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Local Vascularized Flaps for Augmentation of Reinke's Space

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Abstract

Objectives/Hypothesis—The purpose of this study is to describe and test a novel surgical strategy for augmentation of Reinke's space using vascularized flaps: a thyroid ala perichondrium flap (TAP) and a composite thyroid ala perichondrium flap (CTAP) from the anterior larynx. We hypothesized that these specially designed vascularized flaps would remain viable once inset into the lamina propria, and that they would not disrupt rheologic, biomechanical, and histologic properties of the native vocal fold.

Study Design—Experimental. In vivo canine model.

Methods—The length and volume of test flaps harvested in six adult human cadaveric larynges were analyzed to determine suitability for use in augmentation in the lamina propria. Also, 12 beagles randomly underwent unilateral placement of either TAP or CTAP, which were designed in accordance with the human adult cadaveric experiments. Flap perfusion was measured before and after harvest with laser Doppler. After 1 month, the beagles were humanely sacrificed and their larynges subjected to aerodynamic and acoustic evaluation using an excised larynx apparatus. The vocal fold lamina propria of four larynges—two TAP and two CTAP—underwent rheologic evaluation using a simple-shear rheometer. The remaining eight larynges underwent quantitative histologic and immunohistochemical evaluation. The survival and complication (swallowing, airway, local wound) rates of all dogs were noted.

Results—Initial studies with adult human cadaveric larynges established that TAP and CTAP possessed length and volume greater than native lamina propria. In the canine experiments, the perfusion change in the flaps was similar between flap groups. The damping ratio (ζ), dynamic viscosity (η'), elastic shear modulus (G'), and viscous shear modulus (G'') of treated and untreated native vocal folds were not statistically different. The glottic function measures of vocal efficiency, laryngeal resistance, jitter, shimmer, and harmonics-to-noise ratio (HNR) of treated and normal larynges were not statistically different. Similarly, the values for collagen, elastin, and glycosaminoglycans (GAGs) in treated and untreated vocal folds were not statistically different. Also, neither neochondrogenesis nor neoosteogenesis was detected in any treated vocal fold. The

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The University of Texas is the institution where rheologic testing was performed and the resulting data analyzed. All other work was completed at the University of Wisconsin—Madison School of Medicine and Public Health.

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values for vascular and cellular proliferation in treated and untreated vocal folds were not statistically different. All test dogs survived and had no complications related to swallowing, airway distress, or the local wound.

Conclusions—The test flaps described and tested in this study appear to have conceptually attractive features for augmentation of Reinke's space. When placed in an in vivo setting TAP and CTAP did not reveal unfavorable vascular, rheologic, aerodynamic, acoustic, or histologic characteristics. There was no unanticipated morbidity or mortality to the test animals. Long-term viability of these flaps is unknown. TAP and CTAP may open novel pathways for correction of glottic defects and may offer crossover opportunities with tissue engineering techniques.

Level of Evidence—None.

Keywords

Vocal fold; augmentation; Reinke's space; glottic insufficiency; autologous; fat; autologous; lamina propria

INTRODUCTION

Surgical correction of glottic insufficiency has been limited by multiple challenges, including laryngeal access, implant design, morbidity, and durability of outcomes. The many causes of glottic insufficiency—vocal fold paralysis, vocal fold paresis, surgical cancer defects, trauma, and variants of vocal fold scar such as sulcus vocalis—have mandated targeted management approaches. Vocal fold scar has been reported as the most common cause of poor vocal outcome following phonosurgery and, despite numerous approaches for surgical correction, voice outcomes have been inconsistent and inadequate.^{1–5} Some approaches for other causes of glottic insufficiency have proven efficacious, however. For example, type I thyroplasty as modernized by Isshiki has been an extremely successful pathway for correction of large glottic defects from vocal fold paralysis and other causes.^{6–8} However, the implants are usually exogenous, are sometimes costly, can extrude, and can require an external incision for placement into the paraglottic space.^{8,9} These implants are effective in displacing the vocal fold medially to improve glottic insufficiency but do not anatomically correct any lamina propria alterations. Injectable agents such as collagen, and Radiesse™ are widely available, effective, safe, and easily delivered endoscopically in the operating room or office into the paraglottic space.^{10–12} However, they have widely variable durabilities, can be prone to rejection or migration, and can be difficult to revise.^{12,13} Indeed, Teflon™ has been shown to have such strong local tissue reaction that it is essentially no longer used. Moreover, current formulations are not designed to influence lamina propria alterations directly. Autologous tissue implantation with fat and/or fascia has proven effective for medial displacement of the vocal fold but the implants do not possess their own blood supply. They also require a separate harvest site.^{14–16} One might postulate that an improved blood supply to the implant would enhance the durability and predictability of outcomes. Importantly, many surgical approaches have been instituted without substantial testing prior to patient use, potentially leading to unforeseen problems such as extrusion or inflammatory reactions.

Drawing inspiration and caution from the successes and limitations of antecedent surgical approaches and anticipating overlap with the emerging scientific revolution of tissue engineering we have designed two experimental soft tissue flaps that are vascularized, autologous, and locally harvested. They can be applied directly into Reinke's space via a minithyrotomy, as described by Gray.¹⁷ The thyroid ala perichondrium flap (TAP) is derived from the outer cover of the thyroid ala, and the composite thyroid ala perichondrium flap (CTAP) includes additional contiguous investing fascia and fat taken from the anterior

preepiglottic space. Conceptually, TAP and CTAP have numerous potential attractive features and offer opportunities to explore potential limitations before proceeding to clinical application.

TAP and CTAP may have promise to overcome the limited success thus far with introduction of tissue or fillers *directly* into the lamina propria for vocal fold scar. Failure to fill focal lamina propria defects might explain the acceptance of less precise techniques that displace the entire vocal fold such as thyroplasty or fat injection into the paraglottic space.^{14,18–20} TAP and CTAP are designed for direct delivery into the lamina propria when placed through a minithyrotomy. TAP and CTAP might be physically tailored to address subtle lamina propria alterations (e.g., sulcus vocalis, diffuse scar, focal cancer defect), allowing focused augmentation of the free edge of the vocal fold. Viscoelastic properties may also be favorable, especially for the fat-containing CTAP, given that the rheologic properties of fat have been previously demonstrated to be similar to native lamina propria.²¹ Yet it is unclear how TAP and CTAP might alter native viscoelastic properties and glottic function of the vocal fold.

One of the hallmarks of successful treatment is durability. Limited durability of free grafts into the lamina propria or paraglottic space has been in part attributed to poor vascular supply.^{19,20,22} For example, the resorption rate of free fat grafts in the head and neck has been noted to have a wide range of 20% to 90%.²³ However, pedicled thyroid cartilage perichondrial flaps, essentially identical to TAP, have been successfully used since the 1950s to reline the endolarynx following cancer resection.²⁴ More recently, authors employing this thyroid ala perichondrial flap noted no incidence of flap necrosis, suggesting a robust vascular quality.²⁵ No specific study of the flaps' vascularity prior to delivery into the recipient bed has been performed to date. Although durability has been achieved with Teflon injection, robust inflammatory reactions from rejection have been noted limiting the excellent long-term results.^{12,26} Being autologous, TAP and CTAP should resist rejection, potentially allowing better long-term effects. However, their local tissue reactions and vascular and cellular integration have not yet been studied.

Familiar surgical elements of this procedure may help it to be easily adopted. For example, exposure for open tissue harvest from the anterior larynx is nearly identical to that for standard thyroplasty. Exposure required for delivery of the flaps into the lamina propria is that of standard microlaryngoscopy. Also, the minithyrotomy technique has been increasingly adopted by surgeons for access to the lamina propria. Obviation of a separate harvest site for tissue (e.g., fascia lata, abdominal fat) can speed surgical time and limit patient morbidity. Surgical morbidity and mortality with TAP and CTAP has not yet been explored in an in vivo setting.

Because it is unclear if either of these flaps may offer value in glottic reconstruction or if the performance of one is superior, it seems necessary to evaluate the two in an animal model where procedural variables can be controlled and specific characteristics studied.

AIMS/OBJECTIVES

The *long-range goal* is to implement safe surgical strategies for the treatment of vocal fold scar and/or injured lamina propria. The *objective* of this study was to describe and test a novel surgical strategy for augmentation of Reinke's space using vascularized flaps of tissue from the anterior larynx rotated through a minithyrotomy into the lamina propria of the vocal fold. First, we conceived the flaps and performed feasibility testing on six human adult cadaveric larynges. Then we tested the structural and functional properties of the flaps in a biologic setting, using a canine model with several specific aims as outlined below.

Specific Aim 1: Compare the Vascular Supply of the Test Flaps

Hypothesis 1.1—Perfusion change in the test flaps before and after mobilization from their native sites is not statistically different between TAP and CTAP groups.

Specific Aim 2: Compare the Effects of the Delivery of Test Flaps into the Vocal Fold Lamina Propria on Glottic Performance

Hypothesis 2.1—The vocal efficiency of treated larynges is not statistically different from those of normal untreated canine larynges.

Hypothesis 2.2—The laryngeal resistance of treated larynges is not statistically different from that of normal untreated canine larynges.

Hypothesis 2.3—The jitter of treated larynges is not statistically different from those of normal untreated canine larynges.

Hypothesis 2.4—The shimmer of treated larynges is not statistically different from those of normal untreated canine larynges.

Hypothesis 2.5—The HNR of treated larynges is not statistically different from those of normal untreated canine larynges.

Specific Aim 3: Compare the Rheologic Effects of the Delivery of Test Flaps into the Vocal Fold Lamina Propria

Hypothesis 3.1—The damping ratio (ζ) of treated lamina propria is not statistically different from that of untreated lamina propria.

Hypothesis 3.2—The dynamic viscosity (η') of treated lamina propria is not statistically different from that of untreated lamina propria.

Hypothesis 3.3—The elastic shear modulus (G') of treated lamina propria is not statistically different from that of untreated lamina propria.

Hypothesis 3.4—The viscous shear modulus (G'') of treated lamina propria is not statistically different from that of untreated lamina propria.

Specific Aim 4: Compare Selected Histologic Effects of the Delivery of Test Flaps into the Vocal Fold Lamina Propria

Hypothesis 4.1—Neither neochondrogenesis nor neoosteogenesis is induced by the test flaps.

Hypothesis 4.2—Quantitative values for collagen are not statistically different in treated and untreated vocal folds.

Hypothesis 4.3—Quantitative values for glycosaminoglycans (GAGs) are not statistically different in treated and untreated vocal folds.

Hypothesis 4.4—Quantitative values for elastin are not statistically different in treated and untreated vocal folds.

Specific Aim 5: Compare the Viability of the Test Flaps Once Delivered into the Vocal Fold Lamina Propria

Hypothesis 5.1—Treated vocal folds have statistically higher staining values for Ki-67 in treated versus untreated vocal folds.

Hypothesis 5.2—Treated vocal folds have statistically higher staining values for vWF in treated versus untreated vocal folds.

METHODS AND MATERIALS

Fully detailed Materials and Methods can be found in Appendix I.

Human Cadaveric Larynges

After institutional review board approval, six human fresh-frozen cadaveric larynges (three male, three female) with no known history of laryngeal disease were obtained. Each larynx was mounted onto a laryngeal dissection station for manipulation.²⁷ TAP and CTAP were thus conceived and the surgical concepts refined. Once these concepts were outlined and the technique developed and performed, the lengths and volumes of the experimental flaps were tested.

TAP Technique

The flap was outlined along the superior and inferior borders of the thyroid ala and along the contralateral oblique line (Fig. 1A). The flap's attached base was ipsilateral to the vocal fold into which it was inserted. The outline of the flap was sharply incised. Using a Freer elevator the perichondrium was easily separated from the cartilage past midline (Fig. 1B). A minithyrotomy was performed with a 4-mm cutting burr on an otologic drill (Fig. 2A). The site of the minithyrotomy has been described previously and was located approximately halfway between the thyroid notch and inferior border of the midline thyroid ala in the inferosuperior dimension just off of midline. With sharp dissection the inner perichondrium of the thyroid ala was incised and a tunnel within the lamina propria developed for placement of the TAP into the vocal fold lamina propria (Fig. 2B). During the dissection of the tunnel the vocal fold was directly observed by the surgeon to assure that the vocal fold epithelium was not violated. Violation of the epithelium would expose the donor flap to the endolarynx potentially increasing the chance of contamination and/or extrusion. The schematic in Figure 3 of the placement of the TAP within the lamina propria shows its final position in all three orthogonal planes.

CTAP Technique

The overall technique was identical to the TAP technique except in the harvest of the flap. The CTAP extended along the inferior border of the thyroid ala past midline, vertically past the top of the superior border of the thyroid cartilage and into the anteromedial preepiglottic space (PES) (Fig. 4). It was based on the hemilarynx ipsilateral to the vocal fold into which it was inserted. The investing fascia of the PES, some PES fat, and adjacent perichondrium of the interior of the superior thyroid ala were contiguously elevated with the thyroid ala perichondrium using sharp instruments (Fig. 4). Using a Freer elevator and sharp instruments, the CTAP was elevated from the cartilage past the midline to allow for some mobilization of the flap and in anticipation of the ipsilateral minithyrotomy, described above (Fig. 4). After the minithyrotomy was drilled and the lamina propria tunnel created, the CTAP was inserted (Fig. 3).

Canine experiment overview—After approval by the Institutional Animal Care and Use Committee, 12 class A female beagles weighing between 8.4 and 12.6 kilograms (Avg. = 9.6 kg) and between the ages of 11 months and 23 months of age (Avg. = 14 months) were used to evaluate the structural and functional performance of the flaps.

EXPERIMENTAL PROCEDURES

First, all 12 dogs underwent a unilateral experimental vocal fold procedure: six underwent TAP and six underwent CTAP. Side and type of procedure were randomized. Once an appropriate anesthetic plane was reached, a zero degree operating telescope was used to confirm that the laryngeal anatomy and vocal folds were within normal limits. If the canine vocal folds were scarred (e.g., from a debarking procedure) then the dog was awakened and not subjected to further experimentation under the protocol. If normalcy was established, each test subject was prepared in a sterile way for a ventral cervical approach to the thyroid cartilage. A 6-cm horizontal incision at the level of the cricothyroid membrane was made and dissection of the subcutaneous tissue and underlying fascia superiorly to just above the hyoid bone and inferiorly to just below the cricoid cartilage exposed the sternohyoideus muscles.

Perfusion Measurements

Two measurements of blood flow across the anterior larynx were obtained before proceeding with flap harvest using a laser Doppler LISCA Opto-isolation unit (LISCA PIM II Laser Doppler Perfusion Imager (Lisca Development, Sweden) with, and a Transonic Systems, Inc. Team 21 compatible TS 420 Transit Time Perivascular Flow Meter (Lisca Development, Linköping, Sweden). The laser Doppler unit was positioned 10 cm above the thyroid ala with a scanning window of sufficient height and width to encompass the space between the hyoid bone and the cricoid cartilage anteriorly (Avg. size 26 mm × 30 mm). The scans occurred within a 5-minute time period.

The flaps were elevated using identical landmarks and instruments as those used in the human adult cadaveric larynges. Each flap was rinsed with sterile saline and redraped back into “preharvest” position and perfusion measured again in a manner identical to before flap harvest. This measurement occurred between 2 and 5 minutes after completion of the TAP or CTAP flap. Pre- and postflap mean changes in perfusion were compared between TAP and CTAP animal groups using an unpaired *t*-test. *P*-values less than .05 were considered as significant for *all* tests performed in this study. All analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC, USA). A minithyrotomy was performed, a lamina propria tunnel developed and finally the flap was placed into that tunnel. After hemostasis and irrigation, the wound was closed in layers and the dog recovered.

One month after flap placement, the dogs were maintained under Propofol IV until laryngeal endoscopy was performed to check for endolaryngeal anomalies such as hematoma, stenosis, or web formation. Once the endoscopy was completed and photo-documentation was attained, the dogs were humanely euthanized with an overdose of Beuthanasia solution 1 mL/10 lbs (to effect) through their i.v. catheter.

Each dog then underwent a brief post mortem examination prior to laryngeal extraction. Harvested larynges were immediately placed in PBS 1% solution and underwent acoustic and aerodynamic testing. Two experimentally naïve female beagles of similar age and weight also underwent surgical endoscopy, were humanely euthanized and underwent acoustic and aerodynamic testing if their larynges were deemed normal by endoscopy.

Glottic Aerodynamic and Acoustic Measurements in an Excised Larynx Model

To determine vocal efficiency, laryngeal resistance, jitter, shimmer, and HNR for 12 larynges experiments were conducted using an excised larynx apparatus. The aryepiglottic folds and epiglottis were resected down to the petiole. We apposed the arytenoids by passing 3-0 vicryl suture through the body of both arytenoids and back through the vocal processes using a horizontal mattress technique. We then performed a cricothyroid approximation in the midline using 3-0 vicryl suture. Verification of glottic closure along the full length was performed by visual inspection from below through the trachea.

The larynx was then placed on top of the airflow outlet and secured in place with ring lock and an external fixator device as described previously.²⁸ Humidified room temperature air flow through the larynx was increased until phonation threshold pressure (PTP) was attained. Measurements included mean transglottal DC air flow, subglottic pressure, sound intensity, and acoustic parameters recorded through a microphone onto digital audiotape. Measurements started at PTP and increased in even integers for a total of six measurements.

The acoustic signals were transformed into .wav files using the Computerized Speech Lab software (Kay-Pentax, Lincoln Park, NJ, USA) and analyzed for jitter, shimmer, and HNR using PRAAT software (Amsterdam, Holland). Acoustic and aerodynamic measures were compared between TAP, CTAP, and normal animals using an analysis of variance (ANOVA) with pairwise comparisons performed using Fisher's protected least significance difference tests.

Once the acoustic and aerodynamic studies were completed, the vocal fold lamina propria from each hemi-larynx was then removed. Immediately after aerodynamic and acoustic testing, the lamina propria samples of the four larynges (two TAP and two CTAP) destined for rheologic testing were removed, appropriately frozen, and stored until testing.

Rheologic Testing

To determine the rheologic properties of the treated lamina propria of two TAP and two CTAP treated larynges, the four treated vocal folds, and four untreated vocal folds underwent testing using a controlled-strain, linear, simple-shear rheometer system based on the Bose ELF 3200 mechanical testing system (Bose Corporation, ElectroForce Systems Group, Eden Prairie, MN, USA) as described previously.²⁹ With these data, damping ratio (ζ), dynamic viscosity (η'), the elastic shear modulus (G') and viscous shear modulus (G'') of the specimen were calculated according to the theory of linear viscoelasticity.²⁹

For each specimen, the slope and the y-intercept of linear regression were calculated by curve-fitting the data with the log rheologic measure plotted against the log frequency. Because slopes were fairly consistent, we focused on differences between y-intercepts derived from regression analysis assuming equal slopes. These y-intercepts were compared within TAP animals, and within CTAP animals using paired *t*-tests and were compared between TAP and CTAP animals using an unpaired *t*-test.

Histology/Immunohistochemistry

We sought to examine histologic changes between treated and untreated vocal folds in the remaining eight larynges (four TAP and four CTAP). After removal from each animal the larynx was sharply divided in the midsagittal plane and the entire vocal fold from the anterior commissure through the vocal process were removed and fixed in 10% neutral buffered formalin for processing and were embedded in paraffin wax. Serial tissue sections, 5 microns in thickness, were obtained through the musculomembranous portion of the vocal fold in the coronal plane and subsequently stained with routine hematoxylin & eosin (H&E),

and special stains: Alcian Blue pH 2.5 stain kit (American Master*Tech Scientific, Lodi, CA, USA) for glycosaminoglycans, Gomori One-Step Trichrome stain kit (Newcomer Supply, Middleton, WI, USA) for collagen, Verhoeff-Van Gieson Elastic Stain Kit (Newcomer Supply) for elastin. For immunohistochemical staining, 5 micron sections of canine vocal folds as described above were deparaffinized, rehydrated, and antigen retrieval performed. Sections were incubated with primary antibodies (Ki67 [rabbit polyclonal, 1:100 dilution, Abcam, Cambridge, UK], or von Willebrand Factor [vWF] (rabbit polyclonal, 1:500 dilution, Dako)) incubated 1 hour at room temperature. Tissue sections were counterstained with hematoxylin, dehydrated, cleared, and coverslipped for microscopic analysis.

The staining density of each slide was analyzed using Image J software (NIH, Bethesda, MD, USA). Photography of the prepared slides was performed at 4× magnification. For reliability, 10% of the images underwent the same process of “sewing,” masking, and Image J analysis, and their values compared to the original values to generate an interclass correlation coefficient. The staining densities differences of trichrome, alcian blue, EVG, Ki-67, and vWF of treated and untreated vocal folds were compared on a within-animal basis using a paired student's *t*-test.

RESULTS

Specific Aim 1: Compare the Vascular Supply of the Test Flaps

We sought to compare the vascular performance of TAP to CTAP immediately following harvest. The percent decrease in perfusion from before and after flap harvest for TAP and CTAP were 45.4% and 30.3%, respectively. The respective standard errors were 9.3 and 7.6%. There was no statistically significant difference in perfusion change between TAP dogs and CTAP dogs ($P = .24$). We accept the hypothesis that there is no statistical difference for pre- to postmobilization perfusion change between TAP and CTAP groups.

Specific Aim 2: Compare the Effects of the Delivery of Test Flaps into the Vocal Fold Lamina Propria on Glottic Performance

We sought additional assessment of how the flaps performed after the 1 month inset period by measuring aerodynamic and acoustic parameters of test and normal larynges with an excised larynx apparatus. Figures 5 and 6 display the values for the aerodynamic and acoustic measures taken. There were no statistically significant differences between groups for any of the values compared—laryngeal resistance, vocal efficiency, jitter, shimmer, or HNR—either between test groups and normals or between TAP and CTAP groups. Although there were large standard deviations relative to mean values and variable data, we accept the hypothesis that the aerodynamic and acoustic performance of treated larynges is not statistically different from that of untreated larynges. This finding is noteworthy because it suggests no major detriment to glottic performance occurred due to inset of TAP or CTAP, relative to normal larynges.

Specific Aim 3: Compare the Rheologic Effects of the Delivery of Test Flaps into the Vocal Fold Lamina Propria

One month after flap inset into a normal vocal fold, the viscoelastic properties of each animal's treated vocal folds were compared to those of their untreated vocal folds. Values of the four viscoelastic properties measured are presented in Figure 7.

For the damping ratio (ζ), dynamic viscosity (η'), elastic shear modulus (G'), and viscous shear modulus (G''), there was no statistical difference between treated and untreated vocal folds within the TAP or CTAP groups or between TAP and CTAP groups ($P = .38$).

Although there was wide variability in raw numerical data (Fig. 7) with accompanying high standard deviations, we accept the hypothesis that rheologic properties of treated lamina propria are not statistically different from those of untreated lamina propria for all rheologic parameters tested. These findings are significant because they suggest that the inset of these experimental flaps into the lamina propria in a biologic system does not have a major detrimental effect on rheologic properties. We also accept the hypothesis that there is no statistical difference between TAP and CTAP groups for within-animal differences in viscoelastic properties between treated and untreated vocal folds. These findings are significant because one might expect that the differences in tissue type between the flaps would induce different local tissue effects within the lamina propria and ultimately differentially alter rheologic properties.

Although the rheologic properties dictate the functional performance of the vocal folds during vibration, an excised larynx model would allow us to examine the glottic aerodynamic and acoustic performance parameters under flow-induced phonation.

Specific Aim 4: Compare Selected Histologic Effects of the Delivery of Test Flaps into the Vocal Fold Lamina Propria

Neither neochondrogenesis nor neoosteogenesis was detected in any of the treated vocal folds. This finding is significant because neither cartilage nor bone is part of the native musculomembranous vocal fold; induction of either of these types of deposits would likely have a highly detrimental effect on oscillation. We also assessed whether the inset of either of these experimental flaps might induce an unfavorable change in major constituent components of the native lamina propria such as collagen, GAGs, or elastin. The numerical differences of staining density between treated and untreated vocal folds on a within-animal basis are displayed graphically in Figure 8. The means of within-animal differences of staining densities between treated and untreated vocal folds are displayed in Table I.

There were no statistically significant differences between groups for any of the staining densities compared—collagen, GAGs, or elastin—either between within-animal treated and untreated vocal folds or between TAP and CTAP groups. The interclass coefficient for image evaluation was 0.8663, suggesting good reliability. We accept the hypotheses that the experimental flaps do not induce either neochondrogenesis or neoosteogenesis and that there is no detectable difference in collagen, GAGs, or elastin between treated and untreated vocal folds.

Specific Aim 5: Compare the Viability of the Test Flaps Once Delivered into the Vocal Fold Lamina Propria

We used stains for cellular and vascular proliferation to assess the viability of the test flaps, hypothesizing that viable flaps might stimulate ingrowth of cells and blood vessels. Within-animal vocal fold differences are displayed in Figure 9. The means of within-animal differences of staining densities between treated and untreated vocal folds are displayed in Table II.

We found that treated vocal folds did not have statistically higher staining values for Ki-67 or vWF in treated versus untreated vocal folds. We reject the hypothesis that the flaps induced detectable cellular and vascular ingrowth compared to the untreated side. This finding is significant because it suggests that experimental flaps may not be viable.

DISCUSSION

Surgical approaches that *directly* address pathology of the lamina propria (e.g., vocal fold scar and sulcus vocalis) are varied and have included excision, incision, scar manipulation

and implantation of free fat grafts.^{3,4,17,18,30} These techniques can offer vocal improvement, but to date there has not been development of an autologous, vascularized augmentation material that is locally harvested and can be delivered into the lamina propria. The minithyrotomy technique as described by Gray et al.¹⁷ delineates how a direct approach to Reinke's space can be achieved with a surgical skill set that incorporates elements of standard microlaryngoscopy and thyroplasty. Although the minithyrotomy offers attractive access, no appropriate implant has been designed to allow for durable augmentation of Reinke's space once access is obtained. We sought to address this gap by designing two experimental flaps using adult human cadaveric larynges and whose performance parameters we explored in a canine model. We acknowledge that there are limitations of the canine model. Most importantly, the lamina propria does not have the same layered structure as the human adult vocal fold. However, as noted by Garrett et al.³¹ canines are suitable for microsuspension laryngoscopy and their vocal folds vibrated in a similar fashion to human vocal folds with mucosal waves and vertical phase differences under stroboscopic examination. Furthermore, the size of the dog was appropriate for the proposed surgical procedure; a larger animal would have been unwieldy and a smaller one would have prevented successful execution of the procedure.

As noted previously, it was necessary to compare the two procedures given that the fat contained in CTAP, but not in TAP, might offer potential advantages. We postulated that the fat component of CTAP would be less likely to induce additional tissue formation given that free perichondrial and periosteal grafts have been noted to produce cartilage and bone, respectively, when placed in nonnative surroundings.³² Fat, on the other hand, generally does not induce formation of associated tissue. One month was selected because in prior studies, cartilage was easily visible by 2 weeks, suggesting that the 1-month latency used in our study should be sufficient to demonstrate if neochondrogenesis or neoosteogenesis were to happen. Another potential advantage of CTAP relates to the rheologic properties of fat. As noted above, fat appears to have similar, although not identical viscoelastic properties as native lamina propria, which suggests that implanted native fat might perform similarly to a native vocal fold, as has been noted previously.^{18,21} Last, CTAPs are larger than TAPs making them good candidates for larger defects and allowing greater ability of the surgeon to tailor the implant to the defects. However, given that the fat component of the CTAP is at the distal end of the flap and might therefore be susceptible to vascular compromise, we also were interested in the viability of this flap versus the TAP.

Human Cadaveric Studies

After the example of Payr and others, we chose to perform our initial examinations in an ex vivo environment on adult human cadaveric larynges. This approach allowed us to make anatomic measurements not only to establish the fundamentals of surgical technique but also to verify that the proposed procedures might be feasible. We found that both TAP and CTAP were long enough to span the entire ipsilateral vocal fold and that their volume would be sufficient to reconstitute Reinke's space in cases of physical defect. We considered that "extra" length might be important because the inset flap might be prone to migration due to laryngeal movement and because perichondrial flaps are known to contract somewhat over time. We considered that "extra" volume might be important not only because of tissue contraction but also because we anticipated some element of tissue loss of the flap as has been noted with previous reports.¹⁴ Another additional advantage of having extra volume is that of being able to tailor the flap to a defect. For example, a modest vocal fold scar might be best addressed by placement of a thinner TAP within the lamina propria, whereas a deep type II sulcus deformity might be better reconstructed with a larger CTAP. The authors considered technical adjustments to improve the chances of the flap staying in the recipient bed such as suturing the flap to the minithyrotomy site; we believed that this could

compromise the blood supply, however, and chose to avoid this approach. We also had concerns that when the CTAP is rotated into the minithyrotomy tunnel, the fat is directed away from the surface potentially limiting its effect; we believe that fixing the ultimate orientation of the flap would be essentially unfeasible because of laryngeal motion and that ultimately any biologic effect in the lamina propria set into motion by the flap would take place regardless of flap orientation.

Vascular Properties

Laser Doppler perfusion testing performed on all test animals before and immediately following flap harvest revealed a pattern similar to other harvested flaps of immediate perfusion reduction. For example, the pattern of immediate perfusion loss was consistent with a prior study on axial skin flaps in pigs.³³ Comparison of data for perfusion changes between these and other flaps—whether random, axial, or free flaps—is difficult, however. Also, most measurements in the literature on other flaps are either not taken immediately after harvest or if they are, the raw data are not published, making easy comparisons impossible. Furthermore, most Doppler studies are performed on skin, which likely does not adhere to the same vascular principles as perichondrium. Notably, prior studies have shown subsequent increased perfusion to inset flaps and to the recipient bed after the initial perfusion drop.^{34,35} Finally, although the TAP is not novel in use, having been employed since the 1950s, we could find no other similar prior perfusion studies for comparison. There were no statistically significant differences between TAP and CTAP groups for perfusion loss immediately following flap harvest. However, the perfusion loss was 45.4% versus 30.3% for TAP and CTAP, respectively. This trend in the raw data suggests that CTAP may actually have better perfusion than that of the TAP. One might expect the opposite given that the additional tissue at the distal end of the CTAP could cause vascular compromise and would therefore increase the overall perfusion loss. This finding is pertinent because the additional tissue is largely fat, and fat may be highly useful in reconstituting the volume of the lamina propria in cases of injury. There is certainly the possibility of experimental error. For example, previous criticisms of laser Doppler imaging have cited tissue movement and single-site measurements as potential detractors from accurate readings. We attempted to account for this by assuring that the dogs and equipment were immobile and the Doppler unit used a scanning rather than a single-site measurement technique.

Glottic Performance

We performed an assessment of the biomechanical parameters of larynges implanted with TAP and CTAP to assess if there was a substantial alteration in aerodynamic and acoustic performance. Importantly, canine larynges have been noted to possess similar histologic and vibratory characteristics as human vocal folds, suggesting that the canine model is a reasonable model in this study.³¹ Therefore, we compared the vocal efficiency, laryngeal resistance, jitter, shimmer, and HNR to normal larynges. There were no statistically significant differences for laryngeal resistance and vocal efficiency, suggesting general similarity to the normal larynges. However, the standard errors were high relative to the mean values, demonstrating wide variability in animal to animal measures. These high error rates make valid interpretation of the data more difficult and may be explained by factors such as small numbers in each test group, differential healing between animals, and slightly different size and positioning of flaps within the lamina propria. This same pattern of statistically similar, but numerically different data is encountered when examining values for jitter, shimmer, and HNR. It is unclear if the appearance of high jitter and low shimmer values in the test larynges relative to normal vocal folds is accurate; if so it may indicate increased stiffness in the treated vocal folds, suggesting a treatment effect, albeit not a favorable one. For HNR the small number of animals and the wide variability in raw data

may help to explain why the HNR values do not follow the same pattern as the jitter and shimmer values.

These performance measures appear to make sense because the implantation of a soft tissue flap into a normal vocal fold would almost certainly be expected to induce some alteration in oscillation due to asymmetry across the glottis (contour, tension, mass) and to healing effects. For example, if a flap were to have final placement on the subglottic edge rather than the free edge of the vocal fold, then substantial changes in acoustic performance might be anticipated.³⁶ The fact that the measured changes are numerically different but not statistically different between test larynges and normals suggests that there has been a treatment effect in the test groups but that whatever effects have occurred at 1 month are not devastating to glottic performance. Lingering questions about glottic performance may in part be answered by using high-speed digital imaging in future studies.

Rheologic Properties

Many vocal fold implants have been shown to be rheologically dissimilar to native vocal fold mucosa.^{21,37} Given that the oscillation of vocal folds is sensitive to rheologic alterations of the lamina propria, we measured whether the insertion of TAP or CTAP would significantly change any of four rheologic properties—damping ratio, dynamic viscosity, elastic shear modulus, and viscous shear modulus—when compared to the untreated side within the same animal. The values we obtained for damping ratio, dynamic viscosity, elastic shear modulus, and viscous shear modulus were comparable to those found in earlier studies on human cadaveric vocal fold lamina propria, suggesting the validity of our results.³⁸ When we found no statistically significant differences for any group, and for a comparison of TAP to CTAP groups, this was reassuring because substantial alterations in rheology might lead to poor voice outcomes. Importantly, dynamic viscosity has been noted to be strongly correlated to the ease of phonation; increased dynamic viscosity in the treated vocal folds might have been a “red flag” for increased phonatory energy requirements.³⁹

Mere statistical examination, however, may not be sufficient for full data interpretation here. For example, there was substantial variability to the raw data for each of the parameters tested. This variability could be explained by differential healing of the flaps within their recipient beds or even the testing method itself; each sample was tested at 14 frequencies and undergoes tissue alteration over time. Order of magnitude differences in normal human cadaveric vocal folds in the measurement of dynamic viscosity and elastic shear modulus have been previously partially attributed to age differences between subjects as well as differences in the densities and distributions of different vocal fold matrix proteins among the subjects, which could also be the case for the test animals in our study.³⁸ This investigation was limited by a small sample size (4), limiting the confidence that detrimental rheologic change is not likely with this procedure. However, for many parameters, values for treated vocal folds were often both above and below the untreated vocal folds, suggesting that testing a larger number of animals still might not yield a statistical difference due to the large variability.

Histology

Given the risk of disruption of the specific components and layered structure of the vocal fold lamina propria after flap placement, we assessed histologic changes in the lamina propria.⁴⁰ We were particularly concerned that the perichondrially based flaps might induce either neochondrogenesis or neoosteogenesis, which could severely impede vocal fold oscillation. Neither TAP nor CTAP was noted to induce surrounding neochondrogenesis or neoosteogenesis. It may be that a different recipient environment could change the outcome, however. For example, when a clot of blood was applied to free perichondrial grafts placed

into rabbit dermis, the grafts produced abundant cartilage, whereas they did not produce cartilage when the blood clot was omitted.³² Given that graft manufacture of new tissue has been noted previously by 2 weeks and that our examination time point was 1 month, it seems unlikely we would observe cartilage or bone production at a later time point.³²

Another concern was that the inset of the flaps might incite abundant collagenesis and disruption of the amounts of normal lamina propria components such as glycosaminoglycans and elastin, leading to reduced glottic performance. There was no statistically significant difference between the treated and untreated vocal folds, suggesting no detectable changes. Again, perhaps because of the small number of test animals, sampling error in sectioning, variability in healing, and a different time point standard errors were high, making definitive conclusions challenging.

Despite no statistical significance between groups, there were interesting trends. For example, in vocal folds treated with CTAP, there was reduced collagen, increased HA and elastin, relative to untreated vocal folds within the same animal. TAP vocal folds followed the same trend except for a small reduction of HA rather than an increase. One might hypothesize that the interaction of the flaps with the local environment induced these changes because the mere presence of the flap would have been likely to increase collagen amounts, given the perichondrium's collagen content. These findings are both surprising and critically important because an abundant deposition of collagen or reduction of glycosaminoglycans and elastin would create the characteristics of a scarred vocal fold, which is exactly what these experimental approaches are ultimately intended to treat by reducing or eliminating the deposition.

We did not uniformly find obvious visual evidence of the flaps within the lamina propria of the vocal folds into which they were inset. It remains unclear whether the flaps may have retracted out of the vocal fold due to motion of the larynx, whether inadequate vascular supply may have compromised flap survival, or whether the flaps actually began to change their characteristics based upon influence from the recipient bed. The fact that the flap was not consistently seen histologically at 1 month may not necessarily indicate the flap was no longer in place nor that it didn't survive. Given that the dogs were young and healthy with robust healing it is possible that rapid integration of the donor tissue within the recipient bed may have occurred, making the flaps difficult to identify histologically. It is also possible that variable healing due to nonuniformity of the animals could lead to nonuniformity of histologic findings. Furthermore, the flaps are not easily distinguished histologically from the cellular components of the lamina propria because the major cell type in each case is the fibroblast. The CTAP flap might be viewed as an exception to this notion given that it contains fat and the native lamina propria does not; however, as noted elsewhere in the manuscript, the young healthy beagles do not have significant amounts of fat in the preepiglottic space, and it therefore may not be easily seen. Finally, it is unlikely that the flaps did not survive given that these were all class A young healthy dogs, none had wound complications and there was no histologic evidence of tissue necrosis such as giant cell or lymphocytic infiltration. We hope to address these issues in future studies by possibly staining the donor flap with India ink to better show its outlines, making eventual histologic identification easier. We also plan to better secure the test flap in a more stable fashion, helping to reduce confusion about the fate of the donor flap.

There are fascinating questions that arise related to the interaction of the donor flap and recipient bed. For example, mechanical forces have been noted to change the gene expression in fibroblasts subjected to different conditions in a bioreactor, suggesting that the compartment is not static and is subject to alteration by physical forces.⁴¹ One might postulate that the forces of phonation could influence the tissue fate of the flap to appear and

act more like native lamina propria. It may also be that the donor flap influences the recipient bed; for example, adipose-derived stem cells have been noted to exert a paracrine effect in the dermis, suggesting that the fat present in the CTAP could exert an influence on the lamina propria into which it is inset.⁴²

Immunohistochemistry

We sought to evaluate the viability of TAP and CTAP 1 month after inset by staining the lamina propria for markers of vascular (vWF) and cellular proliferation (Ki-67). Flap viability would presumably be demonstrated by higher staining levels for these markers in treated versus untreated vocal folds within an animal. We did not find statistical significance for either TAP or CTAP groups, suggesting questionable viability for either procedure. Higher values for certain animals, however, and the visual evidence of viable flaps within the lamina propria (Fig. 10) seem to point to variable healing rather than uniform flap loss. It may be that influences such as laryngeal movement, flap contraction, or rapid closure of the minithyrotomy around the flap—thereby choking off its vascular supply—could detract from uniform flap survival. One would anticipate thyroid ala perichondrial flaps to survive well, however, because they have been used successfully in relining the endolarynx following extirpation of laryngeal tumors and have been shown to be robust in animal models.^{25,43,44} Technical adaptations such as subepithelial fixation of TAP or CTAP to the arytenoid and a mechanism to reduce the risk of flap constriction at the minithyrotomy site may be useful in future studies.

Technical Observations and Implications

Some observations have informed our understanding of this procedure with implications for the future. The combined endoscopic and open procedure for flap placement is strikingly similar in dogs and humans. Although not necessarily easy, the procedure involves familiar skill sets. Development of the tunnel within the lamina propria requires care not to violate the epithelium because this would compromise easy placement of the donor flap within Reinke's space and would expose it to secretions from the endolarynx, potentially causing infection or even altering its fate. The authors found that development of the lamina propria tunnel was not uniform and that differentiating different layers of the lamina propria was not obvious. The use of instruments borrowed from otologic procedures was helpful, but we believe that more specialized instruments would enhance the precision of this critical element. The authors also found that although placement of the flap into the tunnel was straightforward, assuring that it would stay in place was not. Again, specialized instrumentation, perhaps designed to affix the flap to the arytenoid, would be beneficial.

It is also important to note that there were no canine mortalities in this study, helping to assure the safety of these procedures. Furthermore, no dog needed additional medication for airway compromise or altered diet for postoperative dysphagia. One might have expected dysphagia given the manipulation of the anterior neck structures. There was also concern that preepiglottic space dissection in the CTAP animals might induce significant dysphagia due to dissection near the epiglottis and near the internal branch of the superior laryngeal nerve, which provides sensation to the endolarynx. Dysphagia in the test animals was not observed. No local wound complications were noted.

The authors also noted that endoscopy performed 1 month after the procedure and immediately prior to humane sacrifice did not reveal endolaryngeal trauma such as web formation, hematoma, or superstructural alteration. No superstructural defect was noted in the thyroid ala once the larynx had been excised; one might postulate that removal of the thyroid ala perichondrium could predispose the cartilage to necrosis due to a reduced blood supply. This was not observed and would be consistent with experience in humans during

thyroplasty and open partial laryngectomies. An important observation was made, however, at the minithyrotomy sites, which were all noted to have completely shut. Although one might expect closure of this dead space over the 1-month postoperative period, the authors are concerned that early closure of the minithyrotomy might compromise the vascular supply of either flap. Refinement of this element of the procedure may help to ensure that the vascular supply of the flaps is not choked off by early constriction at the minithyrotomy.

Flap Comparison and Potential

Throughout this study, the small number of test animals has limited our ability to tell a difference between groups treated with TAP and CTAP. However, some trends are notable and may offer insight into future applications. For example, the initial loss of perfusion was less with the CTAP group versus the TAP group, suggesting better perfusion for that flap. If the perfusion is indeed better it helps to alleviate concerns about distal flap perfusion; the distal flap contains the majority of the fat in the CTAP. Rheologically, the CTAP group had lower values (more favorable) than the TAP group for dynamic viscosity, which has been correlated to vocal effort, although the values for G'' , which correlates to required energy for sustaining phonation were the inverse. Intergroup differences, were noted for laryngeal resistance and vocal efficiency but there was no consistent pattern, again making biomechanical conclusions difficult.

Histologically, both groups showed favorable trends overall with increased elastin and decreased collagen. The CTAP group revealed a slight increase in HA, a key component of the lamina propria. Last, although there appeared to be no difference between the groups when measuring vascular proliferation, the cellular proliferation difference between the CTAP group and TAP group approached statistical significance (0.08), suggesting that CTAP may ultimately be more viable.

The modest differences between groups do not prevent us from speculating about potential appropriate uses and pathways. Certainly due to size differences, TAP may be preferable for small defects and CTAP for larger ones. The amount of human preepiglottic space fat is much more abundant than in beagles, allowing for further options for contouring the size and shape of the inset flap. Furthermore, there is recent evidence supporting the presence of adipose-derived mesenchymal stem cells in peripheral fat.⁴⁵ Mesenchymal stem cells have already been shown to have useful properties in the regeneration of damaged vocal folds.⁴⁶ Although it is unknown if the preepiglottic space fat donated to CTAP contains a population of mesenchymal stem cells, certainly the notion of presenting a damaged vocal fold with a fresh population of stem cells is attractive.

Although not seen in this study, one might suspect improved rheologic performance with CTAP, given the rheologic similarities of fat to lamina propria. Also, due to biologic potential of paracrine effects from adipose tissue, one might be inclined to favor the use of CTAP.⁴²

Variations of the CTAP suggest interesting possibilities for laryngeal reconstruction with wide-ranging applications. To wit, if the base of the flap were shifted to an inferior position instead of a lateral position, then simultaneous bilateral CTAPs could be harvested and applied in cases of bilateral pathology (Fig. 11). Furthermore, placement of either unilateral or bilateral flaps through a more laterally positioned minithyrotomy aperture would permit flap inset into the musculature of the paraglottic space. This approach might function well for correction of glottic volumetric defects that are not exclusive to the lamina propria such as paresis or age-related changes or indeed any cause creating a gap at the glottic level. It may also be that the musculature of the paraglottic space has a more robust vascular bed and

might support donor flaps more generously, making this an attractive option for correction of glottic insufficiency in general.

Last, the introduction of a vascularized bed may provide a healthy environment for introduction of stem cells. “Take rates” of injected stem cells are consistently low and, given the relative paucity of cells and vascularity in the lamina propria relative to the paraglottic space musculature, the introduction of an enhanced vascular bed appears attractive. Post hoc alteration of the flaps may also be possible with the introduction of growth factors that have recently made the transition from experimental to human use.⁴⁷

CONCLUSIONS

We present novel application of two autologous soft tissue flaps for glottic reconstruction derived from the anterior larynx. Inset of these flaps into Reinke’s space in canine vocal folds for 1 month appears to have produced no substantial rheologic, biomechanical, or histologic damage to the treated vocal fold when compared with the untreated side, although a treatment effect seems present. Evidence for viability of the flaps was equivocal, suggesting a need for further study and technical modifications. There was no visible untoward local morbidity to the test larynges and the dogs did not have swallowing, airway, or wound complications. Multiple applications can be envisioned for TAP and CTAP both within the lamina propria and within the paraglottic space.

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APPENDIX I: HISTORICAL BACKGROUND

An examination of the history of surgical correction of glottic insufficiency highlights the stepping stones of progress made thus far and the need for further refinements. Whether the approach has targeted the paraglottic space or the vocal fold cover, barriers to the ideal implant have included cost, immune rejection, durability, ease of delivery, malleability of applications and surgical morbidity. Open and endoscopic approaches have competed while variously exchanging the use of alloplastic and autologous substances. The first description of medialization laryngoplasty was by Brünings in 1911 when he reported on the injection of paraffin wax into the paraglottic space of a paralyzed vocal fold.^{A1} Because of the complications arising from the placement of this foreign substance, the technique was eventually abandoned. In 1915, Payr expressed limitations of the paraffin injection technique related to the technical difficulty of performing the procedure, that the size and shape of the injectate was difficult to control, that the injectate might become separated into different deposits and that the result over time might therefore change. He therefore described an open approach for treatment of unilateral vocal fold paralysis where an anteriorly hinged cartilage flap based on the inner perichondrium from the thyroid ala was rotated into the paraglottic space for medialization of both the musculomembranous segment as well as for rotation of the vocal process.^{A2} Payr specifically describes that a tunable effect or “wirkung dosierbaren” was possible as the amount of medial rotation of the implant altered the voice quality since it was performed under local anesthesia and real-time vocal feedback was thus available. Despite his inclusion of a “coffin top” design to prevent the cartilage from pushing out through the thyrotomy window, the final position of the implant was ultimately controlled only by a catgut suture and final results could not be assured. Payr suggested possibly using a free cartilage graft to better affix the rotated cartilage in place although it is unclear if he ever adopted this modification. Nevertheless, the notion of a vascularized, autologous, malleable, low-cost, locally derived implant for correction of glottic insufficiency was born. Pursuing further work with autologous particles in the 1950s and 1960s, Arnold experimented extensively with the injection of cartilage particles and bovine bone dust into the vocal fold and demonstrated minimal tissue reaction with autologous substances but clearly highlighted a limited durability of effect.^{A3,A4}

The advent of the exogenous injectable TeflonTM heralded durable medialization of the vocal fold with reasonable cost, low morbidity and the opportunity for office-based delivery.^{A5} The powerful local tissue reaction with attendant granuloma formation has led largely to its use only under conditions of limited patient lifespan, however.^{A6} Isshiki made a bold stride forward in 1974 by introducing “thyroplasties”, open approaches with alteration of the cartilaginous laryngeal superstructure with or without implants.^{A7} Medialization thyroplasty (thyroplasty type I) has made use of Silastic which is inert and permitted a durable approach.^{A8} Surgical morbidity has been limited, voice results often excellent and the technique has been widely accepted for correction of glottic insufficiency of all varieties.^{A9} Other substances such as titanium, hydroxyl apatite and Gore-TexTM also offer a lasting effect but can be expensive. Alloplastic implants can be subject to extrusion.^{A10,A11}

Autologous paraglottic space expanders have been introduced more recently. Mikaelian, Brandenburg and Koufman introduced the injection of autologous fat into the vocal fold.^{A12–A14} While not subject to immune rejection or limitations of cost, fat has questionable durability and require a separate donor site, increasing surgical morbidity.^{A15,A16} Free fascia has been harvested and injected or placed into the paraglottic space for correction of glottic insufficiency.^{A17,A18} Long term results are often very good.^{A19} These techniques require separate harvest sites however and rely on recipient site vascular supply for maintenance of the transposed tissue.

Donor tissues with more reliable blood supplies have been the subject of much attention, particularly in literature devoted to laryngeal reconstruction often from surgical defects following cancer and airway procedures. Movement of non-native tissues into the endolarynx or paraglottic space was popularized during the growth of partial laryngectomies for cancer. Strap muscles were often placed to simultaneously line the endolarynx and provide a wall against which the contralateral vocal fold could vibrate.^{A20} Composite flaps from the clavicle have been used to reconstruct defects in the trachea.^{A21} Thyroid ala perichondrial flaps have been well described as a vascularized source of coverage of a denuded endolarynx following open partial laryngectomy.^{A22}

The thyroid ala perichondrial flap is likely dependent on small lateral perforating vessels. Delaere has carefully studied perichondrial (“fascia”) flaps and has found the blood supply to be reliable experimentally in rabbits.^{A23,A24} Vascularized rotational flaps of fat and fascia with more substantial volume have been well described for correction of paraglottic space defects following removal of Teflon.^{A25} Free flaps for reconstruction of hemilarynges are well described and have reliable blood supplies but may be too large and difficult to harvest for quotidian use.^{A26} While correction of gross deficiencies of glottic efficiency has been greatly improved, improvements of defects relating to the vocal fold lamina propria have been more modest.

Approaches that *directly* address pathology of the of the lamina propria (e.g. vocal fold scar and sulcus vocalis) are varied and include excision, incision, scar manipulation and implantation of free fat grafts.^{A27–A31} These techniques can offer vocal improvement but to date there has been no development of a vascularized flap that is locally harvested and delivered to the lamina propria. The minithyrotomy technique as described by Gray et al. delineates how a direct approach to the lamina propria can be achieved with a surgical skill set that incorporates elements of standard microlaryngoscopy and thyroplasty.^{A30} While the minithyrotomy is attractive in principle, no appropriate implant has been designed to allow for durable restoration of the lamina propria once access is obtained.

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APPENDIX II: METHODS AND MATERIALS

Human Cadaveric Larynges

After institutional IRB approval, six human fresh frozen cadaveric larynges (three male, three female) with no known history of laryngeal disease were obtained and their demographics noted (Table BI). Prior to use, each larynx was thawed in a warm water bath to room temperature. The hyoid bone and strap muscles were removed and each larynx was mounted onto a laryngeal dissection station (Figure B1).^{B1} TAP and CTAP were thus conceived and the surgical concepts refined. Once these concepts were outlined and the technique developed and performed, the lengths and volumes of the experimental flaps were tested. The techniques were photo documented with an Olympus Camedia C-5050 digital camera (Olympus Imaging America, Inc. Center Valley, CA) at distances between 10 and 30 cm. We used this photo documentation as the basis for the schematic images and artistic sketches included in the manuscript.

TAP Technique

The flap extended along the superior and inferior borders of the thyroid ala and along the contralateral oblique line. The flap's attached base was ipsilateral to the vocal fold into which it was inserted. The outline of the flap was sharply incised. Using a Freer elevator the perichondrium was easily separated from the cartilage past the midline (Figures for TAP and CTAP techniques are provided in the main thesis). A minithyrotomy was performed with a 4 mm cutting burr on an otologic drill. The site of the minithyrotomy has been described previously and was located approximately halfway between the thyroid notch and inferior border of the midline thyroid ala in the infero-superior dimension and just off of midline. With sharp dissection the inner perichondrium of the thyroid ala was incised and a tunnel within the lamina propria developed for placement of the TAP into the vocal fold cover. During the dissection of the tunnel the vocal fold was directly observed by the surgeon to assure that the vocal fold epithelium was not violated. Violation of the epithelium would expose the donor flap to the endolarynx potentially increasing the chance of contamination and/or extrusion.

CTAP Technique

The overall technique was identical to the TAP technique except in the harvest of the flap. The CTAP extended along the inferior border of the thyroid ala past midline, vertically past the top of the superior border of the thyroid cartilage and into the anteromedial pre-epiglottic space (PES). It was based on the hemilarynx ipsilateral to the vocal fold into which it was inserted. The investing fascia of the PES, some PES fat and adjacent perichondrium of the interior of the superior thyroid ala were contiguously elevated with the thyroid ala perichondrium using sharp instruments. Using a Freer elevator and sharp instruments, the CTAP was elevated from the cartilage past the midline to allow for some mobilization of the flap and in anticipation of the ipsilateral minithyrotomy, described above. After the minithyrotomy was drilled and the lamina propria tunnel created, the CTAP was inserted.

Physical characteristics of the flaps

To determine if the flaps were of sufficient length to reconstruct the entire length of the vocal fold, the functional flap length and minimum flap length were measured in mm. The minimal flap length was then subtracted from the functional flap length, leaving supplemental flap length (Table I). If the supplemental flap length was positive, then it was judged to be more than sufficient for lamina propria reconstruction along the entire length of the musculomembranous vocal fold. Measurement of the lengths of the CTAPs was not

performed since the CTAPs possessed additional tissue from the pre-epiglottic space and were therefore always longer than the TAPs.

Next, volume measurements were performed to estimate how much unilateral vocal fold volume replacement might be possible with a TAP. Each TAP was cut off from its base at the site of entry into the minithyrotomy. Then, we used a displacement technique in which the already moist TAP was placed into a graded test tube with saline at a set volume and the volume difference was recorded. The volume difference was judged to be the volume of the TAP (Table I). Measurements of the volumes of the CTAPs were not performed since the CTAPs possessed additional tissue from the pre-epiglottic space and were therefore always larger than the TAPs. TAP volumes were then compared to the calculated volumes of the adult human lamina propria 0.052–0.073 mL (female), and 0.09–0.11 mL (male) (unpublished data- Jack Jiang –personal communication). If the TAP volumes were larger than the calculated volumes then the TAPS were judged to be sufficient for augmentation of Reinke's space.

Canine Experiment Overview

After approval by the Institutional Animal Care and Use Committee, twelve class A female beagles weighing between 8.4–12.6 kilograms (Avg. = 9.6 kgs) and between the ages of 11 months and 23 months of age (Avg.-14 months) were used to evaluate the structural and functional performance of the flaps. The procedural flowchart for the canine studies is presented in Figure B2.

Experimental procedures

First, all 12 dogs underwent a unilateral experimental vocal fold procedure: six underwent TAP and six underwent CTAP. Side and type of procedure were randomized. The dogs received a normal laboratory diet, exercise and social interactions during the duration of the study with the exception of the day or two after surgery when they were fed softer food which is easier to swallow and were exercise restricted. At the start of the procedure each dog's packed cell volume was confirmed to be within normal limits. If confirmed normal, each animal was sedated with an intravenous (IV) injection of Butorphanol (0.2–0.4 mg/kg) and Acepromazine IV (0.02–0.1 mg/kg) through a 20 gauge catheter placed in the cephalic vein. Once sedate, they were induced with IV Propofol (6.6 mg/kg), orally intubated with a 6.5 endotracheal tube (Hudson RCI, Temecula, CA) and maintained under general anesthesia with isoflurane. Each subject was given lactated Ringer's solution IV at 10 mg/kg for the duration of the procedure. The animals were placed in a dorsal recumbent position with both forelegs tied back.

Once an appropriate anesthetic plane was reached, a zero degree operating telescope (Stryker Endoscopy SDC PRO 2) attached to a 3-chip digital camera (Stryker 988) and light source (Q5000 Stryker) (Stryker, Kalamazoo, MI) was used to confirm that the laryngeal anatomy and vocal folds were within normal limits. If the canine vocal folds were scarred (e.g. from a debarking procedure) then the dog was awakened and not subjected to further experimentation under the protocol. If normalcy was established, each test subject was prepared in a sterile way for a ventral cervical approach to the thyroid cartilage. Each subject received Dexamethasone IV (0.07–0.15 mg/kg) and Cefazolin (20–35 mg/kg) IV prior to the start of surgery to prevent post-operative swelling and infection.

A 6 cm horizontal incision at the level of the cricothyroid membrane was made through the skin using a 15 blade scalpel. Dissection of the subcutaneous tissue and underlying fascia superiorly to just above the hyoid bone and inferiorly to just below the cricoid cartilage exposed the sternohyoideus muscles. These muscles were separated in the midline and

retracted laterally along with the skin and subcutaneous tissues. Taking care to preserve and photo document vascular supply to the region, we dissected down to the perichondrium of the thyroid cartilage (Figure B3). Hemostasis was performed as needed using monopolar and bipolar cautery.

Perfusion Measurements

Two measurements of blood flow across the anterior larynx were obtained before proceeding with flap harvest using a laser Doppler LISCA Opto-isolation unit (LISCA PIM II Laser Doppler Perfusion Imager (Lisca Development, Sweden) with, and a Transonic Systems, Inc. Team 21 compatible TS 420 Transit Time Perivascular Flow Meter, (Lisca Development, Linköping, Sweden). The laser Doppler unit was positioned 10 cm above the thyroid ala with a scanning window of sufficient height and width to encompass the space between the hyoid bone and the cricoid cartilage anteriorly (Avg. size 26 mm × 30 mm) (Figure B4). The scans occurred within a five minute time period.

The flaps were elevated using identical landmarks and instruments as those used in the human adult cadaveric larynges. Each flap was rinsed with sterile saline and redraped back into “pre-harvest” position and perfusion measured again in a manner identical to before flap harvest. This measurement occurred between 2–5 minutes after completion of the TAP or CTAP flap. Pre and post-flap changes in perfusion were compared between TAP and CTAP animals using an unpaired t-test. All analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC).

The flap was then moved laterally and a 27 gauge “finder” needle placed through the thyroid ala into the lamina propria of the vocal fold without violating the vocal fold epithelium (Figure B5). The finder needle was placed to correctly identify the appropriate site for creation of the minithyrotomy. Simultaneous inspection of the vocal folds during “finder” needle placement into the lamina propria tunnel and placement of the flaps into that tunnel was performed using the endoscopy system described above; in the human adult cadaveric larynges, vocal fold inspection was achieved with the naked eye as the larynges were excised. A minithyrotomy was performed, a lamina propria tunnel developed and finally the flap was placed into that tunnel. After hemostasis and irrigation, the sternohyoideus muscles were reapproximated with interrupted 4-0 Vicryl suture in the midline. Next the subcutaneous tissues were reapproximated using interrupted 3-0 Vicryl suture. Finally the skin was closed with 3-0 PDS in a subcuticular suture pattern. The subjects received an injection of Buprenorphine (0.005–0.02 mg/kg) IM and the gas anesthesia was concluded. An ice pack was applied over the surgical incision until the subject was extubated.

The dogs received Cephalexin capsules if they were able to swallow 30 mg/kg orally twice a day for 7 days post operatively, or injections of Cefazolin sodium 20–35 mg/kg SQ every 12 hours until able to swallow. If an animal was noted to have difficulty swallowing then a soft diet would be administered. They were also given Buprenorphine 0.005–0.02 mg/kg SQ every 12 hours for post operative pain for 48 hours. The dogs were fed canned dog food and were allowed only restricted exercise for 1–2 days after surgery. The dogs were otherwise left to recover without intervention for one month. They were observed during feedings and exercise for evidence of poor oral intake, airway distress, or death.

One month after flap placement, the dogs were pre-medicated with the same pre-anesthetic cocktail of Butorphanol 0.2–0.4 mg/kg IV and Acepromazine 0.02–0.1 mg/kg IV through a catheter placed in a cephalic vein. They received Propofol as before at 6.6 mg/kg IV. They were maintained under Propofol IV until laryngeal endoscopy was performed to check for endolaryngeal anomalies such as hematoma, stenosis, or web formation. Once the

endoscopy was completed and photo-documentation was attained, the dogs were humanely euthanized with an overdose of Beuthanasia solution 1 ml/10 lbs (to effect) through their IV catheter.

Each dog then underwent a brief post mortem examination prior to laryngeal extraction, Harvested larynges were immediately placed in PBS 1% solution and underwent acoustic and aerodynamic testing. Two experimentally naïve female beagles of similar age and weight also underwent surgical endoscopy, were humanely euthanized and underwent acoustic and aerodynamic testing if their larynges were deemed normal by endoscopy. These two larynges were the control group for the treated dog larynges.

Glottic Aerodynamic and Acoustic Measurements in an Excised Larynx Model

To determine laryngeal resistance, vocal efficiency, jitter, shimmer and HNR ratios for 12 larynges experiments were conducted using an excised larynx apparatus. First, all extraneous soft tissues from the exterior of the trachea/larynx were trimmed off. The aryepiglottic folds and epiglottis were resected down to the petiole. We apposed the arytenoids by passing 3-0 vicryl suture through the body of both arytenoids and back through the vocal processes using a horizontal mattress technique (Figure B6). The false vocal folds were moved away from the glottis by securing them to the thyroid cartilage with a 3-0 vicryl suture. We then performed a cricothyroid approximation in the midline using 3-0 vicryl suture. Verification of glottic closure along the full length was performed by visual inspection from below through the trachea.

The larynx was then placed on top of the airflow outlet and secured in place with ring lock and an external fixator device as described previously (Figure B7).^{B2,B3} The vocal folds were moistened with 2 drops of phosphate-buffered saline (PBS) before every measurement. Humidified room temperature air flow through the larynx was increased until phonation threshold pressure (PTP) was attained. Measurements included mean transglottal DC air flow (L/s) measured by a flowmeter, subglottic pressure (cm H₂O) measured by a manometer, sound intensity (dB SPL) measured by a sound level meter at a measured distance, and acoustic parameters recorded through a microphone onto digital audiotape. Measurements started at PTP and increased in even integers for a total of six measurements.

Laryngeal resistance. was calculated by the following formula:

$$\text{Subglottic pressure/airflow}$$

Subglottic pressure (cmH₂O); Airflow (L/s)

Vocal efficiency was calculated by the following formula:^{B4}

$$4\pi r^2 \times \text{Intensity/Flow} \times \text{Subglottal pressure}$$

r = distance to the sound level meter (cm); Intensity = 10^{dB/10}; Flow (mL/s); Subglottal pressure (KPa)

Jitter L (local) is defined as the average absolute difference between consecutive periods divided by the average period.

Shimmer APQ (11) is the average absolute difference between the amplitude of a period and the average of the amplitudes of it and its ten closest neighbors, divided by the average amplitude.

Harmonics-to-Noise Ratio (HNR) represents the degree of acoustic periodicity and is expressed in dB. For example, if 99% of the energy of the signal is in the periodic part, and 1% is noise, the HNR is $10 \cdot \log_{10}(99/1) = 20$ dB. A HNR of 0 dB means that there is equal energy in the harmonics and in the noise.

The mean latency between removal of each larynx from the animal and termination of the aerodynamic and acoustic testing was 79 minutes with a range of 25–175 minutes.

The acoustic signals were transformed into .wav files using the Computerized Speech Lab software (Kay-Pentax, Lincoln Park, NJ) and analyzed for jitter, shimmer, and noise-to-harmonic ratio using PRAAT software (Amsterdam, Holland). Acoustic and aerodynamic measures were compared between all, TAP, CTAP, and normal animals using an analysis of variance (ANOVA) with pairwise comparisons performed using Fisher's protected least significance difference tests. P-values less than 0.05 were considered as significant. All analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC).

Once the acoustic and aerodynamic studies were completed, the larynges were sagittally transected in the midline. The vocal fold lamina propria from each hemilarynx was then removed. The remaining hemi-larynges were then fixed in 10 % neutral buffered formalin for 1 week and placed in 70 % ETOH for long term storage.

Immediately after aerodynamic and acoustic testing, the lamina propria samples of the 4 larynges (2 TAP and 2 CTAP) destined for rheologic testing were placed in a mixture of 90% PBS (phosphate buffered saline) and 10% DMSO (dimethyl sulfoxide) in an air-tight plastic bag. The bagged tissues were then placed into a container of isopentane pre-chilled with liquid nitrogen for three minutes. Once frozen, the tissues were transferred quickly to a -80°C freezer for storage. They were then shipped via overnight express packaged on dry ice for rheologic testing.

Rheologic testing

To determine the rheologic properties of the treated lamina propria of two TAP and two CTAP treated larynges, the four treated vocal folds and four untreated vocal folds underwent testing using a controlled-strain, linear, simple-shear rheometer system based on the Bose ELF 3200 mechanical testing system (Bose Corporation, ElectroForce Systems Group, Eden Prairie, MN) (Figure B8).^{B5} In brief, each specimen was subjected to a translational linear, simple shear between two parallel, rectangular acrylic tissue plates. A displacement (x) was prescribed to the specimen via the upper plate, and it was measured by a linear variable differential transformer (LVDT). The shear force (F) resulting from the viscoelastic response of the specimen was detected by a piezoelectric force transducer attached to the lower plate. The specimens were tested at a frequency range of 1–250 Hz, covering phonatory frequencies.^{B5} A normal load transducer measured the compressive force between the specimen and the plates. Mechanical testing was performed in a transparent acrylic environmental chamber. A digital camera was mounted directly above the chamber and photos of the specimen mounted between the tissue plates taken. Scaled images of the specimen in the chamber were examined with image analysis software (IMAGE J, National Institutes of Health, Bethesda, MD), in order to determine the area of contact between the specimen and the upper plate.

Data collection was performed on the displacement signal output of the LVDT and the force signal output of the piezoelectric force transducer, digitized at a rate of 8196 samples/s. The digitized signals were processed by the WINTEST software after the experiments, for calculating the amplitudes of the two signals and the phase shift between them. With these data, damping ratio (ζ), dynamic viscosity (η'), the elastic shear modulus (G' and viscous shear modulus (G'') of the specimen were calculated according to the theory of linear viscoelasticity.^{B5}

The dynamic viscosity (η') is proportional to the energy dissipated or lost in the viscoelastic material, typically as heat. It characterizes the mechanical opposition to (shear) flow in the material and is often a monotonically decreasing function of frequency for viscoelastic and polymeric materials. The elastic shear modulus G' is proportional to the elastically stored strain energy (or internal energy) in the viscoelastic material over one cycle of oscillation. It quantifies the elasticity or stiffness of the material in shear. The viscous shear modulus G'' , on the other hand, is defined as the imaginary part of the complex shear modulus G^* , and it quantifies the viscous (energy dissipation) behavior of a viscoelastic material. The damping ratio (ζ) (also called loss tangent or loss factor) can be defined as the tangent of the phase shift delta (phase shift between shear stress and shear strain), which is simply the ratio of the viscous shear modulus to the elastic shear modulus (G''/G').

For each specimen, the slope and the y-intercept of linear regression were calculated by curve-fitting the data with the log rheologic measure plotted against the log frequency. Since slopes were fairly consistent, we focused on differences between y-intercepts derived from regression analysis assuming equal slopes. These y-intercepts were compared within all dogs, TAP dogs, and CTAP dogs using paired t-tests and were compared between TAP and CTAP animals using an unpaired t-test. P-values less than 0.05 were considered as significant. All analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC).

Histology/Immunohistochemistry

We sought to examine histologic changes between treated and untreated vocal folds in the remaining 8 larynges (4 TAP and 4 CTAP). After removal from each animal the larynx was sharply divided in the mid-sagittal plane and the entire vocal fold from the anterior commissure to the vocal process were removed and fixed in 10% neutral buffered formalin for processing and were embedded in paraffin wax. Serial tissue sections, 5 microns in thickness, were obtained through the musculomembranous portion of the vocal fold in the coronal plane and subsequently stained with routine Hematoxylin & Eosin (H&E), and special stains: Alcian Blue pH 2.5 stain kit (American Master*Tech Scientific, Lodi, CA) for glycosaminoglycans, Gomori One-Step Trichrome stain kit (Newcomer Supply, Middleton, WI) for collagen, Verhoeff- Van Gieson Elastic Stain Kit (Newcomer Supply, Middleton, WI) for elastin.

For immunohistochemical staining, 5 micron sections of canine vocal folds as described above were deparaffinized, rehydrated and antigen retrieval performed using heat induced epitope retrieval with 10mM sodium citrate buffer, pH6.0 in a Decloaking Chamber (Biocare Medical, Walnut Creek, CA). Non-specific staining was blocked using 10% bovine serum albumin, followed by 5% non-fat milk. Sections were incubated with primary antibodies [Ki67 (rabbit polyclonal, 1:100 dilution, Abcam), or von Willebrand Factor [vWF] (rabbit polyclonal, 1:500 dilution, Dako)] incubated 1 hour at room temperature. The sections were washed with TBS/Tween and endogenous peroxidase quenched with 3% H₂O₂. Antibodies were detected using MACH 2 goat anti mouse IgG or Rabbit on Rodent (Biocare Medical, Walnut Creek, CA) biotin-free HRP polymer detection systems (15–40

min) and the signal visualized with DAB substrate (Dako). Tissue sections were counter-stained with hematoxylin, dehydrated, cleared and coverslipped for microscopic analysis.

The staining density of each slide was analyzed using Image J software (NIH, Bethesda, MD). Photography of the prepared slides was performed at 4X magnification with a Nikon Eclipse E600 microscope with an Olympus DP70 microscope digital camera supported by DP70-BSW software (DP manager, Version 3.3.1.222 and DP Controller, Version 3.3.1.292, Olympus Corporation). To capture the vocal folds in their entirety at 4X required a range of 2–6 photos per vocal fold, with an average of 3. These individual photos were “sewn” together with no overlap, using the layering and flattening features of Adobe Photoshop CS4 Extended Version 11.0. The vocal fold images were then masked using the lasso function of Adobe Photoshop such that the only remaining histologic image was lamina propria. The fully automated process of k means clustering from the plugins menu of Image J 1.42q was used to calculate the total area of the lamina propria as well as the individual staining densities for the respective stains. For reliability, 10% of the images underwent the same process of “sewing”, masking and Image J analysis and their values compared to the original values to generate an interclass correlation coefficient. The staining densities of tri-chrome, alcian blue, EVG, Ki-67 and vWF of treated and untreated vocal folds were compared on a per-animal basis using a paired student’s t-test. P-values less than 0.05 were considered as significant. All analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC).

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Fig. 1. Sketches of adult human larynges from an anterior view. (A) The outline of the TAP is noted in red dotted lines. (B) The perichondrium of the anterior surface of the thyroid ala is elevated to mobilize the TAP (black arrow). The lateral aspect of the TAP remains attached to the thyroid cartilage and local soft tissues.

Fig. 2.

(A) This sketch depicts an adult human larynx from an anterior view. The TAP (black arrow) is held laterally and a minithyrotomy (white arrow) created with a drill. (B) This schematic depicts an adult human larynx in axial section through the level of the vocal folds. A small instrument is used to inset the TAP (thin black arrow) through the minithyrotomy into the lamina propria (thick black arrow) of the ipsilateral vocal fold.

Fig. 3.

(A) Schematic of development of a lamina propria tunnel through a minithyrotomy (sagittal view). (B) Schematic of placement of a CTAP (thick black arrow) into the lamina propria through a minithyrotomy (axial view). (C) Schematics of three vocal folds in midcoronal section. The first image depicts the three layers of the lamina propria—superficial (small arrow head), intermediate (medium arrow head), and deep (large arrow head). The second image depicts a CTAP (thick black arrow) inset into the superficial layer of lamina propria. The third image depicts a TAP (thin black arrow) inset into the superficial layer of lamina propria.

Fig. 4. (A) This image depicts a human adult cadaveric larynx from an anterior view. The outline of an unharvested CTAP is noted in green ink on the larynx. (B) This image depicts the same human adult cadaveric larynx from a superior view. The CTAP has been mobilized and is being retracted by forceps. The ample supply of preepiglottic space fat is noteworthy (white arrow). (C) This drawing depicts an adult cadaveric larynx from an anterior view. The CTAP (black arrow) has been mobilized and is being retracted by forceps. The preepiglottic space defect is noted (black arrowhead).

Fig. 5. Bar graphs of mean vocal efficiency and mean laryngeal resistance of three groups of canine larynges: TAP-treated, CTAP-treated, and normal. Standard error bars are marked above each mean bar.

Fig. 6. Bar graphs of mean jitter L, shimmer APQ and HNR of three groups of canine larynges: TAP-treated, CTAP-treated, and normal. Standard error bars are marked above each mean bar.

Fig. 7. Plots of rheologic measures of four treated and four untreated vocal folds versus frequency in the physiologic range.

Fig. 8. Histologic staining values for treated and untreated vocal folds. Column 1 on the x-axis displays values of treated vocal folds, and column 2 displays values of untreated vocal folds. Straight lines connect values from the same animal.

Fig. 9.

Immunohistochemical staining values for treated and untreated vocal folds. Column 1 on the x-axis displays values of treated vocal folds, and column 2 displays values of untreated vocal folds. Straight lines connect values from the same animal.

Fig. 10.

Histologic images of canine vocal folds in coronal section stained with EVG (4× magnification). (A) TAP-treated vocal fold. The black arrow points to a viable TAP within the lamina propria. (B) Untreated vocal fold. (C) CTAP-treated vocal fold. The black arrow points to the adipose component of a viable CTAP within the lamina propria. (D) Untreated vocal fold.

Fig. 11. Sketch of a human adult larynx from an anterior view. Bilateral inferiorly based CTAPs are mobilized. The left CTAP is held by forceps.

Fig. B1.

Photo of a Laryngeal Dissection Station with an affixed cadaveric canine larynx. This station was highly useful in the conception and development of the experimental flaps using human cadaveric larynges. The ex-vivo environment allows the surgeon and-or colleague to use both hands for tissue manipulation as well as rotate the specimen for different visual perspectives.

Fig. B2.
Flowchart of canine procedures.

Fig. B3. Intraoperative photo of the anterior view of a surgically exposed canine larynx. A reliable inferolateral vascular pedicle (arrow) was identified at the level of the cricothyroid membrane in all dogs. CT-cricothyroid muscle. T- thyroid cartilage.

Fig. B4.
Schematic of laser Doppler equipment.

Fig. B5.

Endoscopic photo of canine endolarynx with an endotracheal tube in the posterior glottis. A “finder” needle (arrow) has been inserted through the thyroid cartilage into the vocal fold lamina propria with care taken not to violate the vocal fold epithelium. The placement of this needle helps to identify the appropriate site on the thyroid ala surface for creation of the minithyrotomy.

Fig. B6.

Photo of a cadaveric canine larynx from a superior view. The larynx has undergone the placement of a mattress suture through the arytenoids to close the glottis (arrow). This suture is essential in securing the closure of the glottis during aerodynamic and acoustic testing.

Fig. B7. Schematic of the excised larynx apparatus. Components are indicated by small arrows. DAT-digital audiotape.

Fig. B8. Schematic of simple-shear rheometer. LVDT- linear variable differential transformer. F- shear force.

TABLE I

Means of Within-Animal Differences of Staining Densities between Treated and Untreated Vocal Folds by Group.

Mean Values	TAP	CTAP
Trichrome (SD) %	−0.2 (4.7)	−1.5 (7.8)
EVG (SD) %	0.40 (2.3)	2.3 (3.1)
Alcian Blue (SD) %	−4.0 (21.7)	2.2 (16.5)

SD = standard deviation; TAP = thyroid ala perichondrium flap; CTAP = composite thyroid ala perichondrium flap.

TABLE II

The Means of Within-Animal Differences of Staining Densities between Treated and Untreated Vocal Folds by Group.

Mean Values	TAP	CTAP
Ki-67 (SD) %	-83.7 (17.1)	-1.1 (18.4)
vWF (SD) %	0.05 (0.4)	-0.07 (0.64)

SD = standard deviation; vWF = von Willebrand Factor; TAP = thyroid ala perichondrium flap; CTAP = composite thyroid ala perichondrium flap.

TABLE B1

Larynx number	Functional flap length (mm)	Minimum flap length (mm)	Supplemental flap length (mm)	TAP 3-D volume (mL)	Sex	Age (yrs)
1	26	19	7	0.20	F	unknown
2	27	17	10	0.20	F	78
3	33	17	16	0.12	F	70
Mean	29	18	11	0.17		74
4	39	21	18	0.30	M	89
5	33	23	10	0.30	M	33
6	33	17	16	0.18	M	72
Mean	35	20	11	0.26		65
Mean (all)	32	19	13	0.22		68

Physical characteristics of adult human cadaveric larynges and their experimental flaps. TAP- thyroid ala perichondrium flap. CTAP- composite thyroid ala perichondrium flap.