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Vocal Fold Cover Replacement: Imaging Analysis Of Intermediate And Long-Term Outcomes

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Abstract

VOCAL FOLD COVER REPLACEMENT: IMAGING ANALYSIS OF INTERMEDIATE AND LONG-TERM OUTCOMES

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The voice is an important source of communication. Voice disorders, dysphonia, has an estimated 30 percent lifetime prevalence in the United States with a significant economic and social cost. Severe vocal fold scarring can lead to persistent dysphonia, has no optimal treatment and negatively impacts those affected by impairing their quality of life and general health. We propose a novel approach to treating sever vocal fold scarring by replacing the entirety of the vocal fold mucosa with a tissue engineered implant, the cell-based outer vocal fold replacement (COVR). We hypothesized that intermediate and long-term implantation of human stem-cell derived COVR implants would lead to regeneration of vocal fold epithelium and lamina propria and establish an extracellular matrix (ECM) approximating normal parameters. We aimed to assess the development of these structures by assessing active factors in wound healing including white blood cell presence, collagen and fibrin deposition, and glycosaminoglycan incidence. We resected the vocal fold mucosa and implanted COVR gels, COVR gel components including a decellularized gel scaffold (DECELL) and human-derived adipose stem-cell injection (hASC Injection), or performed no treatment following intervention (SCAR) in 49 New

Zealand white rabbits with intermediate, 6-weeks, postoperative recovery and three Yucatan Mini Pigs with long-term, 6 months, post-operative recovery prior to laryngeal harvest. Vocal folds were sectioned and assessed through light microscopy, fluorescent microscopy, and/or nonlinear scanning laser microscopy (NLSM) to assess outcomes of the intervention. Analysis of light microscopy of the intermediate implantation groups showed that the COVR implantation had the lowest levels of collagen deposition (50% in COVR, 67% in DECELL, 82% in hASC Injection, and 83% in SCAR), greatest glycosaminoglycan incidence (50% in COVR, 8% in DECELL, 9% in HASC injection, and 25% in SCAR), equivalent white cell presence (50% in COVR, 83% in DECELL, 45% in hASC Injection, and 42% in SCAR) at laryngeal harvest. Additionally, fluorescent microscopy showed persistence of HLA-staining cells in the COVR and hASC Injection groups and none in the DECELL or SCAR treatment groups. NLSM imaging of the long-term implantation of COVR showed increased collagen deposition in the superficial lamina propria in the hASC Injection group, while the SCAR had no change in collagen levels, and the COVR implant animal had decreased collagen levels compared to untreated control folds. The superficial lamina propria also had increased elastin deposition in the COVR group, decreased levels in the SCAR animal, and no change in the hASC Injection animal. In the rabbits, COVR implantation promotes improved healing and decreased scarring at six weeks, as evidenced by increased glycosaminoglycan deposition and decreased collagen development. Additionally, there is evidence of xenograph ASC grafts persist for several weeks with continued immunomodulatory effects. Our data suggest decreases of overall elastin levels are a more sensitive marker of fibrosis in NLSM imaging than increases in collagen levels.

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INTRODUCTION:

The voice is an important source of communicating between people. It is vitally important for many professions in addition for typical socialization through verbal communication. Voice disorders, or dysphonia, annually affects an estimated 1.7 percent of population in the United States, over 5.5 million people, and has an estimated 30 percent lifetime prevalence.[1,2] There is also a substantial cost to diagnosing, managing, and treating patients with dysphonia, estimated at 12 billion dollars annually.[3] The diagnosis for a majority of these cases is acute laryngitis, however a significant portion of dysphonia etiologies arise from benign vocal fold lesions (ie. polyps, nodules) and malignant neoplasms.[1] Patients who have damaged vocal fold tissue, either through benign lesions, trauma, or surgical resection, can develop persistent dysphonia due to vocal fold scarring. Severe scarring leaves those afflicted with impaired phonation including chronically hoarse voice, vocal fatigue, and risks or ends professional careers.[4]

Dysphonia has been described by patients as impairing their quality of life, impacting their work performance and employment, and has also been associated with depression.[5-9] Voice disorders have also been found to profoundly affect general health impacting the mental, global and social health independent of and with greater association than other life-threatening comorbid pathologies including malignancy, pulmonary, cardiac disease.[10] Thus it has been suggested that the effects of dysphonia and subsequently the allocated budgets for treatment is lacking. To make matters worse, treatment options for dysphonia are imperfect. In the case of severe vocal fold scarring, there is no optimal treatment.

Physiology of the Vocal Fold

To understand the difficulty of finding a treatment for severe vocal fold scarring, it is important to understand the physiology of the vocal folds. The vocal folds are paired symmetric structures within the larynx. Each vocal fold is made of three-layers: the non-keratinized stratified squamous epithelium, lamina propria, and muscle. In the human vocal fold, the lamina propria is subdivided into three distinct layers based on the amounts of collagen and elastic fibers in each layer.[11,12] The lamina propia layers are the superficial, intermediate, and deep lamina propria. The superficial lamina propria has a higher proportion of elastin and a narrow band of collagen. Whereas the intermediate and deep layers of lamina propria together form the 'vocal ligament' or 'elastic conus,' which has densely organized collagen.[12] Other mammal vocal folds including the rabbit, pig, and canine, common animal models used for vocal fold research, have a two-layer lamina propria, the superficial and deep layers only.[13,14] The vocal fold layers in cross-section can be found in **Figure 1.**[15]

'Body-cover' theory – 'Body' explained

The landmark 'body-cover' theory, originally proposed by Hirano et al. in 1974, describes a functional distinction of the vocal fold based on the interactions between these two structures. In his theory, Hirano describes the 'body' consisting of the vocal conus and underlying musculature, primarily the thyroarytenoid muscle, which work together as a functional unit. The shape and structure of the 'body' is further influenced by the vocalis and cricothyroid muscles, external to the vocal fold proper. This in turn, alters the mechanical properties of the vocal folds including position, shape, and tension, ultimately affecting the quality of phonations produced.

'Body-cover' theory – 'Cover' explained

The 'cover' is the mucous membrane which sits on the 'body' and is comprised of both the superficial lamina propria and overlying squamous epithelium. This 'cover' is the vibratory layer of the vocal fold. The superficial lamina propria is particularly important for its role in vibration. The biomechanics of vibration rely on the superficial lamina propria's extracellular matrix (ECM) protein composition, the concentrations of proteoglycans and glycosaminoglycans and their relative densities, to maintain the pliability and elasticity necessary for phonation.[16] Of these, the glycosaminoglycan, hyaluronic acid, has been identified as having the greatest influence on maintaining the viscoelasticity properties necessary for normal phonation.[16-18]

The biomechanics involved in the production of phonation underly the importance of appropriate structure and pliability of the vocal folds. The cover portion of the vocal fold vibrates over the body in a mucosal wave motion to create phonation.[19] During normal phonation, the vocal folds will oscillate and collide in an incredible range of 20Hz to 3kHz, with an average fundamental frequency of an adult male around 100 Hz and an adult female at 200 Hz.[20,21] Unlike other specialized stratified squamous epithelium, the vocal cords withstand cyclical tensile, shear, and impact tissue stress requiring continual repair of the epithelium while maintaining its viscoelastic properties.[22] An in depth discussion on the mechanism of phonation including acoustics and biomechanics of vocal fold oscillations is beyond the scope of this project. However, it should be noted that finding an optimal treatment for vocal fold scarring requires a robust replacement able to withstand the stresses of normal vibratory function.

Pathophysiology of Vocal Fold Scarring

Despite the vocal folds impressive ability to heal, both normal and impaired healing can lead to fibrosis of the vocal folds. While incidence and prevalence statistics of vocal fold scarring is still lacking in the literature, vocal fold scarring and resultant dysphonia has been studied for nearly 30 years.[23] The pathophysiology of vocal fold scarring has been illuminated in this time.

Causes of vocal fold scarring can be divided between two sources of damage: trauma (e.g. phonotrauma from overuse, intubation, iatrogenic during surgery) or inflammation (e.g. laryngitis, exposure to environmental irritants, laryngopharyngeal reflux).[24-30] In particular, vocal fold damage and scar formation due to squamous cell carcinoma of the vocal fold and iatrogenic damage to the vocal fold during laryngeal cancer resection can lead to significant scar formation.[31]

The microstructure of the vocal fold scar tissue has been of particular interest in scar formation and treatment options. Histologic analysis of scarred folds has demonstrated fibrotic changes in the extracellular matrix (ECM) particularly evident in the lamina propria. These include deposition of excessive and disorganized collagen, loss of elastin fibers, and changes in hyaluronic acid, fibronectin, and procollagen densities.[25,32-34]. Collagen deposition and loss of elastin affects the mechanical properties of the vocal fold, such that the cover layer stiffens and is unable to form a mucosal wave impairing optimal vibration. Additionally, severe scar formation can undergo a cicatricial process which leads to loss of vocal fold bulk in the affected fold that then causes glottal insufficiency, such that the two folds are not able to collide.[35,36] Together, these changes to the vocal fold ECM manifest into difficulty phonating properly and appear clinically as dysphonia.

Current Literature for Scar Treatment

Together with the longstanding interest in the pathophysiology of vocal fold scars, treatment options have been studied. Non-surgical interventions to improve dysphonia include voice therapy. As a treatment it has had questionable success for non-organic vocal dysphonia, such as with phonotrauma, but lacks evidence of efficacy for severe vocal fold scarring.[37-39]

Surgical intervention has instead become the treatment regimen of choice for severe scar tissue. Phonosurgery, microsurgery of the vocal folds to improve vocal qualities, was the initial surgical option for vocal fold scarring and has continued to improve with technological advancements, yet has inconsistent, often suboptimal results.[37,40] Additionally, surgical interventions have extended recovery periods, up to one year, in order to achieve maximum effect with ongoing voice rest and rehabilitation needed during recuperation.[38,41] The requirements of recuperating from cold steel surgical interventions with unpredictable, unsatisfactory results following scar resection has created fertile ground of research in laryngology to find a more suitable treatment option.

Treatments involving injectable therapies to bolster results of surgery have been studied concurrently with direct surgical intervention. These treatments have many benefits in common, including the ability to provide in-office injections, ease of use, cost-effectiveness, and availability of the injectable substances.[42] Steroid injections directly into the scarred vocal fold is a common first-line treatment for vocal fold scars. Steroids are thought to control the inflammatory response by modifying cytokine secretion, but there is limited evidence that they are an effective treatment for severe vocal fold scarring.[43-45] Furthermore, inflammation and wound healing can cause contracture of the vocal fold, as mentioned previously, which can be worsened by the steroid's atrophic

effects on the vocal folds.[45] Research into injectables materials that ameliorate these side effects has steadily continued. Biologic materials including fascia, autologous fat, and collagen, commonly used for augmentation and medialization of the vocal fold, have been attempted to improve vocal scarring with minimal desired effects.[46-49] As such, while steroid injections and biologic material injections currently make up the common treatment for vocal fold scarring, they leave much to be desired in terms of outcomes.

Bioactive chemicals found in the normal ECM of the vocal cord mucosa have also been studied extensively as a treatment for vocal fold scarring. The glycosaminoglycan, hyaluronic acid (HA), was initially found to be important for scarless wound healing in fetuses, and given its active properties functionally and biologically has become an active area of research and treatment modality.[50-52] HA within the vocal fold minimizes the mechanical stress of the folds during oscillations, acting as a shock absorber and tissue damper.[53,54] HA also has immunomodulating properties important for cell migration and wound healing.[50] HA has been used to prevent vocal scar formation following injury.[55-57] Finck et al. 2010 reported on long-term outcomes of esterified HA gels implanted following surgical resection of scar, with improvements in vocal fold videostroboscopy assessments, including vibration amplitude, mucosal wave symmetry, glottic gap, and voice quality assessments.[58] Provided its perceived and documented benefits, future therapies that incorporate HA as a component are highly likely.

The development and growth of regenerative medicine–constructing tissue to replace or repair existing tissue–has led to the creation of sophisticated materials that can be used to repair scarred vocal folds and regenerate the ECM microstructure. Synthetic polymer scaffolds, cell transplantation, growth factor injections, and other state-of-the-art

biomaterials have created additional opportunities to find a solution for regenerating the larynx with varying success. [59-63] These studies are too numerous to include here, however cell therapies within the folds have included pluripotent stem cells, mesenchymal stem cells, and fibroblasts. Pre-clinical *in vitro* animal studies have shown that adipose-derived mesenchymal stem cells have multiple applications within the larynx, including becoming cartilage-like grafts, developing into lamina propria, epithelium, and ECM.[64-66]

Cell and biologic therapies have shown the greatest promise, with some therapies advancing to human clinical trials. Hirano et al. 2017 injected hepatocyte growth factor into the vocal folds for four weeks with assessment at six months post-procedure. They found treatment to be safe and effective, with significant improvement in objective (maximum phonation time, vocal fold amplitude) as well as subjective (voice handicap index-10 [VHI], voice grading and expert voice quality assessment) findings.[67] Ma et al. 2019 injected autologous cultured fibroblasts in patients with vocal fold scarring and age-related atrophy, these patients then received graded assessments at four, eight, and twelve months. Treatment groups had significantly improved mucosal waves at four and eight months, and improved vocal quality, assessed by professional voice raters, at twelve months.[68] Hertegård et al. 2020, injected bone-marrow derived autologous mesenchymal stem cells following scar resection. This also showed safety and efficacy improving vibration and elasticity at one year, along with improvement in subjective patient surveys.[69] These studies have focused on the introduction of a single component to improve the vocal fold repair through immunomodulation of the healing process through either direct introduction to scar tissue or following scar tissue resection.

Introduction to COVR

This laboratory instead proposes a novel approach of replacing the entire vocal fold mucosa. To this end, we developed the cell-based outer vocal fold replacement (COVR) implant, a tissue-engineered treatment designed to be implanted directly following removal of native scarred vocal folds.[70] The COVR implant is created from a multipotent adipose-derived mesenchymal stem cell tissue embedded within a three-dimensional fibrin gel scaffold. Previous work by this laboratory has shown the epithelization by adipose-derived stem cells to create a bi-layered structure similar to the epithelium and lamina propia.[64] Additionally, this structure was found to exhibit biomechanical and functional vibration mechanics suitable for vocal fold structure replacement.[65]

Long et al. 2018 showed initial success of COVR implantation within an *in vivo* rabbit study.[70] This pilot study involved unilateral cordectomy of sixteen rabbits within three treatment arms: fibrin glue alone, autologous mucosal replants of vocal fold tissue and COVR implants. Four weeks after surgery the rabbits were euthanized, and larynges harvested. The COVR treatment group achieved excellent graft implantation, regeneration of vocal fold epithelium and lamina propria, and induced phonation as determined histologically and by mucosal wave symmetry, respectively. Importantly, at this timepoint the COVR ECM structure remained immature with impaired vibration compared to the mucosal transplant treatment group.

The structure of the ECM, particularly changes to the fibrin and collagen deposition within the lamina propria, have been found to reflect the degree of damage and healing of the vocal folds. Previous COVR implant larynges were harvested at four weeks and had immature ECM with mature elastic fibers not identified within the implant

sites and diminished mucosal-polysaccharide staining when compared to mucosal replants. It is possible that additional post-operative recovery time would allow for ECM maturity and therefore regenerate a more normal composition.

Statement of Purpose and Specific Aims

The goal of this project was to identify the intermediate and long-term outcomes of human-derived stem cell COVR implants within animal models. We included a large animal model with a COVR implant developed using human stem cells and long-term follow up to create realistic conditions of the COVR implant as a future human therapeutic.

We hypothesized that both intermediate and long-term implantation of human cell based COVR will also lead to regeneration of the vocal fold epithelium and lamina propria and establish an ECM structure that approximates normal ECM structure.

AIM 1:

The assessment of the development of the epithelium, lamina propria, and the ECM following intermediate-term implantation of COVR in vocal folds through histology. We determined intermediate effects in COVR implants compared to individual components of the COVR implant by assessing active factors in wound healing, such as white blood cell presence, collagen and fibrin deposition, and glycosaminoglycan incidence.

AIM 2:

To develop a large, voicing animal model for the assessment of longer term COVR implantation to ultimately determine the effect on voice quality following COVR implantation.

METHODS

All animal activities were done in accordance with the Institutional Animal Care Committee, following USDA guidelines for animal ethics and under veterinarian supervision. The protocols of the studies were submitted to and approved by the UCLA Animal Research Committee IRB.

COVR development

Human adipose-derived stem cells (hASC) were used to construct the COVR implants. For development of rabbit COVR gels, rabbit fibrinogen was used, and pig fibrinogen was used for pig COVR gels. Otherwise, production of COVR gel implants were equivalent for both groups. Size of the implant was adjusted for the two species, with rabbits receiving one-half a gel per vocal fold, and pigs receiving a whole gel.

As described previously, COVR development used animal-specific fibrinogen, bovine thrombin, and hASC in suspension were mixed in a 4:1:1 ratio.[70,71] Final hASC content was of $6x10^5$ cells/gel. The mixture was placed within a 12 mm Corning Transwell culture insert to create fibrin gels embedded with ASC. Total volume was 900 ul per gel. Following gelation, additional hASC were pipetted onto the surface to emulate the epithelial layer. The insert conserved the air interface on the hASC covered surface of the gel intended to become the epithelial layer and media diffused through the remainder of the gel. The COVR gels were incubated and maintained with culture medium containing 10% fetal bovine serum and 10 ng/mL epidermal growth factor for 2 weeks. Media was replaced every 2-3 days.

Decellularized Gel (DECELL) development

DECELL production was identical to initial COVR development. Following the 2 weeks of incubation, the COVR gel was placed in sterile Sodium Dodecyl Sulfate (SDS)

1% solution on a shaker for 3 days, with daily solution changes. DECELL gels were then washed for 24 hours with sterile saline prior to implantation.

Rabbit Laryngeal Surgery and COVR Implantation

49 New Zealand white rabbits, weighing 3-3.5 kg, underwent survival surgeries as described previously.[70-72] Anesthetization was achieved with combined mask and chemical induction and maintenance on isoflurane. In brief, a vertical midline neck incision was made to expose the larynx and trachea. A lower tracheostomy was performed, and pediatric endotracheal tube was placed to maintain intra-operative anesthesia. A laryngofissure through the midline thyroid cartilage exposed the vocal folds. A cordectomy meeting the definition of a European Laryngological Society type II was then conducted.[73] The membranous cover layer of the vocal fold was resected bilaterally by sharp dissection, with reflection of the epithelium and lamina propria to superior and inferior edges of the vocal fold confirmed with exposure of the thyroarytenoid muscle. Surgery was performed by the same surgeon in all cases. Intraoperatively, the COVR implant was divided in half, with one half of the gel placed on either vocal fold for the rabbit implantations, while maintaining appropriate directionality of the developed COVR epithelial layer.

Immediately following surgical resection, rabbits received one of four treatments: 11 rabbits received hASC Injection directly into the remaining vocal fold structure, receiving approximately 600,000 cells per side. 13 rabbits received COVR implants, which were placed superficially over the thyroarytenoid muscle with the bulk of the gel overlying the medial vocal fold. The COVR implant was then secured with sutures at the corners of the gel. 12 rabbits received DECELL implants which were placed similarly. 12 rabbits in the SCAR treatment group had removal of the mucosal membrane only with

cupped forceps and served as injured controls. Care to achieve appropriate hemostasis was taken. Following the intervention, the rabbits received corticosteroid, proton pump inhibitor, and subcutaneous pain medication for 48 hours in addition to a 10-day antibiotic course. At 6 weeks the rabbits were euthanized, and larynges were harvested. Two of the animals died prematurely. One COVR group animal died 2 days after implantation. At autopsy, food was found at the laryngeal inlet, and cause of death was attributed to aspiration and asphyxiation. One animal in the SCAR group died immediately post-operatively and was attributed to ketamine reaction. Further animals did not receive this medication.

Pig Laryngeal Surgery and COVR implantation

Three Yucatan Mini pigs, aged 12-18 weeks and weighing 13-26 kg, were anesthetized via combined masked and chemical induction with intraoperative maintenance of isoflurane. The preferred transoral endoscopic approach for intervention under direct visualization was attempted initially. Both the SCAR and hASC Injection treatments were successfully completed via direct endoscopy. However, the anatomy of the pig's airway, particularly the pig's deep larynx and narrow pharynx, made it difficult to perform the fine physical maneuvers required for optimal positioning of COVR implantation. The COVR implantation was converted to an open transcervical approach as described in the rabbit surgery above. Surgical interventions of the vocal fold mucosa were unilateral with a single, unaltered COVR gel implanted to the vocal fold. The remaining vocal fold was unaltered and served as control vocal fold.

Following surgical dissection, the three pigs were each placed into a treatment group. One pig received hASC Injection of approximately 600,000 cells in the injured vocal fold, to provide equivalent cell number to the COVR. One pig received no

treatment and served as a SCAR control. The last pig received the COVR implant placed superficially with four sutures at the corners of the COVR implant to secure it in position. At 6 months, terminal surgery included intraoperative *in vivo* phonation recorded via high-speed camera. Pigs were subsequently euthanized, and larynges were harvested.

The COVR pig developed rapid neurologic decline and death at 6 weeks post-op. On autopsy, the cause of death was determined to be an endemic porcine infection, most likely a strain of Streptococcus suis. The pig had been immunosuppressed which was thought to be a factor in its death. Because of the circumstances, in vivo phonation was not available for this animal.

Microscopy

All rabbit larynges were formalin-fixed, paraffin-embedded, and sectioned. Histological sections of the bilateral vocal folds were stained with hematoxylin and eosin (H&E), MOVAT pentachrome (MOVAT), Anti-Human Leukocyte Antigen (HLA), Anti-Bovine Serum Albumin (BSA), and 4′,6-diamidino-2-phenylindole (DAPI). MOVAT stain is commonly used for connective tissue staining and includes a mix of five stains: alcian blue, Verhoeff hematoxylin, crocein scarlet combined with acidic fuchsine and saffron.

Light Microscopy

The H&E and MOVAT pentachrome sections were evaluated by light microscope to assess the presence of collagen, glycosaminoglycans (GAG), fat, fibrin, infiltrate, and irregular epithelium. Infiltrate was described as none, present, or infiltrate plus dependent on the amount of white blood cells found within the section. Secondary review of select slides were reviewed by a blinded head and neck pathologist for histological

confirmation. The COVR group rabbit that died prematurely following the implantation was not included in the results or analysis.

Fluorescent microscopy

Rabbits were stained with DAPI fluorescent dye, used to visualize nuclei and additionally dyed with either HLA or BSA. The hASCs were confirmed to stain and fluoresce with HLA. As the COVR and hASC Injection treatments both used human derived stem cells, HLA fluorescence was used to determine survivability of the hASCs. BSA served as a negative control.

Nonlinear scanning laser microscopy (NSLM)

NLSM imaging can capture both second harmonic generation (SHG) and two-photon fluorescence (TPF). SHG imaging captures images based on the scattering of photons that are collected, while TPF uses fluorescent emissions for image detection and capture.[74] NLSM has been previously used in pig vocal folds to visualize, quantify, and define orientation of both Type I and Type III collagen, as well as elastin.[75,76] For NSLM imaging unstained, paraffin fixed slides of pig vocal fold tissue were created from both the treated and untreated cords of each pig. NSLM imaging was performed on a custom Leica Sp8-MP-DIVE confocal microscope system with imaging of both SHG and TPF image detection and capture recorded simultaneously. SHG was used to visualize collagen and TPF to visualize elastin. Images were collected using a 20x dry lens, with 10 percent overlap of images with live imaging averages during acquisition. SHG was captured at 400-420nm and TPF was captures at 497-567 nm wavelengths. Microscope imaging and capture details, including power of SHG and TPF lasers, line and frame averages, and acquisition depth were based on trial and error. The hASC Injection and SCAR treated pig vocal folds were imaged to include all scannable section from the

epithelium to just beneath the transition of the musculature, capturing the entirety of the vocal fold cover layer. These pig cover layers areas of interest were confirmed with H&E and MOVAT pentachrome stained sections. The COVR treated pig vocal fold sections were imaged in their entirety.

We performed a modified approach to the imaging and data analysis initially described in in Miri et al. 2012.[75] Specimen imaging of the mucosa layer included all epithelium and lamina propria layers up to and including the interface of the thyroarytenoid musculature. Image analysis was completed using Image J software. The images were processed through Image J using the preinstalled automated image thresholding and then processed by the automated percent area fraction programs and outputs were recorded. Superficial and deep LP layers were distinguished based on anatomical relationships to epithelial and musculature borders, respectively, as described in the literature.[12,77] Vocal fold images that were either purely musculature or between confirmed superficial and deep lamina propia layers were labeled indeterminant. Raw images were processed manually to include regions of interest in the superficial and deep lamina propria to exclude any portion of epithelium or musculature within the captured images. A representative image of the SHG and TPF NLSM captured images and the Image J processed regions of interest can be found in **Figure 2.**

Collagen and elastin levels, as reflected by the percent area fraction, in both the superficial lamina propia (SLP) and deep lamina propria (DLP) were recorded. A collagen-to-elastin ratio, reflecting percent area fraction collagen divided by percent area elastin for each image was also collected. Direct intra-animal comparison between treated and untreated vocal fold of each pig was analyzed. Representative images of the H&E,

MOVAT pentachrome, and NLSM imaging of collagen and elastin in the SLP and DLP layers can be found in **Figure 3.**

RESULTS

Aim 1 Results:

Light microscopy representative images of rabbit vocal fold sections stained with H&E and MOVAT pentachrome can be found in **Figure 4.** Representative fluorescent microscopy images of rabbit vocal folds stained with HLA + DAPI and BSA + DAPI can be found in **Figure 5.**

COVR (n=12):

Collagen was present in 50 percent of the sections and was generally described as either thin or wavy and rarely thick and bundled. Appreciable fibrin was present in only 17 percent of vocal folds, was found deep to the epithelium, was small, with well-defined borders, and slightly eosinophilic. GAGs were found in 50 percent of the sections and was present throughout the vocal fold sections. Fat was present in 25 percent sections. Infiltrate was found in 50 percent of the sections, all infiltrates were described as present with no infiltrate plus noted. Irregular epithelium was noted on 25 percent of sections. All twelve sections of the COVR group rabbits stained positive for HLA and negative for BSA.

DECELL (n=12):

Collagen was present in 67 percent and was described as wavy, aligned, and occasionally thick and bundled. Fibrin was present in 67 percent of the sections. The fibrin within the DECELL sections were large, stained darkly eosinophilic, had clearly defined edges, and were located at or near remnant sutures. In only one section, 8 percent, had GAG stain positive within the vocal folds. Fat was not present in any

laryngeal sections. Infiltrate was present in 83 percent of sections with two sections, 17 percent, described as infiltrate plus. Irregular epithelium was noted in 25 percent of sections. No sections of the DECELL group stained positive for either HLA or BSA.

hASC Injection (n=11):

Collagen was present in 82 percent of sections and was primarily described as deep, dense, and bundled. Fibrin was present in 18 percent of sections and described as amorphous and slightly eosinophilic. In only one section, 9 percent, stained positively for GAGs. Fat was present in 55 percent of sections. Infiltrate was present in 45 percent of all sections, with 27 percent described as infiltrate plus and the remaining 18 percent as infiltrate present. Irregular epithelium was noted in one section, 9 percent. All twelve hASC Injection sections stained positive for HLA, no section stained positive for BSA.

SCAR (n=12):

Collagen was present in 83 percent of the sections and was primarily described as heavy and disorganized. Fibrin was present in one section, 8 percent, and described as amorphous and slightly eosinophilic. GAGs were stained positive in 25 percent of the sections. Fat found in 42 percent of sections. Infiltrate was noted in 42 percent, with no sections described as infiltrate plus. Irregular epithelium in was found in 17 percent of sections. No sections of the SCAR group stained positive for either HLA or BSA.

Aim 2 Results:

Nonlinear scanning laser microscopy (NLSM) values of percent area fraction of collagen and elastin, and the calculated collagen-to-elastin ratio of each Pig vocal folds in the three treatment arms, hASC Injection, SCAR, and COVR, were compared directly.

hASC Injection treatment pig:

hASC Injection treated vocal folds (hASC) yielded 38 total images which were categorized as superficial lamina propia (SLP) (n=12), deep lamina propria (DLP) (n=6),

and indeterminant lamina propria (n=20). The control vocal fold (CTL) yielded 42 total images: 6 SLP images, 6 DLP images, and 30 indeterminant images. Percent area fraction findings of both elastin and collagen, and collagen-to-elastin ratio data found in **Figure 6.**

SLP and DLP percent area fractions of both the CTL and hASC treated vocal folds were compared. The CTL fold had significantly greater collagen area fraction in the DLP than in SLP (10.1±3.6 and 5.0±2.5%, $p<0.01$ two-way ANOVA). The collagen-to-elastin ratio was significantly higher in the DLP than the SLP (0.40±0.07 and 0.21 ±0.31, $p<0.01$ two-way ANOVA). The hASC vocal folds had significantly increased collagen area fraction in the SLP than DLP (27.1±6.4% and 14.6 ±4.6%, $P<0.001$ two-way ANOVA). The hASC Injection collagen-to-elastin ratio was significantly greater in the SLP than in the DLP (1.2±0.3% and 0.2±0.08%, $p<0.001$ two-way ANOVA). Elastin was not significantly different between the SLP and DLP in either the CTL or hASC vocal folds.

Comparisons between the CTL and hASC treated vocal fold SLP and DLP were analyzed. In the SLP, collagen percent area fraction was significantly greater in the hASC group than in the CTL fold (27.1±6.4% and 5.0±2.5%, $p<0.001$ two-way ANOVA). Additionally, the SLP collagen-to-elastin ratio was greater in the hASC Injection fold than in the CTL fold (1.2±0.3% and 0.2±0.08%, $p<0.001$ two-way ANOVA). There was no significant difference in the elastin percent area fraction in SLP. Within the DLP, the hASC Injection treated vocal fold had a higher collagen percent area fraction than the CTL fold, that was approaching but did not reach significance ($p = 0.07$, two-way ANOVA). However, the collagen-to-elastin ration in the hASC was greater significantly greater than the CTL fold DLP (0.5±0.1 and 0.04±0.07%, $p<0.05$ two-way ANOVA).

SCAR treatment pig:

Scarred vocal folds (SCAR) imaging yielded 54 total images, 18 SLP, 20 DLP, and 16 indeterminant images. CTL yielded 58 total images, 18 SLP, 13 DLP, and 27 indeterminant images. Percent area fraction findings of both elastin and collagen, and collagen-to-elastin ratio data can be found in **Figure 7.**

Within the CTL vocal fold, the SLP had significantly increased collagen percent area fraction (12.9±4.6% and 4.5±2.2%, $p<.001$ two-way ANOVA) and elastin percent area fraction (31.0±9.2% and 16.1±4.2%, $p<0.001$ two-way ANOVA) compared to the DLP. The collagen-to-elastin ratio in the SLP was increased compared to the ratio DLP but did not reach significance (0.4±0.2 and 0.3±0.2, $P = 0.054$ two-way ANOVA).

Pig 2's SCAR treatment had significant differences in both the superficial and deep lamina propia. Compared to CTL the SLP elastin was significantly lower (21.0±5.9% and 31.0±9.2, $p<0.001$ two-way ANOVA). The collagen-to-elastin ratio was also significantly increased in the SCAR SLP than in the CTL fold (0.7±0.3% and 0.6±0.2, $p<0.001$ two-way ANOVA). There was no significant difference in the collagen levels between the SCAR and CTL within SLP. In the deep lamina propia, the SCAR fold had increased collagen percent area fraction (12.8±5.1% and 4.5±2.2%, $p<0.001$ two-way ANOVA). The collagen-to-elastin ratio was also significantly increased in the SCAR fold compared to the CTL (0.7±0.3% and 0.3±0.2, $p<0.001$, two-way ANOVA). There was no significant difference in DLP elastin levels between the SCAR and CTL folds.

COVR treatment pig:

COVR treated vocal folds (COVR) imaging yielded 1048 images, 64 SLP, 28 DLP, and 956 of the remaining images were indeterminant or contained only

musculature. CTL yielded 334 total images, 26 SLP, 24 DLP, 284 were indeterminant or musculature only. Percent area fraction findings of both elastin and collagen, and collagen-to-elastin ratio data can be found in **Figure 8** Additionally, representative H&E and MOVAT pentachrome stained sections and NLSM imaging of both control and COVR treated vocal folds can be found in **Figure 9.**

Within the CTL fold, the collagen percent area fraction within the SLP was significantly higher than the DLP (21.4±9.8% and 11.2±4.5%, $p<0.001$ two-way ANOVA). The elastin percent area fraction was significantly greater in the DLP than the SLP (20.7±6.9% and 11.3±10.2%, $p<0.001$ two-way ANOVA). The collagen-to-elastin ratio was significantly larger in the CTL SLP than the DLP (4.4±4.7% and 0.7±1.1%, $p<0.001$ two-way ANOVA). Within the COVR folds, there were no significant differences in the percent area fraction of either the collagen and elastin, nor in the collagen-to-elastin ratio between the SLP and DLP.

Pig 3's COVR treatment had significant differences in both the superficial and deep lamina propia, compared to the CTL fold. Comparing the SLP, the collagen was significantly increased in the CTL fold compared to the COVR fold (21.4±9.8% and 8.1±3.4%, $p<0.001$ two-way ANOVA). The elastin in the COVR SLP was also higher than in the CTL (17.7±8.6% and 11.3±10.2, $p<0.01$ two-way ANOVA). The collagen-to-elastin ratio was significantly larger in the CTL SLP than the COVR SLP (4.4±4.7% and 0.7±1.1, $p<0.001$ two-way ANOVA). The DLP collagen percent area fraction was significantly larger in the COVR group than the CTL (11.2±4.5% and 7.5±3.3%, $p<0.01$ two-way ANOVA). There was no significant difference between the COVR and CTL DLP with respect to the elastin and collagen-to-elastin ratio.

A comparison image of percent area fractions and collagen-to-elastin ratio of the superficial layers of all three pigs can be found in **Figure 10.**

DISCUSSION

Dysphonia has a sizable health care cost burden and has significant negative effects in the lives of those affected. Optimal treatment for the disruption of the vocal fold microstructure in dysphonia caused by severe vocal fold scarring remains elusive and attempts at improving the healing process to improve vocal quality has proved challenging. Surgical resection and cold steel interventions often redevelops scar tissue through normal wound healing, while injectable therapies of biologic and bioactive materials have led to limited success in the development of appropriate extracellular matrix and improved functional outcomes.

Despite decades of attempts, scarred vocal folds remain difficult to repair. Regenerative medicine techniques to restore native tissue or replace structures with functionally identical tissue, provides an avenue of therapeutics that may best address the challenge of vocal fold scarring. Multiple cell and biologic therapies have shown preclinical success, with three recent human clinical trials demonstrating encouraging safety profiles and improved objectively gathered results.[67-69] Overall, subjective improvements in vocal quality, as reported by patients on the vocal health index (VHI) has also been encouraging, though less consistent. In patients who received injected autologous fibroblasts as treatment, VHI improved, but failed to reach statistical significance.[68] In patients who received autologous mesenchymal cell injections, only half of them showed improvement on the VHI.[69] Some stem cell injections lack persistence, including some mesenchymal stem cells that have been noted to die within 24 hours following injection. Furthermore, treatment is limited by scar size, such that

larger defects cannot be fully regenerated by injection.[78-80] Together, this provides a limited therapeutic treatment window and time-limited effectiveness of immune modulation. The approach we take on replacing the damaged vocal fold mucosa entirely with the COVR implant directly addresses the concerns raised by these clinical trials.

Aim 1 - Histology of COVR and Components

Collagen

We compared the histologic findings of the intermediate-term implantation of COVR, and the COVR components, a decellularized fibrin matrix and the human adipose-derived stem cells within rabbit vocal folds to elucidate the benefits and method of action that the COVR implant has in wound healing and scar formation. When comparing hematoxylin and eosin, as well as Movat pentachrome stained sections, patterns emerge within the healing cycle. Overall analysis of the rabbit laryngeal section histology provide evidence that intermediate term COVR implantation inhibits scar formation when compared to the separated COVR components, decellularized scaffold and direct hASC Injections, as well as non-treated scar control. On review, the COVR and DECELL group had notably fewer total sections stain positively for increased collagen than the hASC Injection and Scar treatment groups. The collagen in both the DECELL and COVR groups was also more often described as thin and wavy, while the hASC Injection laryngeal sections were more often described as thick and bundled or disorganized, the hallmark description of histologic fibrosis.[18] It is possible that the wavy collagen will continue remodeling to become organized and cross-linked, functionally normal collagen, however, scar tissue formation and collagen remodeling can take up to 2 months.[25] Thus longer implantation and histological follow-up is required to confirm the ultimate fate of this collagen. Nonetheless, the decreased collagen levels and favorable

description of this collagen supports the finding that the COVR implant attenuates scar tissue formation that is maintained after intermediate-term implantation.

Inflammatory Response

In comparing the inflammatory response, an infiltrative process was present in a majority of the DECELL group and half or less of all sections in the COVR, hASC Injection, and SCAR treatment groups. However, the hASC Injection and DECELL groups had multiple sections described as having a continued robust white cell presence at six weeks, while both the COVR and SCAR group both had no sections with this level of immune response. The DECELL group's inflammatory response raises the question of an inflammatory response to the decellularization process. Indeed, residual SDS can cause a severe inflammatory response, however, the infiltrate in our sections lacks the encapsulation, giant cells, or hemosiderin-laden cells as previously described, making this explanation unlikely.[81] Instead, we propose that the lymphocytic reaction in the DECELL group more likely resembles a foreign body immune response. Mesenchymal stem cells have low immunogenicity, while fibrin as a standalone therapy and as a medium for cell introduction, is known to promote faster wound healing through angiogenesis, improved cell viability, proliferation and differentiation, and excellent integration into surrounding tissue.[82,83] Our data indicates that xenographic ASCs implants maintain an immunomodulatory effect on surrounding tissue up to six weeks and have an improved attenuating influence when embedded within a fibrin matrix than when introduced into tissue by direct injection alone.

Glycosaminoglycans

As mentioned previously, the levels of GAGs in the vocal fold are critically important for normal function. HA is the most abundant glycosaminoglycan in the folds,

is a reliable marker of good healing in the vocal folds, and stains with the pentachrome used in this study.[33] The COVR group sections stained positively for HA in six of the twelve sections sections and the SCAR group stained positively for HA in a three of twelve sections, while the DECELL and hASC Injection groups both stained positively for HA in only one section. The mechanism of GAG deposition, whether GAG derives from the introduced ASC or native tissue, is unclear. Mesenchymal stem cells have HA synthase and have been implicated in HA deposition in autologous bone marrow derived MSC transplants in mice larynges and allogenic laryngeal mucosa derived MSC transplants in canine larynges.[84,85] Given that this was a xenographic treatment after removal of native tissue, it is likely that introduced ASCs synthesize their own GAG, but we cannot rule out stimulation of intrinsic GAG synthesis. The relative deficit of GAG in the hASC Injection and DECELL groups may be explained by the prolonged incubation during COVR development, during which GAGs may be synthesized and retained within the scaffold. Additionally, the fibrin gel may play a protective role of the embedded cells, limiting the lymphocytic response and clearing of the COVR cells, from which the injected cells do not benefit. In either case, our data indicates that ASCs embedded within a scaffold, but not directly injected into damaged mucosa, leads to improved GAG deposition into the ECM at six weeks, reflecting improved wound healing and a more normal ECM microstructure. This improvement of microstructure suggests improved biomechanical properties which may have implication on functional outcomes of the COVR treated vocal folds. The significance of the six COVR sections that did not stain for HA is unclear, further studies are needed to confirm if the HA positivity has an effect on voicing.

HLA staining

Previous research in this laboratory has shown persistence of autologous adipose-derived stem cells at four weeks embedded in fibrin gel scaffold.[86] The rabbit fluoroscopy imaging of positive HLA-staining cells indicates that xenographic implantation of human ASC implanted in rabbit vocal folds persists up to six weeks whether embedded within a fibrin gel structure or directly injected into the vocal fold. Additionally, there was a greater HLA positivity in the COVR fluorescent images than in the hASC Injection sections. There was no such positive staining in the DECELL gel structure. The increased stem cell persistence in the COVR group compared to hASC Injection further supports a protective benefit of the fibrin scaffold in overall cell survival and may suggests increased proliferation of embedded cells, previously described in keratinocytes and fibroblasts.[87] Further analysis is required to understand whether these cells arise from improved survivability of originally embedded cells, increased proliferation of cell lineages, or a combination of both.

Fibrin

There were clear differences in the fibrin appearance and amount between the treatment arms. The fibrin within the DECELL sections had well circumscribed, deeply eosinophilic structures. When compared to the H&E laryngeal sections of the animal that received COVR implant and died post-operatively, the DECELL larynges appeared identical. We suspect then that the DECELL fibrin is retained fibrin from the implanted gel structures. The fibrin in the COVR treated laryngeal sections had less clear gel remnant and more closely resembled the fibrin found in both the hASC Injection and SCAR groups. The COVR sections likely had a mixture of the remnant fibrin scaffold

and fibrin of residual wound healing, while the SCAR and hASC Injection was solely lingering wound healing fibrin.

All treatment arms had presence of other findings, such as a presence of dispersed fat cells and irregular epithelium, though there were too few cases to make any conjecture on their implications, if any.

Aim 2 -NLSM imaging of pig vocal folds

Nonlinear scanning laser microscopy (NSLM), also called two-photon microscopy or multi-photon microscopy, pulses two photons that simultaneously interact with a molecule causing excitation, the resulting emission is then recorded as normal.[88] Each of the NSLM photons are typically half the energy of a single-photon system, such as in fluorescent microscopy, which minimizes the damage caused to tissue by higher energy photons while simultaneously improving image detection.[89] This allows for excellent imaging of the vocal fold microstructure including the elastin and collagen within the vocal folds, without the need to alter the tissues for imaging. Analysis of treated vocal fold extracellular matrix collagen and elastin levels and calculated collagen-to-elastin ratio were compared to the control, unaltered contralateral folds in the three treatment arms: hASC Injection, SCAR, and COVR implantation. Notably, there was no attempt or intervention to enforce voice rest in these animals following their operation, which is usual treatment in humans following vocal fold scar resection.

hASC Injection

The pig that received hASC Injection had control vocal folds with collagen, elastin, and collagen-to-elastin ratios in the layer and concentrations distribution described in the literature. The control fold had significantly greater collagen levels in the deep lamina propria than in the superficial layer. The vocal fold that received hASC

Injection had significantly increased collagen deposition in the superficial lamina propria layer, similar to that seen in the rabbit histology, however this was not appreciated on light microscopy of the pig vocal fold. The fibrosis indicates an increased inflammatory response during healing, although no white blood cells were present in light microscopy or in the NLSM images. This is likely due to the longer post-operative healing period prior to laryngeal harvest. Voice rest during acute wound healing with gradual vocal fold use has been noted to improve vocal fold wound healing and voice quality outcomes in humans, while uncontrolled voice use worsens outcomes.[90-92] We propose that the unrestricted animal's spontaneous phonations led to increased stress at the vocal fold surface prior to epithelialization, which worsened acute wound healing and led to more more fibrosed superficial lamina propria.

When comparing the hASC Injection treated and untreated vocal folds we found significantly increased collagen deposition in the superficial layer and an increased and non-significant though increased collagen deposition within the deep layer. Interestingly, the magnitude of collagen increase in the superficial layer was much greater than in the deep, suggesting that the hASC does have wound healing benefits that are more present within the deep but not superficial layers of lamina propria. This is further evidenced by an improved collagen-to-elastin ratio within the deep lamina propia and worsened collagen-to-elastin ratio in the superficial lamina propria. The decreased fibrosis may signal that the ASCs have their greatest immunomodulatory benefit at or near their injection site, the thyroarytenoid muscle, and a weakened effect more superficially. There were no significant differences between the elastin levels in the control versus treatment vocal folds in either the superficial or deep lamina propria. This is consistent with

previous findings of elastin levels returning to baseline at 6 months following injury.[34] Overall, the increased collagen deposition indicates increased fibrosis and poor healing following hASC Injection alone. Functionally, the superficial lamina propria is more directly involved in sound production and vocal quality and we would expect a poorer functional outcome in this pig.

SCAR Pig

The pig that received mucosal resection without intervention had an interesting baseline vocal fold structure. NLSM imaging of the control fold showed significantly increased baseline collagen levels and elastin levels in the superficial lamina propria, and an increased collagen-to-elastin ratio that was also approaching significance. The scarred vocal fold was more uniform, lacking any significant difference in total collagen or elastin between the two lamina propria layers. Comparing the scar and control folds, the elastin levels in the scarred fold was significantly decreased in the superficial lamina propria. Within the deep lamina propria, the scarred fold showed significantly increased collagen levels, as expected. Our findings support previously document decreases in elastin levels in scarred folds and collagen deposition in the deep lamina propria.[34,48] Moreover, the baseline findings in this animal's control fold illustrate the importance of evaluating collagen structure and organization, not purely overall levels, when determining scar tissue in histology.

COVR Pig

The pig that received the COVR implant died six weeks after the intervention from an indolent *Streptococcus Suis* infection that was exacerbated by postoperative immunosuppression. NLSM imaging analysis of the control vocal fold identified significantly increased collagen in the superficial layer and significantly increased elastin

in the deep lamina propria, which we believe reflects injury to the control vocal fold. This is not surprising as we transitioned to an open surgical approach during COVR implantation which we may have led to some collateral damage, not seen in the other pigs that underwent an endoscopic intervention.

Interestingly, the COVR treated vocal folds were more uniform, as there were no significant differences between the superficial lamina propria compared to the deep layer. While epithelialization occurs during COVR development, this data supports a delayed division of the mucosal layers that occurs beyond six weeks following implantation. Comparing the treated and untreated folds, the superficial lamina propria of the COVR fold had significantly decreased collagen deposition, increased elastin levels, and an improved collagen-to-elastin ratio than the control. While mature scar formation may take up to a year in the vocal folds, postinjury collagen synthesis is greatest during the first six weeks of healing, and the SHG imaging modality is sensitive to both Type I and Type III collagen, which predominate early scar formation.[25,34,75] Thus our data suggests that COVR implantation improves wound healing at six weeks, which we expect will improve longer durations of healing as well.

In the deep lamina propria, the COVR implant had increased collagen deposition compared to the control fold. During COVR development, a layer of hASCs is added to the superior, non-immersed surface of the gel, which becomes the epithelial layer. As this animal had only six weeks of COVR implantation, we would expect continued persistence of hASCs greater at the epithelium and superficial lamina propria, as seen in the rabbit HLA histology. A greater number of bioactive stem cells would therefore have improved healing effects within the superficial lamina propria and less of an effect within

the deep lamina propria. In addition, initial healing and implant grafting would occur at the juncture of thyroarytenoid musculature and COVR implant, which would help explain the increased collagen deposition in the deep lamina propria. Although long-term COVR implantation is needed to confirm that fibrosis indeed remains limited in the superficial lamina propria with this therapy, our preliminary findings are encouraging. Furthermore, additional methodologies are needed to understand the long term structural and functional improvements that COVR treated larynges have on voice production and vocal quality.

Superficial Lamina Propria treatment comparisons

Contrasting the superficial lamina propria in the three treatment arms and their respective controls further produced interesting findings. Contrary to expectation, the hASC Injection pig, but not the SCAR pig, had a significantly increased amount of collagen at six months. hASC cell injections as a treatment for vocal fold scarring leads to improved collagen deposition in preclinical and clinical applications and is associated with improvement in functional outcomes. A limitation of the NLSM imaging analysis in this study is our reliance on quantifying the amount of collagen present, but not describing nor account for the organization of the collagen fibers measured. As such, we believe the percent area fraction of collagen, to infer levels of collagen, is a poor marker of fibrosis if collagen organization is not taken into account. The calculated collagen-to-elastin ratio is also heavily influenced by the collagen percent area fraction and was not found to be a useful as a marker of fibrosis or comparison between animals. Instead, we argue that decreased elastin is a more sensitive marker of fibrosis and improved healing in NLSM imaging. In comparing elastin levels, we find the SCAR pig has the worst outcome, as expected. The hASC Injection elastin levels are equivalent to control fold levels and therefore suggest improved healing compared to scar. The COVR treatment

had a significantly increased elastin level in the treated vocal fold also suggesting improved healing, albeit at a shorter post-operative endpoint.

Comparisons of the deep lamina propria layers between the treatment and control for the three treatment arms had increased collagen deposition for the SCAR and COVR treated folds only. While statistically significant, vocal fold functionality and voice production relies heavily on the cover portion of the vocal fold and less so on the body, to which the deep lamina propria belongs. Thus, we expect that these increases in collagen deposition would have minimal influence on voice quality and production, if any. We suggest that future NLSM microstructure imaging focus on the superficial lamina propria, and intermediate lamina propria where applicable.

Our preliminary findings suggests that the COVR implant, as a complete mucosal replacement, improves overall wound healing with decreased fibrosis and intermediate-term ECM microstructure. This histologic analysis is limited by the subjective nature of image analysis and the imperfect method of translating images to an absolute value; thus, biochemical analysis is underway of the rabbit harvested larynges. Through enzymatic cell digestion of all harvested rabbit vocal fold sections, we hope to identify cytokine, mRNA, and ECM components and determine absolute quantitative values of glycosaminoglycans, collagen, and fibrin, to correlate with the imaging analysis performed here. Together, this will better elucidate the method of actions of the COVR components, particularly the hASC's role in immunomodulation and wound healing. Additionally, ECM collagen and elastin levels within the pig laryngeal sections will be identified and quantified by cell digestion to correlate with the imaging data presented here.

It remains to be seen if the histological improvements in the rabbit and pig laryngeal sections translate to functional improvement following COVR implantation. This laboratory is currently pursuing additional methods of analysis of pigs involved in this study. Pig spontaneous *in vivo* phonations were recorded prior to their operations and during the weeks to months following their interventions until their termination. During the terminal surgery, the animals underwent induced *in vivo* phonation during which we recorded the sound production and concurrent high-speed video recordings of the vocal folds, with the notable exception of the COVR pig. These high-speed video recordings will be analyzed for stroboscopy and mucosal wave analysis, commonly used methods of vocal fold functional assessment both clinically and in animal research. The acoustic analysis of the audio recordings at various timepoints pre- and post-surgery, will allow us to determine what acoustic improvements, if any, occur following each intervention and how these change over time. It is the hope of this laboratory to create a robust voicing animal model for COVR implantation to advance this treatment as a therapeutic option for patients.

CONCLUSION

Animals that received COVR, a human-derived adipose stem cell-based gel implant, better regulate healing and scar development following intervention than those which received cells only, fibrin gel only, or were scar controls. In rabbits, COVR implantation promotes improved healing and decreased scarring at six weeks, as supported by the increased hyaluronic acid deposition and decreased collagen development. Xenograph ASCs grafts persist weeks and have continued immunomodulatory effects up to six weeks following implantation. ASC immunomodulatory effects are increased effects when embedded in a fibrin gel than

when directly injected into damaged vocal folds. Decreases of overall elastin levels are more sensitive marker of fibrosis in non-linear scanning microscopy imaging than increases in collagen levels. Further research is needed to determine if histologic improvement translates to improved functional improvements.

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