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In Vivo Vocal Fold Cover Layer Replacement

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Abstract

Objectives/Hypothesis: An animal vocal fold replacement model is needed to investigate treatments for vocal fold scarring. We developed a rabbit surgical model, hypothesizing that orthotopic vocal fold cover implants would attach and survive. We further hypothesized that superficial scarring would be limited, allowing unimpeded vibration.

Study Design: Translational research: animal surgical study.

Methods: Rabbit vocal fold covers were excised and immediately reimplanted. After 4 weeks, rabbits were phonated and vibration was recorded with high-speed videography. Larynges were then excised, elastic moduli measured by indentation, and covers sectioned for histology.

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J.L. was responsible for the concept, planning, implementation, and manuscript drafting. J.S. and S.R. were responsible for experimental procedures. G.L. was responsible for kymography. Z.Z. was responsible for experimental procedures and data analysis. D.C. was responsible for concept and planning. All authors contributed to manuscript editing and approval.

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Results: Five of six rabbits survived. Phonation was achieved in all, with mucosal waves evident. Elastic modulus did not differ significantly from contralateral uninjured control vocal folds. Histology demonstrated epithelial integrity, partial preservation of elastic fibers, and variable degrees of collagen deposition.

Conclusions: Vocal fold cover implantation in rabbits is feasible, and grafts survived. Attachment onto the thyroarytenoid muscle prevented excessive scarring, maintained tissue mechanics, and preserved mucosal vibration.

Level of Evidence: NA

Keywords

Phonation; rabbit; elastic modulus; indentation; vocal fold wound healing

INTRODUCTION

There is currently no satisfactory substitute for the vocal fold cover layer, the vibrating portion of the vocal cord comprising epithelium and lamina propria, and synonymous with the mucosa. As such, there has been substantial recent interest in creating a three-dimensional vocal fold cover replacement using tissue-engineering techniques.[1] However, implantation of a cell-populated, three-dimensional cover replacement has not yet been reported. The feasibility of such an approach remains in question.

Two issues pertain to any vocal fold mucosa replacement. First, can a three-dimensional tissue attach and survive when applied to the underlying thyroarytenoid muscle or vocal ligament? Second, will the new tissue heal without scarring that would impair its function? A gold standard of cover replacement would address these questions about vocal fold implantation, and would provide a benchmark for tissue-engineered constructs to meet or exceed. The native cover layer itself can be considered the gold standard, because its cells and microstructure are completely tolerated by the organism and are capable of phonation. The purpose of this study was to develop a gold standard implantation technique for in vivo cover replacement, using the ideal normal vocal fold cover layer. Issues related to the replacement procedure itself can then be studied without confounding by properties specific to a particular tissue-engineered implant.

Rabbits were selected as the model animal. Normal vocal fold mucosae were resected, replaced in situ, and allowed to heal. Viability, scarring, and vibratory function, the critical outcomes of vocal fold replacement, were studied in this normative model of cover layer reimplantation.

MATERIALS AND METHODS

Cover Replacement Surgery

The Institutional Animal Care and Use Committee approved this study. Six New Zealand white rabbits underwent survival surgery for vocal fold cover removal and reimplantation. Rabbits were anesthetized via mask induction, and the larynx exposed through a midline neck incision. A size 3 pediatric endotracheal tube was inserted through the cricothyroid

membrane for anesthesia. A laryngofissure through the midline thyroid cartilage exposed the vocal folds. Under loupe magnification, the membranous cover layer was resected from the left true vocal fold (also known as the inferior division of the thyroarytenoid fold in rabbits) by sharp dissection with microscissors. Dissection began at the anterior commissure, where the interface between thyroarytenoid muscle and lamina propria could be easily visualized in crosssection. Cuts were made at the superior and inferior edges of the vocal fold, and the entire cover layer was elevated back to the vocal process of the arytenoid cartilage. The final posterior cut at the cartilage freed the rectangular membranous vocal fold cover. The cover was then immediately replaced in its natural position. To ensure adhesion, a few microliters of fibrin glue prepared from rabbit fibrinogen and thrombin was applied at the interface of the vocal fold and the replanted cover. Four sutures of 6–0 fast-absorbing gut were placed at the corners of the replanted tissue to secure the cover in position. The right vocal fold was kept as an untreated control. The laryngofissure and tracheotomy wound were closed with 4–0 Prolene suture, and the rabbit converted back to mask anesthesia for neck wound closure. Intravenous dexamethasone and antibiotics were administered during the procedure. Intramuscular dexamethasone, antibiotics, and analgesics were administered twice daily for 3 days postoperatively.

In Vivo Phonation and Analysis

One nonoperated control rabbit underwent in vivo phonation with high-speed video (see Supporting Figure 1 in the online version of this article for images from one glottal cycle). Five operated rabbits then underwent in vivo phonation at 4 weeks after the implantation surgery. Rabbits were anesthetized via mask anesthesia to perform a cricothyroidotomy. The vocal folds were exposed via thyrohyoid pharyngotomy. All supraglottic structures, including the epiglottis, superior portion of the thyroid cartilage, and superior division of the thyroarytenoid folds (analogous to the human false vocal folds) were resected for clear visualization of the inferior thyroarytenoid folds.[2] A second endotracheal tube was inserted to supply airflow at 100 mL/s through the glottis, whereas manual pressure on the thyroid cartilage adducted the vocal folds. Vibration was recorded with a high-speed digital video camera (Phantom v210; Vision Research Inc., Wayne, NJ) at a capture rate of 8,000 frames per second. Each rabbit was recorded for at least five segments of 2 seconds each. Acoustic output was digitally recorded with a cardioid condenser microphone positioned approximately 15 cm from the glottis. After phonating, the rabbit was euthanized and bilateral vocal folds excised.

High-speed video of phonation was reviewed using Phantom Video Player software, assessing vocal fold appearance and mucosal wave symmetry. A 2-second segment of each rabbit's vibration was converted to a kymogram, performed at the midpoint of the membranous vocal folds. The entire 2-second kymogram was inspected for each rabbit. Acoustic output was analyzed with WaveSurfer software (Royal Institute of Technology, Stockholm, Sweden; http://www.speech.kth.se/wavesurfer/) to identify fundamental frequency.

Elastic Modulus Measurement

Young's modulus of the bilateral excised vocal folds were measured by an indentation method described previously.[3] Epithelial surface indentation was performed with a 1-mm-diameter cylindrical indenter mounted onto a force transducer, at steps of 6 μm each second, to a final depth of 80 μm. Output force (F) was measured at each imposed indentation depth (h). The initial unloading slopes of five force-displacement curves (dF/dh) were averaged using Matlab software (MathWorks Inc., Natick, MA) to calculate elastic modulus from a standard Hertzian model.

Histologic Analysis

After indentation, vocal folds were fixed in formalin, embedded in paraffin, and sectioned for histology. Stains included hematoxylin and eosin, Verhoeff's elastic stain, Masson's trichrome for collagen, and Alcian Blue stain for mucopolysaccharides. Sections were taken from the midmembranous portion of the vocal folds.

RESULTS

Cover Replacement Surgery and In Vivo Phonation

Six rabbits underwent cover replacement surgery. One rabbit (animal 2) died during immediate postoperative recovery from an obstructing tracheal hematoma. The remaining five rabbits survived without complication and proceeded to phonation at 1 month. Each phonated rabbit produced sound at fundamental frequencies ranging from 600 to 1,000 Hz, at airflow rates of 100 mL/s (Table I).

Vocal fold vibration was observed on high-speed video and kymography. Figure 1 shows selected still images from a single glottal cycle from animal 5. Figure 2 shows a segment of the resulting kymogram (see Supporting Video 1, in the online version of this article). Both the left (operated) and right (control) vocal folds participated in phonation in each rabbit, with mucosal waves on both sides proceeding from inferior to superior. Membranous glottal closure was complete, whereas the posterior cartilaginous glottis remained open. Vocal folds vibrated in phase with each other. Figures 1 and 2 demonstrate a left-right vibrational asymmetry with faster inferior-superior progression of the mucosal wave on the right side. However, minor left-right asymmetries were also noted in the nonoperated control rabbit's phonation (see Supporting Video 2, in the online version of this article).

Elastic Modulus Measurement

Young's moduli for the 10 vocal folds are shown in Table I. Means were 7.8 kPa for the control vocal folds and 7.7 kPa for the operated vocal folds, with no statistically significant difference by paired *t* test.

Histologic Analysis

Example micrographic images from one rabbit (animal 5) are shown in Figure 3. Epithelium was continuous in all vocal folds. Elastic fibers were present in all vocal folds, although some sections had a disorganized appearance as in Figure 3, which demonstrates interruption of the fine transverse elastic fibers seen in the control. Two of the five rabbits

(animals 1 and 6) were judged to have increased collagen throughout the operated vocal fold lamina propria relative to the contralateral control, based on Masson's trichrome staining; the other three rabbits did not have increased collagen staining. Mucopolysaccharides were preserved, based on Alcian Blue staining. Histology at the graft borders could not be assessed from these sections taken at the midmembranous vocal fold. The deep interface of the cover with TA muscle showed no extracellular matrix alteration versus controls.

DISCUSSION

As tissue-engineered vocal fold replacements advance, an animal implant model will be needed. This work developed a surgical model for complete replacement of the vocal fold mucosa in rabbits. Rabbits offer the minimum larynx size that would accommodate this fine surgery, while still needing only simple and economical vivarium care. They do have a laryngeal ventricle, although unlike humans the thyroarytenoid muscle underlies both the superior and inferior folds.[2] For the greatest similarity with humans, this surgery was performed on the inferior folds. Like other animals, rabbit vocal folds lack the "vocal ligament" or dense deep collagen layer adjacent to the thyroarytenoid muscle, presenting instead a bilayered lamina propria with elastin and collagen together in the deep layer.[4] Nonetheless work done in rabbits has been central to our current understanding of vocal fold scar physiology. Endoscopic rabbit scar studies demonstrated the disorganized collagen throughout the lamina propria and the loss of elastic fibers that are now considered to be characteristic of vocal fold scarring.[5] Rabbit vocal folds also vibrate with a mucosal wave, which allows functional assessment of the healing vocal folds by high-speed videography.[6] This experimental series studied the histology, tissue mechanics, and phonatory vibration of operated and control vocal folds in rabbits after cover replantation surgery. The goal was to develop a surgical technique and gold standard by which future tissue-engineered implants can be judged.

The laryngofissure and vocal fold replacement surgery described here is feasible in small animals. Vocal fold resection and replacement was performed with loupe magnification and microvascular instruments, and was technically on par with many otolaryngologic procedures. Larger animals and humans could presumably undergo an endoscopic approach, but the rabbit airway is simply too small. Intra-operative airway management required a temporary tracheotomy. Maintaining a tracheotomy for airway protection postoperatively was considered, because the laryngofissure and vocal fold manipulation placed the rabbit at some risk of airway obstruction. Of course, a tracheotomy would drastically increase the rabbits' care needs and potentially endanger the vocal fold graft by increasing pathogen exposure. This pilot study therefore attempted the surgery without tracheotomy, with good success considering the learning curve while developing the procedure. The first operated rabbit exhibited prolonged postoperative stridor requiring additional perioperative steroid dosing. The only death occurred in the second rabbit, due to poor hemostasis causing an obstructive laryngeal blood clot. No complications occurred in the subsequent four rabbits. Animal 6 was operated on by a different surgeon. Pictorial results shown here are all from animal 5.

After surgical feasibility, the next hurdle in three-dimensional cover replacement surgery is graft survival. We hypothesized that the thyroarytenoid muscle bed would offer adequate nutrition to support the very thin graft tissue. Fibrin glue assisted in adhering the graft, and the silent rabbits had little risk of dislodging the graft by speaking or coughing. Under these conditions, all the grafts did adhere well. Histology demonstrated complete epithelial coverage without evidence of ulceration or seroma formation. Mucopolysaccharides were present in a normal distribution.

Scar formation is a major concern after the successful adherence of a cover replacement graft. We hypothesized that limiting the surgery to the muscle interface would minimize extracellular matrix disruption in the vibratory cover layer. Results were variable. Histology in three of the rabbits did show grossly equivalent collagen content in the operated and control vocal folds. However, animals 1 and 6 were judged to have greater collagen content in the operated fold than in the untouched control side. These results highlight the learning curve of the surgery and the critical importance of minimizing trauma to the graft before and after implantation. Elastic fibers, the other fibrous extracellular matrix component altered in scarring, were disrupted. Elastic fibers were present in all rabbits, but there were discontinuities in the fine continuous transverse network seen in control vocal folds. Further immunofluorescent, electron microscopic, and quantitative analysis must be performed to fully appreciate the impact on these important structures.

Even with some extracellular matrix alteration, all rabbits had good vibratory function. Phonation was produced in all five rabbits, with fundamental frequencies similar to normal rabbits.[7] All rabbits produced bilateral inferior-to-superior mucosal waves as viewed on high-speed video. Membranous glottal closure was complete, and the two vocal folds vibrated in phase. Asymmetries in mucosal wave speed and lateral amplitude occurred and are of uncertain significance. The control larynx also exhibited minor asymmetries, which may reflect the phonation system rather than an inherent left-right difference.

Young’s modulus was measured by indentation, and demonstrated no significant difference between operated and control vocal folds. This simple mechanical test is a screen for excessive scarring that would be expected to increase modulus. However, because the vocal fold is very soft, accuracy is hampered by noise in the measurement. More sensitive assessment of mechanical properties may be useful in future studies.

This work demonstrates that replanting the entire vocal fold mucosa en bloc partially preserves its complex microstructure and function. The impact of wounding is detected in the disrupted elastic fibers, but the degree of collagen deposition and functional deficit are far less than expected for such an extensive resection (analogous to a European Laryngological Society Type II cordectomy). The mechanisms for the improvement require further basic investigation, but we propose three theories. First, the plane of injury is deep, whereas the lamina propria and epithelium are not violated. This reduces the cellular activation that launches the normal wound healing process and leads to scarring. Second, presence of an intact epithelial barrier during the early phases of wound healing can protect the underlying tissues from pathogens and chemical insults. Third, maintaining a biologic dressing over the wound may reduce fibroblast contraction that causes dense fibrosis.

Questions remain regarding the generalizability of these findings to a clinical setting of severe vocal fold scar, which is the intended recipient of a tissue-engineered vocal fold cover replacement. The scar environment may differ in its healing capacity than these normal rabbits due to modulated fibroblast and inflammatory cell phenotypes,[8] or to vascular insufficiency in the wound bed. Also the rabbits, being silent creatures, were completely compliant with 1 month of postoperative voice rest. Even short phonatory exposures are known to alter epithelial layer integrity, so the impact of phonotrauma on a healing implant cannot be neglected.[9] Finally, normal human vocal fold mucosa is not available as a graft tissue. Any tissue-engineered replacement is expected to have different mechanical and wound healing properties than native tissue. However, the challenge of producing a biologically identical vocal fold replacement may actually be an opportunity. With growing knowledge of vocal fold wound modulators, a graft could be designed specifically to mitigate scar formation while restoring vibratory function. The wound-healing behavior is likely to play as great a role in ultimate graft function as its intrinsic similarity to the vocal fold.

CONCLUSION

Vocal fold cover replacement surgery is feasible in rabbits. Although some extracellular matrix disruption did occur, the degree of scar formation and functional impairment was limited. This model will be useful for future studies of tissue-engineered vocal fold replacement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of California–Los Angeles.

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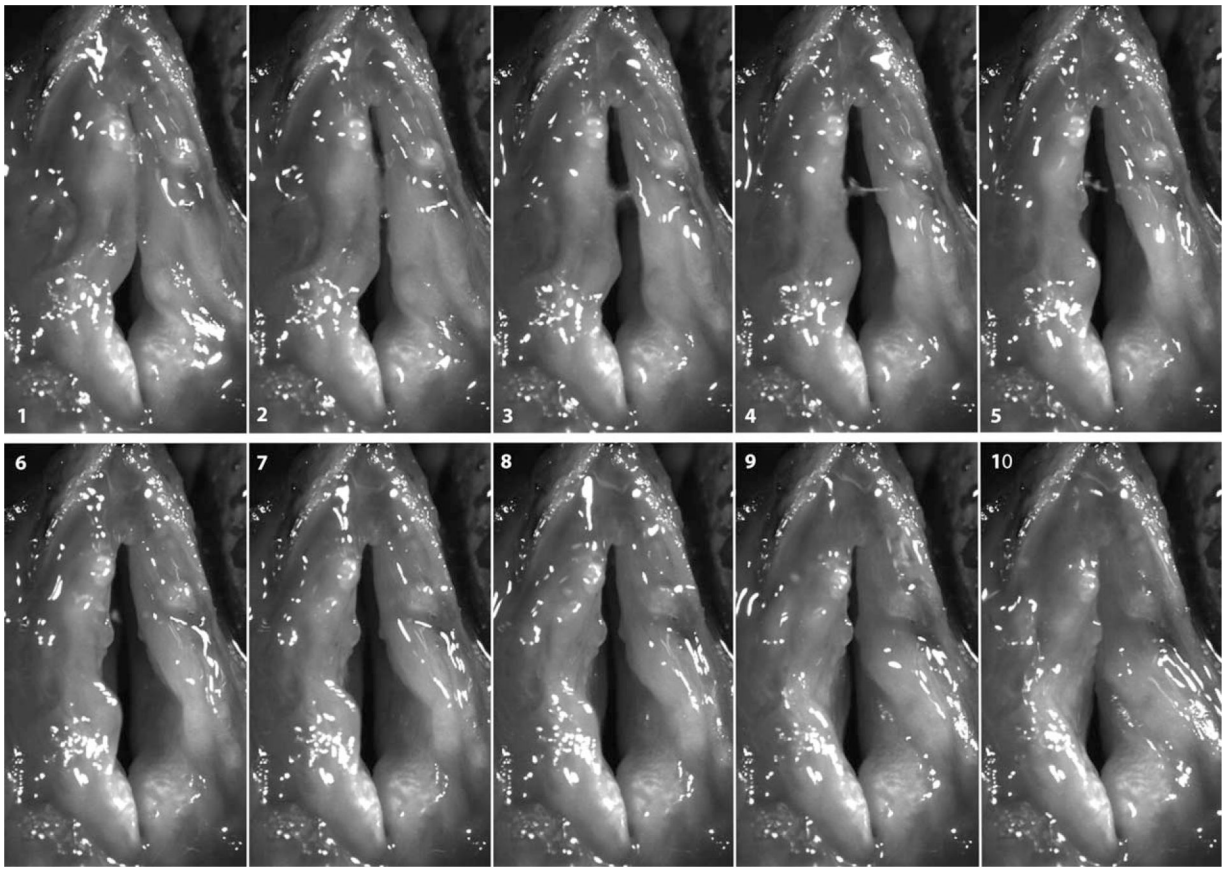

Fig. 1.

Phonation 1 month after left vocal fold cover replacement surgery. High-speed images were selected from a single glottic cycle recorded at 8,000 fps. Superior thyroarytenoid folds have been excised.

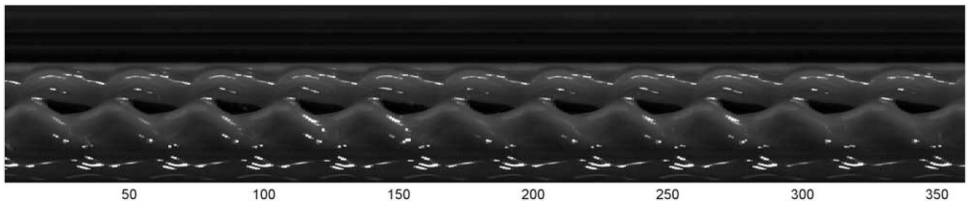

Fig. 2.

Kymogram of phonation 1 month after left vocal fold cover replacement surgery. The left vocal fold movement appears at the bottom of the image, and the right vocal fold at the top. Eleven glottic cycles are shown.

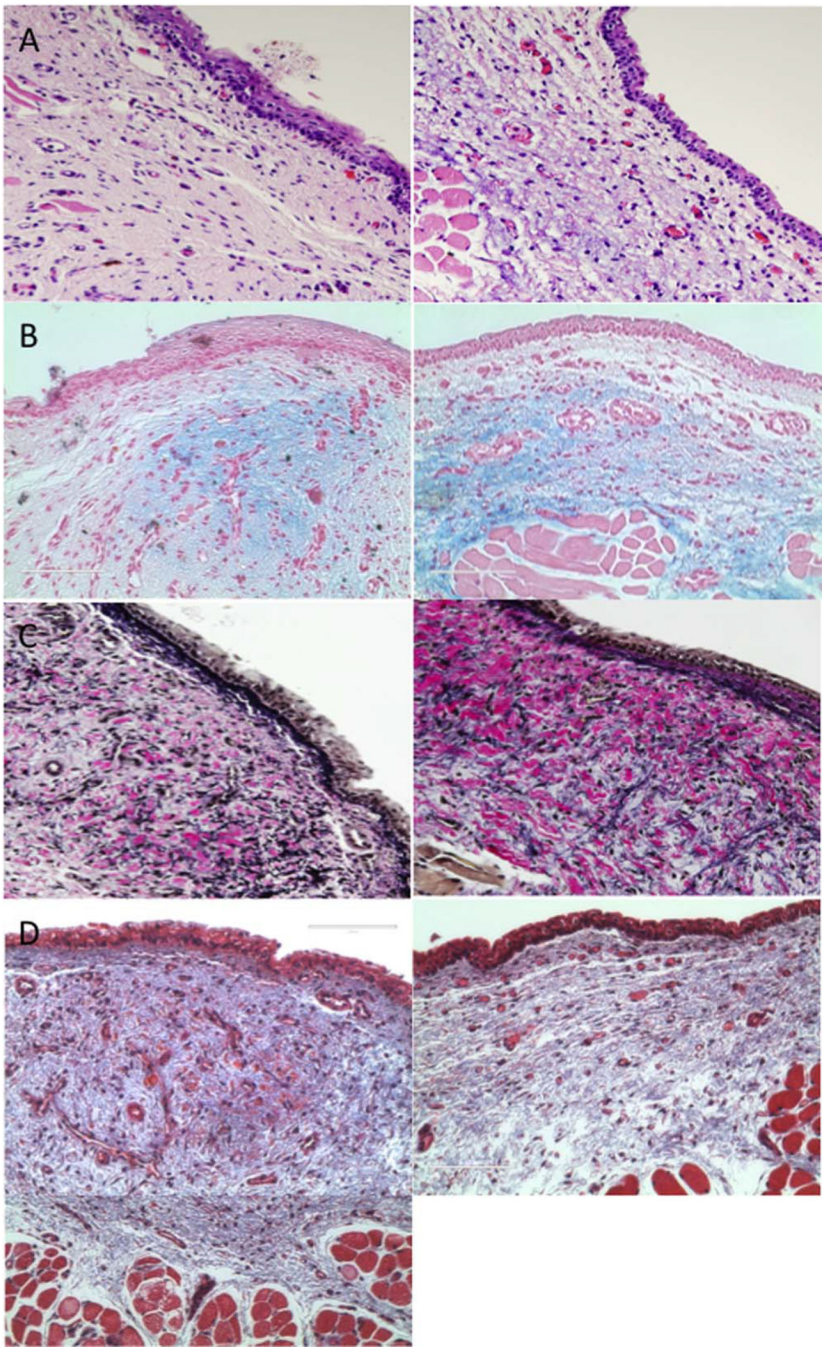

Fig. 3.

Vocal fold histology 1 month after vocal fold cover replacement surgery (all at 40×). The left column is the surgical left vocal fold, and the right column is the unoperated right vocal fold. (Row A) Hematoxylin and eosin. (Row B) Alcian Blue stain, with hyaluronic acid appearing blue. (Row C) Verhoeff's elastic stain, with elastic fibers appearing black. (Row D) Masson's trichrome stain, with collagen appearing blue. The left vocal fold in row D is a panoramic image to include the muscle-graft interface.

TABLE I.

Elastic Modulus and Phonation Frequency After Unilateral Vocal Fold Cover Replacement.

	Animal No.					
	1	**2**	**3**	**4**	**5**	**6**
Young's modulus, control VF, kPa	8.9	N/A	2.4	10.7	13.1	4
Young's modulus, surgical VF, kPa	4.3	N/A	2.6	10.9	13.1	7.7
F0 range, Hz	590	N/A	900–960	700–760	640–700	980

F0 = fundamental frequency; N/A = not applicable; VF = vocal fold.