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Biochemical Basis of Vocal Fold Mobilization After Microflap Surgery in a Rabbit Model

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Abstract

Objectives/Hypothesis—To investigate phonation-related extracellular matrix (ECM) changes in the vocal fold lamina propria after microflap surgery using an in vivo rabbit phonation model.

Study Design—Prospective animal study.

Methods—Twenty-four New Zealand White rabbits were used in this study. Quantitative polymerase chain reaction and immunohistochemistry were used to investigate alterations in vocal fold ECM proinflammatory and profibrotic gene, and protein expression from a control group of animals receiving a microflap without phonation and a separate group of animals receiving experimentally induced phonation on postmicroflap days 0, 3, and 7.

Results—IHC demonstrated the highest concentration of CD45 in vocal folds on postoperative day 0. Staining for CD45 was absent by postoperative day 7, with no differences in CD45 staining between groups. Fibronectin gene expression increased significantly on postoperative day 3 in the control and experimentally induced phonation groups, with maximal staining of fibronectin around the microflap incision on postoperative day 7. No alterations in cyclooxygenase-2, interleukin-1 β , and transforming growth factor- β 1 gene expression were observed between groups.

Conclusions—Results of the present study revealed an acute inflammatory response in the vocal fold at the time of microflap (day 0) and up to 3 days post-microflap. By post-operative day 3, staining of CD45 positive cells decreased, with essentially no evidence of inflammation by post-operative day 7. With the end of the acute inflammatory response occurring around day 3, these data may provide support for mobilizing tissue after inflammation has subsided and the process of active tissue remodeling has ensued (days 3–7).

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Keywords

Gene expression; immunohistochemistry; microflap; phonomicrosurgery; vocal fold; wound healing; voice rest

INTRODUCTION

Postoperative vocal restrictions are often recommended for patients who undergo phonomicrosurgery for benign vocal fold disease during the early stages of healing to improve tissue repair and reduce vocal fold trauma. Although there is a shortage of empirical studies in the literature on the efficacy of voice rest, it is thought that immobilization of the vocal folds promotes healing and reduces scar formation by providing biologic protection to the fibroblast-bearing layers of the vocal fold lamina propria.¹ Unfortunately, there is great variability among practicing otolaryngologists regarding the type (i.e., absolute vs. relative) and duration of postoperative voice rest.² For instance, complete vocal rest may be recommended for anywhere between 3 and 14 days following the removal of a vocal fold lesion, such as a cyst or polyp, whereas some otolaryngologists are unlikely to recommend any type of vocal restrictions following the removal of these lesions.²

Many factors have been proposed to explain the inconsistency in clinical practice, including beliefs about the physiology of vocal fold mucosal healing, lesion characteristics, surgical techniques, pre- and postoperative voice therapy, and patient adherence to treatment. Consequently, it appears that such variability may be due to the lack of empirical evidence to guide clinical management, poor patient adherence to treatment, and a negative impact of vocal restrictions on a patient's overall quality of life.³ These inconsistencies in clinical practice highlight the need for basic science investigations to investigate long-standing assumptions regarding the physiology of vocal fold mucosal healing and responses to contemporary surgical treatments.

The lamina propria is a multilayered structure with layers that have been characterized by the density and distribution of elastin and collagen.⁴ Fibroblasts make up the main cellular components of the lamina propria, whereas glycosaminoglycans and proteoglycans occupy the interstitial spaces within the extracellular matrix (ECM).⁴ The overall goal of contemporary phonomicrosurgery is to restore optimal biomechanical function by removing vocal fold disease, while providing minimal disruption to the surrounding vocal fold layered structure. The microflap technique is one such approach that has been used in the treatment of benign vocal fold lesions. Recently, our laboratory demonstrated the feasibility of performing microflap surgery in a rabbit model, with the goal of investigating the effects of tissue vibration on repair of vocal fold microflap defects after experimentally induced phonation.⁵⁻⁹ To better understand the effects of tissue vibration on healing of vocal fold microflap incisions (surgical technique described by Courey et al.¹⁰), there is a critical need to examine the molecular activities and phonation-related ECM changes after microflap surgery. Thus, the purpose of the present study was to investigate the effects of early versus delayed vocal fold mobilization on ECM gene and protein expression of the vocal fold after

microflap surgery using an in vivo rabbit phonation model. We focused on the transmembrane protein tyrosine phosphatase CD45 and known profibrotic and proinflammatory mediators involved in homeostasis of the ECM, including transforming growth factor- β 1 (TGF- β 1), interleukin-1 β (IL-1 β), cyclooxygenase-2 (COX-2), and fibronectin.^{6,8,11,12}

MATERIALS AND METHODS

Animals

The animal use protocol used in this study was approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Animal Welfare Act (7 U.S.C. et seq.). Twenty-four New Zealand White breeder rabbits, weighing 3 to 5 kg were used in this study. All animals underwent bilateral microflap surgery (see microflap procedure below). Animals were randomized into six groups (Fig. 1) with four animals per group. The experimental group received experimentally induced phonation after microflap surgery, and a separate control group received the microflap procedure without experimentally induced phonation. Animals were euthanized after the completion of study procedures, and larynges were harvested for further analysis. Within each larynx, one vocal fold was used for gene-expression experiments and the contralateral vocal fold was used for immunohistochemistry (IHC) staining.

Surgical Procedure

Animals were injected with ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (0.75 mg/kg) for anesthesia. Vital signs (i.e., heart rate, oxygen saturation level, body temperature) were monitored during the procedure to ensure the overall well-being of the animals. Transoral direct-suspension microlaryngoscopy was performed with an 11-cm Holinger-Tucker pediatric anterior commissure side-slotted laryngoscope⁸ (Karl Storz Endoscopy-America, Inc., El Segundo, CA).

Microflap Procedure

Surgical procedures were documented using a 30°, 2.7-mm, rigid endoscope coupled to a Telecam-C camera (Karl Storz Endoscopy-America). Using routine microlaryngeal instruments (Fig. 2) modified only to shorten the length to better match the distance needed to approach the rabbit larynx, a 3-mm incision was made through the epithelium and mucosal cover of one vocal fold, and elevation was performed using a microlaryngeal fine-angled spatula (Fig. 3). The microflap was redraped and allowed to heal by secondary intention. The same procedure was performed on the contra-lateral side.

Phonation Procedure

Phonation was elicited using an in vivo rabbit phonation model.⁵⁻⁸ The on/off duty cycle consisted of 3-second trains delivered every 7 seconds for 30 minutes.⁵⁻⁸ Rabbits underwent experimentally induced modal phonation as described in detail elsewhere,^{6,8} on postoperative days 0, 3, and 7. Tissues were harvested 4 hours after completion of the

phonation experiments to allow for gene expression and protein transcription prior to harvest of the larynx. Animals in the control group underwent the microflap procedure and humidified air flow delivered to the glottis for 30 minutes without experimentally induced phonation.

Gene-Expression Analysis

Known profibrotic and proinflammatory genes were investigated, including TGF- β 1, IL-1 β , COX-2, and fibronectin. At the time of laryngeal harvest, a 1-mm tissue specimen of vocal fold epithelium and lamina propria was obtained. The specimen included the mucosal layers of the vocal fold above the thyroarytenoid and did not include muscle. Specimens were immediately stabilized in RNAlater (Ambion, Austin, TX). The animals were then euthanized without recovery from anesthesia using an overdose intracardiac injection of pentobarbital sodium (150 mg/kg). An ultrasonic homogenizer (Fisher Scientific, Pittsburgh, PA) was used to homogenize the vocal fold tissue. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) and treated with deoxyribonuclease I (Qiagen) to minimize contamination from genomic DNA. Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) per the manufacturer's recommended reaction protocol. Reactions were performed using a Veriti Thermal Cycler (Applied Biosystems) with the following parameters: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. Primers for IL-1 β , TGF- β 1, COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Sigma-Aldrich Corporation (St. Louis, MO). Real-time polymerase chain reaction (PCR) was performed in a final volume of 20 μ L reaction mixture composed of 2 μ L of template complementary DNA, 0.9 μ L of forward primer (20 pmol/ μ L), 0.9 μ L of reverse primer (20 pmol/ μ L), 10 μ L of Power SYBR Green PCR Master Mix (Applied Biosystems), and 6.2 μ L of ribonuclease-free water. The following protocol was used for real-time PCR: 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Fluorescence was detected using a StepOnePlus Real-time PCR System (Applied Biosystems). The Ct method was used to determine the relative ratio of gene expression. Target gene ratios from each sample were normalized using expression ratios of the internal control gene (GAPDH). Normalized gene-expression ratios were then compared between the vocal folds that underwent microflap surgery and phonation, and the control group receiving a microflap without phonation.

IHC Analysis

Vocal folds were prepared for IHC as described previously.⁸ Following euthanasia, one side of the larynx was dissected. The specimens were soaked in 4% paraformaldehyde in phosphate buffered saline for 4 hours at 4°C, and then in 15% sucrose solution overnight and then 30% sucrose solution overnight at 4°C for dehydration. The larynges were then transferred into embedding medium (optimal cutting temperature compound; Tissue-Tek, Torrance, CA) frozen, using a combination of acetone and dry ice, and stored in a deep freezer (-80°C) for later analysis. In preparation for immunohistochemistry, 10- μ m-thick frozen sections were made using a cryostat (Leica, Bannockburn, IL). After performing the blocking procedure for 1 hour at room temperature, sections were incubated in primary

antibody solution for 2 hours at room temperature. After washing, sections were incubated in the secondary antibody for 1 hour at room temperature.

For CD45 staining, mouse anti-CD45 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:100 was used for the primary antibody and DyLight 594 conjugated anti-mouse goat antibody (Abcam, Cambridge, MA) at 1:500 was used for the secondary antibody. For fibronectin staining, mouse anti-fibronectin (Abcam) at 1:100 was used for the primary antibody and DyLight 488 conjugated anti-mouse goat antibody (Abcam) at 1:250 was used for the secondary antibody. Nuclear staining was performed using 4', 6-diamidino-2-phenylindole (20 ng/ μ L). Images were captured using a Nikon Eclipse 90i microscope (Nikon, Tokyo, Japan), and image analysis was performed using NIS-Elements software (Nikon). The percentage of CD45-positive cells in the lamina propria was measured using the percentage of CD45-positive cells divided by the total number of cells in the lamina propria.

Statistical Analysis

Separate Kruskal-Wallis tests were used to investigate the overall main effects for gene expression and IHC expression across groups. The threshold of significance was set to $P < .05$. If the overall main effect was significant, post hoc pairwise comparisons were performed to investigate differences between groups using nonparametric Mann-Whitney U testing using a threshold for significance of $P < .05$.

RESULTS

Gene-Expression Analysis

Results revealed a significant overall main effect for fibronectin expression ($P = .037$). Post hoc pairwise comparisons revealed a significant increase in fibronectin gene expression in animals receiving experimentally induced phonation on postoperative day 3 compared to postoperative day 0 ($P = .043$) (Fig. 4), and in control animals undergoing microflap surgery without phonation on postoperative day 3 compared to postoperative day 0 ($P = .029$) (Fig. 4). There were no significant differences in COX-2, IL-1 β , or TGF- β 1 gene expression between groups (Fig. 4).

Immunohistochemical Analysis

Figures 5 and 6 display representative sections of a vocal fold after microflap surgery with IHC staining for CD45 and fibronectin. Staining for CD45 revealed an acute inflammatory response at postoperative day 0, characterized by dense staining of CD45-positive cells (Fig. 5). Fibronectin was localized to the basal layer of the epithelium around the microflap incision, with dense staining at postoperative days 3 and 7 compared to postoperative day 0 (Fig. 6).

By postoperative day 3, staining of CD45-positive cells decreased, with essentially no CD45-positive staining present at postoperative day 7. Statistical analysis revealed a significant main effect of CD45-positive cells ($P = .008$) across groups. Post hoc testing revealed that the percentage of CD45-positive cells was significantly decreased on post-

operative day 7 compared to postoperative day 0 in animals receiving a microflap and experimentally induced phonation ($P = .029$) and in the control animals receiving a microflap without phonation ($P = .029$). There were no between-group differences in CD45-positive cells between the phonation and control groups at postoperative days 0, 3, and 7 (Fig. 7).

DISCUSSION

Optimal postoperative voice outcomes are dependent on preservation of the vocal fold lamina propria. It is believed that some degree of vocal fold immobilization may be necessary to optimize microflap adherence and tissue repair. A fundamental question is whether prescribed tissue immobilization (e.g., voice rest) promotes an optimal environment for wound healing. The current study investigated the effects of tissue mobilization on wound healing of the vocal fold after receiving a microflap in animals undergoing microflap surgery without phonation (control group) and animals receiving a microflap and experimentally induced phonation on postmicroflap days 0, 3, and 7.

In many connective tissues throughout the body, fibroblasts play an important role by contributing to deposition, degradation, and rearrangement of the ECM.⁴ It has been suggested that appropriate therapeutic control of each of these processes may result in optimal vocal fold wound healing.¹³ Although all of the necessary ECM components may be present in the healing wound, a factor of chief importance is the overall organization of the remodeling ECM. It is generally believed that vibratory forces may be essential for the orderly remodeling of collagen fibers during postoperative tissue healing.¹⁴ This appears to suggest that even with the best surgical technique, the potential for postoperative edema, scar, and persistent dysphonia are nonetheless possible in the absence of appropriate postoperative management. The rationale behind postoperative vocal restrictions is to immobilize the tissue for a specified duration to minimize the inflammatory process and promote subsequent tissue remodeling. Although it has been posited that long periods of tissue immobilization may be necessary to achieve optimal healing of the basement membrane in an earlier study involving a canine model, this idea has fallen out of favor in modern day approaches to voice care.¹⁵

Previously, our laboratory measured the effects of a short-time dose (30 minutes) of modal and raised intensity phonation on vocal fold inflammatory gene expression in the normal rabbit vocal fold.⁸ Results revealed a significant increase in TGF- β 1, IL-1 β , and COX-2 gene expression following a 30 minute time dose of raised intensity phonation compared to modal phonation and control, substantiating the effects of phonotraumatic phonation on the vocal fold inflammatory cascade in normal tissue. Importantly, a short-time dose of modal phonation did not lead to changes in inflammatory gene expression. However, it remains unclear whether the same time dose of modal phonation is detrimental to the healing process after phonosurgery. Therefore, in the current study, we replicated a vocal fold microflap incision in a rabbit model and induced modal phonation for 30 minutes at the time of microflap surgery (postoperative day 0), and on postoperative days 3 and 7 to investigate the effects of a short-time dose of modal phonation on healing of vocal fold microflap incisions. The time points chosen were selected based on current clinical practice, as well as the need

to investigate time points coinciding with the inflammatory, proliferative (0 and 3 days) and remodeling phases of tissue repair (7 days).²

CD45-positive cells were densely stained throughout the vocal fold on postoperative day 0 in vocal folds receiving a microflap without phonation (control group) and vocal folds receiving a microflap and a 30-minute time dose of experimentally induced modal phonation (phonation group) at the time of microflap surgery. The presence of CD45-positive cells on day 0 provides confirmation of an acute inflammatory process with an onset at the time of microflap surgery. By postoperative day 3 staining of CD45-positive cells decreased, with essentially no CD45-positive staining present at postoperative day 7 and no differences in CD45 between groups. Further, gene expression data revealed no differences in TGF- β 1, IL-1 β , or COX-2 gene expression between the control and experimentally induced phonation groups. Thus, modal phonation initiated on postoperative days 0, 3, and 7 did not appear to increase or prolong the inflammatory response. The increase in fibronectin gene expression on postoperative day 3 and dense staining of fibronectin in the vocal folds on postoperative days 3 and 7 likely suggest that the vocal folds are undergoing active remodeling characterized by chemotaxis and the recruitment of cells to orchestrate repair. Tateya et al.¹⁶ demonstrated that fibronectin was present on postoperative day 1 and remained strongly present and unchanged from postoperative day 3 through 14 in rats after a vocal fold stripping procedure. Similarly, in the present study, fibronectin was localized in the basal layer of the epithelium around the microflap incision on postoperative days 3 and 7. Results revealed no differences in the density of fibronectin staining between the control and phonation groups.

Studies have demonstrated the usefulness of the microflap technique in preserving the vocal fold mucosa with exceptional voice outcomes after phonomicrosurgery.^{1,17} A microflap takes advantage of the natural histologic planes of the vocal fold to carefully remove lesions with minimal disruption to vibratory mechanics.¹⁸ Because dissection within the vocal ligament is avoided, inflammation and scar formation are minimized.⁹ This is in contrast to injury by microcup forceps or vocal fold stripping, another common form of injury used in animal wound healing literature that results in a significant and immediate inflammatory response with corresponding changes in ECM production.^{19–23} Although the microflap procedures performed in the present study did not involve the removal of underlying vocal fold pathology, extensive dissection was performed in this model study in the subepithelial plane to more closely simulate an actual microlaryngeal surgical approach. The minimal mucosal gap that was our end result is arguably comparable to an optimal surgical outcome, with close reapproximation of the mucosal edges. However, in microflap procedures involving more extensive surgical dissection with a larger residual mucosal gap, sound clinical judgment should be appropriately used to guide postoperative voice use recommendations beyond the 3-day minimum discovered in the present study. Additionally, although laryngopharyngeal reflux (LPR) was not a variable controlled for in the present study, the role of LPR on the healing microflap and the importance of controlling for objectively documented sources of laryngeal irritation during the acute stages of wound healing should not be discounted.²⁴ LPR, inhaled pathogens, and other sources of laryngeal

irritation have the potential to exacerbate the wound healing response and result in poor voice outcomes.

CONCLUSION

Results of the present study revealed an acute inflammatory response in the vocal fold at the time of microflap surgery (day 0) and up to 3 days postmicroflap surgery. By postoperative day 3, staining of CD45-positive cells decreased, with essentially no evidence of inflammation by postoperative day 7. Fibronectin staining for up to 7 days after microflap surgery revealed a process of active tissue remodeling characterized by chemotaxis and the recruitment of cells to orchestrate repair. However, no differences were observed in fibronectin or CD45 staining between phonation and control groups. Thus, 30 minutes of experimentally induced modal phonation initiated on postoperative days 0, 3, and 7 did not appear to increase or prolong the inflammatory response. With the end of the acute inflammatory response occurring around day 3, these data may provide support for mobilizing tissue after inflammation has subsided and the process of active tissue remodeling has ensued (days 3–7). These data have the potential to inform future investigations and recommendations regarding postoperative voice use protocols. However, sound clinical judgment cannot be replaced and should always be exercised in cases of microflap procedures involving extensive dissection and in controlling for sources of laryngeal irritation that may exacerbate and delay the wound healing response.

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	Control	Experimental
Day 0	+mf, -phonate	+mf, +phonate
Day 3	+mf, -phonate	+mf, +phonate
Day 7	+mf, -phonate	+mf, +phonate

Fig. 1.

All animals received a microflap (mf) in the six different study groups. Four rabbits were in each group. The control animals did not receive experimentally induced phonation and were harvested on their randomized postoperative day. The experimental group differed by the presence of experimentally induced phonation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2.

(A) Microlaryngeal instruments used for rabbit microflap surgery including sickle knife and elevator. (B) Laryngeal instruments modified in length to accommodate the pediatric laryngoscope used in rabbit microsurgery compared to the longer length needed to approach the human larynx. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 3. (A) Illustration of microflap incision and elevation. (B) Sickie knife making incision into left vocal fold. (C) Elevation of mucosa using a microelevator to develop the flap. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 4.

Log-transformed mRNA expression ratios for fibronectin, transforming growth factor- β 1 (TGF β 1), cyclooxygenase-2 (COX-2), and interleukin-1 β (IL-1 β) in the control and phonation groups at postoperative days 0, 3, and 7. C = control; P = phonation.

Fig. 5. Representative coronal sections of the vocal fold stained for CD45 (pink staining) in the (A) control and (B) phonation group at postoperative day (POD) 0, 3, and 7 (bar =100 μ m). Pink staining represents CD45-positive cells. Blue counterstain represents staining of cell nuclei.

Fig. 6. Representative coronal sections of the vocal fold stained for fibronectin (green staining) in the (A) control and (B) phonation group at postoperative day (POD) 0, 3, and 7 (bar =100 μ m). Green staining represents fibronectin. Blue counterstain represents staining of cell nuclei.

Fig. 7.

Percentage of CD45-positive cells in the lamina propria at postoperative days 0, 3, and 7. C =control; N.S. =not significant; P =phonation.