold injury.

## CONCLUSION

Some individual variation was observed in the temporal changes of vocal function of scarred vocal folds after cordectomy. However, in terms of vibratory and aerodynamic functions, this study suggests that it takes at least 6 months for maturation of vocal fold scarring.

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# Glottal Reconstruction With a Tissue Engineering Technique Using Polypropylene Mesh: A Canine Experiment

Yamashita, Masaru; Kanemaru, Shin-ichi; Hirano, Shigeru; Umeda, Hiroo; Kitani, Yoshiharu; ###.
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## Abstract

OBJECTIVES: The larynx must be resected in some cases of cancer or stenosis, and various techniques are generally employed to fill the resulting defect. No ideal way, however, has been established to restore vocal function after this form of insult. The aim of this preliminary feasibility study in a canine model was to investigate the effectiveness of a polypropylene-based tissue engineering approach to repair a partial glottal defect. METHODS: Eight dogs were used in this study. A laryngeal defect involving resection of the left vocal fold was created through a thyroid cartilage window. A scaffold made of polypropylene and collagen was preclotted and wrapped with autologous fascia lata, inserted through the window, and sutured to the laryngeal defect in 5 dogs. The defect was reconstructed with an adjacent sternohyoid muscle flap in 3 control dogs. The surgical site was evaluated 3 months after operation by fiberscopic examination, computed tomographic imaging, histologic evaluation, and study of excised larynges. RESULTS: On fiberscopic examination, the experimental group implants were completely covered with regenerated mucosa in all cases, and a favorable vocal fold contour was found in 4 of the 5 cases. One case was characterized by a concave vocal fold shape and red granulation. In the control group, the muscle flap was replaced by scarred mucosa with a concave vocal fold contour in 2 cases, and there was soft white granulation at the anterior resected edge in the third case. The histologic data revealed the regeneration of lined epithelium, subepithelial tissue, and muscle structure in both groups. The excised larynx phonatory data revealed reduced vibratory amplitude in the experimental group compared with the control group; however, excised phonation was not achieved in 2 of the 3 cases in the control group. CONCLUSIONS: This polypropylene-based tissue engineering technique appears to be a viable tool for glottal reconstruction; however, additional refinement is required to maximize long-term phonatory function.

## Full text

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in the experimental group compared with the control group; however, excised phonation was not achieved in 2 of the 3 cases in the control group.

Conclusions: This polypropylene-based tissue engineering technique appears to be a viable tool for glottal reconstruction: however, additional refinement is required to maximize long-term phonatory function.

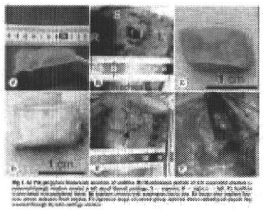
Key Words: fascia, laryngeal defect, polypropylene, reconstruction, tissue engineering, tissue regeneration.

## INTRODUCTION

Many surgical procedures have been used to reconstruct laryngeal defects after partial resections due to malignancy, but no currently available surgical technique is ideal. Current procedures include the use of the autologous muscle flap,1"3 myocutaneous flap,4·5 fascial graft,69 cartilage graft,11112 thyroid gland flap,13·14 and mucosal graft,15·'7 and all demand a high degree of skill and involve complicated surgical techniques. Additionally, most functions remain suboptimal after these procedures. For example, poor vocal function is inevitable after repeated surgeries, because these graft tissues are poor substitutes for the anatomic and biomechanical properties of the native vocal fold.

Remarkable recent progress in regenerative medicine has resulted in the ability to regenerate differentiated tissues and certain organs under appropriate conditions by use of tissue engineering techniques. Many published articles have addressed regenerative approaches for laryngeal tissues, but relatively few have focused on regeneration of the vocal fold structure following a gross glottal defect.18"20 Tissue engineering techniques hold promise for the regeneration of functional vocal fold tissue without the need for complicated surgical procedures. The feasibility of this approach for vocal fold reconstruction, however, has not yet been established.

Our previous research in which a polypropylene mesh scaffold coated with collagen sponge enabled us to successfully regenerate tracheal and cricoid defects2'"27 supports the value of this scaffold as a promising tool for regenerative medicine in the head and neck region. Subsequent work using this scaffold seeded with bone marrow-derived stromal cells showed promise in the treatment of a glottal defect20; however, the reepithel ialization rate was suboptimal , and outcome data were limited to endoscopic and radiographic findings.



View Image - Fig LA) Polypropylene framework structure of scaffold. B) Membranous portion of left vocal fold structure is removed through window created in left ala of thyroid cartilage. S - superior; R - right; L - left. C) Scaffold is preclotted with peripheral blood. D) Implant covered with autologous fascia lata. E) Image after implant fixation. Arrow indicates fixed implant. F) Operative image of control group. Asterisk shows sternohyoid muscle flap inserted through thyroid cartilage window.

In this study, therefore, glottal restoration following partial resection of the larynx was attempted in a canine model by a modified in situ tissue engineering approach, based on the introduction of an artificial scaffold constructed of polypropylene and autologous fascia lata. Endoscopic, radiographic, histologic, and vibratory data were compiled to evaluate the performance of this tissue engineering technique.

Our hypothesis was that reepithel ialization, vocal fold contour, and functional vibratory performance would be superior after this tissue engineering intervention, compared with traditional muscle flap reconstruction.

MATERIALS AND METHODS

Preparation of Scaffold. A single polypropylene mesh sheet with a pore size of 260 μm (Marlex mesh; CR Bard Ine, Billerica, Massachusetts) was used as a scaffold framework (Fig IA). A 1% porcine dermal atelocollagen (supplied by Nippon Meatpackers Ine, Ibaraki, Japan) preparation comprising type I (70%) and type III (30%) collagens dissolved in aqueous hydrochloric acid (pH 3.0) was coated on both sides of this polypropylene framework. After collagen-coating, freeze-drying with a freeze dryer (FDU-810, Tokyo Rikakikai Co Ltd, Tokyo, Japan) and cross-linkage with a vacuum dry oven (VOS300SD, Tokyo Rikakikai Co Ltd) were performed. The resultant spongy collagen matrix was designed to enhance cellular attachment and ingrowth into the scaffold.

Animals and Surgical Procedures. Animal care, housing, and experimental procedures were conducted according to the Guidelines for Animal Experiments of Kyoto University. Eight adult beagle dogs weighing 9 to 1 1 kg were anesthetized with subcutaneous injections of ketamine hydrochloride (5.0 mg/kg; Sankyo Co Ltd, Tokyo) and xylazine hydrochloride (2.0 mg/kg; Bayer Ltd, Tokyo). They were divided into groups of 5 experimental animals and 3 control animals.

After a cervical longitudinal skin incision, the left ala of the thyroid cartilage was exposed. A 1 .2 ? 0.7cm cartilage window was created with a scalpel, and the membranous portion of the left vocal fold, including the vocalis and thyroarytenoid muscles, was removed via this cartilage defect (Fig 1 B). The vocal process was kept intact, and the anterior edge of the left vocal fold was physically inaccessible through the created window defect.

In the experimental group, a scaffold implant, preclotted with 2 mL of arterial blood, was trimmed to match the size of the cartilage window. The clotting procedure rendered the implant completely infiltrated with blood and eliminated all air spaces (Fig IC). Next, the scaffold was wrapped in a 4 ? 2-cm autologous fascia lata graft harvested from the quadriceps muscle (Fig ID). Fascia lata was chosen because of its thickness and accessibility for harvesting an area large enough to cover the scaffold completely. Pilot experimental data (unpublished) demonstrated that the addition of fascia lata produced favorable outcomes compared with the use of the collagen-treated polypropylene scaffold alone.

In the control group, the left sternohyoid muscle was cut and the superior aspect was used as a reconstructive flap (Fig IF). The fully prepared implant in the experimental group or the muscle flap in the control group for each animal was inserted through the window defect and anastomosed according to the resected boundaries of the thyroid cartilage with 3-0 absorbable sutures (Vicryl, Ethicon Ine, Somerville, New Jersey; Fig IE).

Ampicillin sodium (Meiji Seika Kaisha Ltd, Tokyo; 250 mg per animal, subcutaneous) was administered in both groups for 7 days to prevent postoperative infection.

Endoscopic Evaluation. Endoscopic examinations were undertaken weekly with a video-endoscopy system consisting of a video bronchoscope (BF type 1T240, Olympus Co Ltd, Tokyo) and a video processor (CV-240, Olympus Co Ltd) coupled to a light source (CLV-U40D, Olympus Co Ltd). All examinations were performed under general anesthesia induced with ketamine and xylazine at the previously noted dosages.

Tissue Harvest and Outcome Measurements. Three months after surgery, the animals were humanely sacrificed with a cardiac injection of pentobarbital sodium (50 mg/kg; Dainippon Sumitomo Pharma Co, Ltd, Osaka, Japan) after the induction of general anesthesia. Laryngés were harvested en bloc and subjected to 3-dimensional (3-D) computed tomography (CT), excised larynx phonation, and processing for histology. The 3-D CT scanning was performed with a helical CT scanner system (Legato Duo, GE Yokogawa Medical Systems, Tokyo). Judgments were made from the CT images by an otolaryngologist and 2 radiologic technologists.

The vibratory function of the repaired glottis was evaluated with an excised larynx setup. Each larynx was mounted on an artificial trachea that delivered warmed (37°C) and humidified (more than 90% humidity) air. Bilateral arytenoid adduction and closure of the posterior glottal space were achieved with a 3-0 nylon string,28 and vocal fold vibration at maximum amplitude was recorded with a high-speed video camera system (Memrecam Ci, NAC Image Technology, Ine, Tokyo). Using frame-by-frame analysis, we compared the vibratory amplitude ratio (R) of the operated side to that of the control side using the formulae A = (D max - D min)/L and R = (A op/A cont) ? 100%, where A = vocal fold vibratory area, D max = distance from the vocal fold medial surface to the glottal midline at the moment of maximum vibratory amplitude, D min = distance from the vocal fold medial surface to the glottal midline at the moment of minimum vibratory amplitude, and L = length of the glottis extending from the anterior commissure to the vocal process.28

The phonation threshold pressure of each harvested larynx was also recorded with an arterial pressure sensor (22 18 A, NEC San-ei Instruments, Ltd, Tokyo) located in the subglottis, 3 cm below the vocal folds.

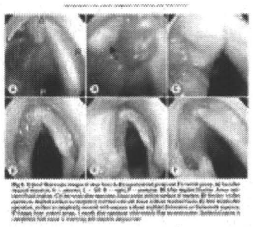
After 3-D CT and excised larynx phonation, the laryngés were processed for histologic analysis. Each larynx was fixed with formalin, and 4-um axial paraffin sections were prepared. Histologic assessment of hematoxylin and eosin-stained sections under light microscopy was performed to evaluate the status of each surgical site.

RESULTS

All 8 dogs recovered well from the initial surgical procedure, with no local or systemic complications. An oral diet was administered to each dog. No lifethreatening symptoms were seen in any dog during the observation period.

Endoscopic Evaluation. The fiberscopic findings in the 5 dogs in the experimental group were negative for stenosis or scaffold dislocation. The 3 animals in the control group were also negative for the dislocation of muscle flaps. No aspirated food was observed by endoscopy between the trachea and the main bronchi in any animal during the observation procedures in either group.

Figure 2A-E illustrates typical fiberscopic images of an operated larynx with a successfully reconstructed vocal fold at 1 month after surgery (Fig 2E), compared with an image immediately following surgery (Fig 2A). Figure 2C, taken 7 days after surgery, indicates that fascia still exists on the implant surface at this time point. The preclotted and fixed scaffold implant was an adequate size match for the laryngeal defect, and the implant surface was covered with soft tissue by day 14 (Fig 2D). Figure 2E, taken 1 month after surgery, illustrates regenerated mucosa without dislocation of the scaffold. The regenerated mucosa had a convex contour analogous to the anatomy of the native vocal fold. Figure 2F shows an image from a case in the control group; soft tissue was seen overlying the left, concave vocal fold.



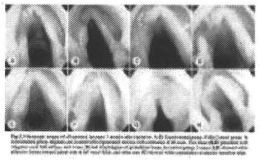
View Image - Fig 2. Typical fiberscopic images of dogs from A-E) experimental group and F) control group. A.) Just after surgical resection. A - anterior; L - left; R - right; P - posterior. B) After implant fixation. Arrow indicates fixed implant. C) One week after operation, fascia exists still on surface of implant. D) On day 14 after operation, implant surface is completely covered witfi soft tissue without residual fascia. E) One month after operation, surface is completely covered with mucosa witliout scaffold dislocation or framework exposure. F) Image from control group, 1 month after operation with muscle flap reconstruction. Epithel ialization is completed. Soft tissue is overlying left concave surgical site.

Figure 3 contains fiberscopic images from all operated laryngés at the 3-month time point. The implanted scaffolds were completely covered with newly regenerated mucosa with capillaries in all cases in the experimental group (Fig 3 A-E). Two of the 5 cases (Fig 3BJJ) presented an irregularly regenerated vocal fold surface, and 1 case (Fig 3D) presented a small degree of granulation tissue. In the control group, 2 cases showed white adhesive lesions toward the lateral side of the left vocal folds (Fig 3FJrI), and the third case showed a white granulation at the anterior resection edge (Fig 3G). No sign of infection or fistula was seen in either group.

CT Examination. Axial (Fig 4A) and 3-D CT images (Fig 4B) of the surgical sites revealed a clear luminal view of the reconstructed vocal fold, with no evidence of cartilage formation, in all cases in the experimental group. The control group also showed no evidence of cartilage formation.

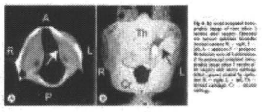
Excised Larynx Examination. The vibratory amplitude of the reconstructed vocal fold (normalized to that of the contralateral control vocal fold) was evaluated with a high-speed video camera system. Excised larynx phonation was not achieved in 2 cases from the control group because of persistent glottal gaps, even with bilateral arytenoid adduction and closure of the posterior glottis. The other case in the control group required granulation removal before data collection.

The mean vibratory amplitude of the reconstructed vocal fold in the experimental group was 12.20% (SD, 8.80%) of that of the contralateral fold. (This value should be 100% in a normal larynx.) The l successfully phonated larynx in the control group had 39.2% as the vibratory amplitude (average, 13.07%; SD, 22.64%). Statistical analysis using the Wilcoxon rank sum test showed a significant decrease in vibratory amplitude in the reconstructed fold compared to the contralateral fold ($p = 0.009$) in the experimental group. The mean phonation threshold pressure in the experimental group was 6.9 cm H2O (SD, 2.3 cm H2O), and the pressure in the 1 successfully phonated larynx in the control group was 7.0 cm H2O. The phonation threshold pressure in a normal canine larynx ranges from 4 to 6 cm ?2?.28



View Image - Fig 3. Fiberscopie images of all operated laryngés 3 months after operation. A-E) Experimental group. F-H) Control group. In experimental group, implants are covered with regenerated mucosa with capillaries in all cases. Two cases (BJD) presented with irregular vocal fold surface, and I case (D) had small degree of granulation tissue. In control group, 2 cases (F,H) showed white adhesive lesions toward lateral side in left vocal folds, and other case (G) showed white granulation at anterior resection edge.

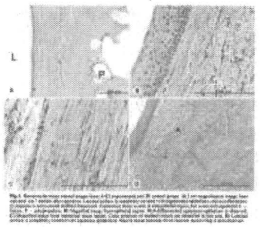
Histologic Assessments. Figure 5 shows hematoxylin-eosin-stained images taken 3 months after operation from the experimental group (Fig 5A-C) and the control group (Fig 5D). Histologic evaluation revealed a complete squamous epithelial lining in both groups (Fig 5A3J3). Disorganized connective tissue was observed in the subepithelial region (Fig 5BJD). No evidence of an inflammatory reaction was found in any specimen in either group. A small amount of skeletal muscle tissue was observed in the space between the epithelium and the polypropylene scaffold framework in 2 of the 5 cases in the experimental group (Fig 5C). In the control group, muscle tissue from the inserted muscle flap was identified. No cartilage formation was observed in any case in either group.



View Image - Fig 4. A) Axial computed tomographic image of case taken 3 months after surgery. Operated site (arrow) indicates favorable luminal contour. R - right; L - left; A - anterior; P - posterior. B) Anterior view of 3-dimensional reconstructed computed tomographic image taken 3 months after surgery still shows cartilage defect (arrow) created by operation. R - right; L - left; Th - thyroid cartilage; Cr - cricoid cartilage.

## DISCUSSION

Various treatment options to reconstruct the larynx after partial surgical resection and/or structural damage have been pursued for more than 50 years.29·30 The established treatments have remained controversial, as surgeons continue to encounter poor postoperative voice function and the need for complicated repeat surgeries to achieve adequate laryngeal reconstruction. Factors that contribute to unsatisfactory surgical outcomes include 1) difficulty reconstructing delicate tissues and structures, which as a whole are dynamic and influenced by movements necessary for swallowing and phonation; 2) difficulty reconstructing the native contour of the laryngeal luminal surface; and 3) infection or foreign body reaction.



View Image - Fig S. Hematoxylin-eosin stained images from A-C) experimental and D) control groups. A) Low-magnification image from operated site 3 months after operation. Luminal surface is completely covered with regenerated epithelium, and no inflammatory response is seen around scaffold framework. Connective tissue is seen in subepithelial region, but is not well organized. L - lumen; P - polypropylene. B) Magnified image from epithelial region. Well-differentiated squamous epithelium is observed. C) Magnified image from interstitial tissue region. Cross-striations of skeletal muscle are identified in this case. D) Luminal surface is completely covered with squamous epithelium. Muscle tissue (asterisk) from inserted muscle flap is still observed.

Although autologous tissue and homografts have been employed as implant materials for laryngeal reconstruction,117 damage inflicted on the donor site and/or the difficulty of these surgical procedures highlights the need for a more clinically efficient treatment approach. Moreover, in cases of tumor invasion into the larynx, deformities of the reconstructed site often render it difficult to monitor tumor recurrence. Regenerative approaches to tissue reconstruction in this area hold promise for overcoming these problems.

Regenerative medicine is a powerful clinical discipline with the potential to enhance the quality of life of patients who undergo organ reconstruction. This technique generally exploits 3 fundamental components: 1 ) cells acting as "seeds" for tissue regeneration; 2) a scaffold on which cells can proliferate and grow; and 3) regulatory factors that mediate cell behavior.3 ' The approach reported here represents in situ tissue engineering. Under this paradigm, scaffolds are the key component, as providing a well-prepared scaffold to the target site in vivo can lead to successful tissue regeneration even without cells or regulation factors under favorable conditions. Using this in situ tissue engineering approach, we have successfully achieved the regeneration of cricoid cartilage,24-27 trachea,23·25·27 and peripheral nerves.32 These procedures have already been applied to clinical cases in our institutions.23·26

Because polypropylene is a widely used polymer with high levels of biocompatibility and morphological plasticity, it is well suited as a scaffold material. Polypropylene has already been used clinically in reconstruction surgery for the abdominal wall.33 Using a columnar-shaped prosthesis with porous-type collagen and polypropylene as a scaffold, Nakamura et al21 and Okumura et al22 reported favorable outcomes in canine tracheal regeneration, as shown by cellular invasion to intact collagen, epithelialization of the luminal surface of the implants, and complete integration of the scaffold into the recipient's tissue. Yamashita et al25 demonstrated the possibility of regenerating a layered tissue structure, namely, epithelium, subepithelial tissue, and cartilage, in a canine tracheal resection model. Omori et al also demonstrated encouraging results with this scaffold for regeneration of the human trachea23·26 and human cricoid cartilage.26

Huber et al18 and Ringel et al19 reported favorable outcomes in the treatment of partial and complete hemilaryngectomy models using a xenogeneic extracellular matrix derived from decellularized porcine urinary bladder tissue. Their histologic data suggested the possibility of tissue regeneration in the larynx; however, their techniques require donor tissue from other animals. Also, functional vocal fold performance was not evaluated.18,19

The goal of this study was to evaluate the effectiveness of a similar scaffold composed of polypropylene mesh, collagen from porcine skin, and autologous fascia, to reconstruct a functional vocal fold after a partial laryngeal defect. Autologous fascia was incorporated here, as polypropylene-treated scaffolds without fascia produced poor outcomes in preliminary experiments (unpublished data), as evidenced by a lack of epithelialization, framework exposure, and early detachment of the coated collagen sponge. As fascial tissue contains a robust fibrous protein network and is frequently used as a coating material in the area of plastic and reconstructive surgeries, we used fascia lata to wrap the scaffold in the present experiment.

The fiberscopie and histologic data reported here demonstrate the viability of the scaffold in vivo 3 months after implantation, without infection. We observed restoration of epithelium, some muscle

tissue ingrowth, an adequate anatomic contour in the majority of cases, minimal granulation, and no cartilage formation in the experimental group. The vibratory performance of the reconstructed vocal fold, although present, was suboptimal. However, considering vibratory data from the control group, this tissue engineering technique appears to hold greater functional potential than a traditional muscle flap reconstruction method. Additional research is required to engineer tissues that can closely mimic the native biomechanical properties of an intact vocal fold.

It is important to note that although the focus of this study was the application of a tissue engineering scaffold, complete restoration of a functionally intact glottis is a demanding challenge and may depend on the addition of therapeutic cell populations and/or growth regulation factors.

## CONCLUSIONS

This preliminary study demonstrated that a polypropylene-based scaffold infiltrated with arterial blood and wrapped in autologous fascia lata is a viable tool for glottal reconstruction after partial resection of the larynx. Useful outcomes and other advantageous reconstructive factors may eventually favor the present tissue engineering approach over conventional surgical approaches, although further investigation and refinement are needed to maximize long-term phonatory function.

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