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Modulation of Inflammatory and Profibrotic Signaling in a Rabbit Model of Acute Phonotrauma Using Triamcinolone

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Abstract

Objective—To investigate the hypothesis that prophylactic triamcinolone modulates acute vocal fold inflammatory and profibrotic signaling during acute phonotrauma.

Study Design—In vivo rabbit phonation model.

Setting—Academic medical center.

Subjects and Methods—Forty New Zealand white breeder rabbits were randomly assigned to 1 of 4 groups: control (no intervention), no treatment (30 minutes of raised intensity phonation), sham treatment (bilateral intralaryngeal triamcinolone acetate injection at 0 µg/25 µL followed by 30 minutes of raised intensity phonation), or steroid treatment (bilateral intralaryngeal triamcinolone acetate injection at 400 µg/25 µL followed by 30 minutes of raised intensity phonation). Quantitative polymerase chain reaction (qPCR) was used to investigate gene expression levels of cyclooxygenase-2 (COX-2), interleukin (IL)–1β, and transforming growth factor (TGF)–β1.

Results—Results revealed a significant main effect for COX-2 ($P = .002$). Post hoc testing revealed that rabbits receiving no treatment (15/10) had higher COX-2 gene expression than

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Author Contributions

Joseph E. Hall, conception and design, IACUC application and approval, data acquisition, data analysis, drafting and editing manuscript, final approval; **Atsushi Suehiro**, conception and design, data acquisition, data analysis, drafting and editing manuscript, final approval; **Ryan C. Branski**, conception and design, drafting/revising manuscript, final approval; **C. Gaelyn Garrett**, conception and design, drafting/revising manuscript, final approval; **Bernard Rousseau**, conception and design of study, data acquisition, data analysis, drafting and revision of manuscript, final approval.

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control (5.90; $P < .001$). There were no significant differences in COX-2 expression between treatment groups. Results revealed a significant main effect for IL-1 β ($P < .001$). Post hoc testing revealed that rabbits receiving no treatment (14.70) had higher IL-1 β gene expression than control (6.30) ($P = .001$). There were no significant differences in IL-1 β gene expression between treatment groups. There were no significant differences in TGF- β 1 gene expression ($P = .525$) between treatment and control groups.

Conclusion—Given conflicting evidence, further studies are necessary to investigate vocal fold steroid injections prior to and following the induction of phonotrauma. Prophylactic administration of triamcinolone immediately prior to acute phonotrauma resulted in no significant changes in COX-2, IL-1 β , and TGF- β 1 gene transcript levels.

Keywords

phonotrauma; triamcinolone; acute; voice disorders; rabbit; vocal cords

Voice disorders affect approximately 3% to 9% of the population at any given time.¹ These disorders are associated with an increased incidence of stress, depression, anxiety, and the inability to perform occupational tasks.^{2–5} Although the etiology of dysphonia is multifactorial, nearly 22% of patients seeking treatment present with organic vocal fold lesions.⁶ Unfortunately, many commonly used treatments lack empirical evidence to support their widespread clinical use.

Injectable corticosteroids, including triamcinolone acetonide, are frequently used to enhance favorable healing in various dermatologic conditions such as hypertrophic scar and keloids.^{7,8} Triamcinolone injections into the vocal folds have been reported to reduce inflammation, granulation tissue, and hypertrophic scarring.⁹ The postulated mechanism of action is alteration of inflammation and wound repair. However, the data supporting the usage of steroids in the management of vocal fold inflammation are conflicting. For example, Coleman et al¹⁰ found that steroids actually caused a delay in vocal fold wound healing following microflap surgery in a canine model. Results revealed increased inflammatory infiltrate around the steroid-treated microflap at 2, 4, and 6 weeks; a 12-day delay in the presence of inflammatory infiltrate; and a 21-day delay in the neovascular response from triamcinolone treatment at the time of microflap.¹⁰ A more recent investigation of vocal fold surgical incisions injected with or without dexamethasone found no difference in the inflammatory response but did reveal a decrease in the rate of collagen deposition in steroid-treated vocal folds at 3 and 7 days postinjury ($P = .002$).¹¹ Despite these data, prophylactic use of steroids at the time of vocal fold microsurgery continues to be advocated to minimize the inflammatory process and improve healing outcomes.¹²

Given the conflicting evidence regarding triamcinolone, further studies are needed to investigate the effects of triamcinolone on modulation of inflammatory and profibrotic signaling in the treatment of laryngeal disorders. In the present study, we investigated the hypothesis that triamcinolone modulates acute vocal fold inflammatory and profibrotic signaling during acute phonotrauma. An in vivo rabbit phonation model was used to investigate the effects of prophylactic administration of triamcinolone acetonide on

transcript levels of the vocal fold inflammatory and profibrotic genes cyclooxygenase-2 (COX-2), interleukin (IL)-1 β , and transforming growth factor (TGF)- β 1.

Materials and Methods

Animals

The animal care and use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Vanderbilt University Medical Center, in accordance with Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq). Forty New Zealand white breeder rabbits weighing between 3 and 5 kg were randomly assigned to receive no intervention (n = 10), 30 minutes of raised intensity phonation (n = 10), bilateral intralaryngeal sham injection followed by 30 minutes of raised intensity phonation (n = 10), or bilateral triamcinolone acetonide injection of 400 μ g in 25 μ L total volume followed by 30 minutes of raised intensity phonation as described previously (n = 10).^{13,14} Sham injection consisted of sodium chloride for isotonicity, 0.99% (w/v) benzyl alcohol, 0.75% carboxymethylcellulose sodium, and 0.04% polysorbate 80 with pH adjusted to between 5.0 and 7.5 with sodium hydroxide or hydrochloric acid. This was consistent with the triamcinolone acetonide carrier without active medication. All investigators were blinded to the substance injected until final analysis. An appropriate level of anesthesia was obtained using ketamine 35 mg/kg, xylazine 5 mg/kg, and acepromazine 0.75 mg/kg injected intramuscularly. Additional anesthesia (ketamine 17.5 mg/kg, acepromazine 0.375 mg/kg) was delivered intramuscularly as necessary throughout the procedure to maintain a surgical plane of anesthesia. Anesthesia monitoring included heart rate, temperature, and oxygen saturation levels to assess the animal's general well-being and state of anesthesia.

Surgical Procedure

The neck was shaved and prepped from the submentum to the chest. Animals were placed in the supine position, and an incision was made from the hyoid bone to the sternal notch. The larynx and trachea were exposed. The trachea was transected proximal to the sternum, and sutures were used to suspend the lower portion of the trachea to the sternal fascia. A 3.5-mm cuffed endotracheal tube (RUSCh, Kernen, Germany) was placed into the upper portion of the trachea approximately 2 cm from the vocal folds. The cuff of the endotracheal tube was then inflated to seal off the trachea and deliver airflow through the glottis. For animals undergoing laryngeal injection, a 25-gauge spinal needle was used to inject superficially into the vocal folds. All dilutions of injected substance were in a total of 25 μ L of solution and were injected bilaterally. Two electrodes were inserted into the cricothyroid membrane bilaterally to serve as anodes for electrical stimulation. Two electrodes were also inserted into the cricothyroid muscle bilaterally to serve as cathodes for electrical stimulation, as described previously.¹³ A Neptune humidifier (Teleflex Medical Incorporated, Triangle Park, North Carolina) and Gilmont Instruments flowmeter (GF-8522-1700; Barrington, Illinois) were used to deliver compressed humidified air heated to 37°C to the glottis.

A Grass S-88 stimulator (SA Instrumentation, Encinitas, California) and constant current isolation unit (Grass Telefactor, model PSIU6; West Warwick, Rhode Island) provided electrical stimulation to the larynx. The total train duration was 10 seconds (3 seconds on; 7 seconds off), as described previously.¹³ A 5-degree 2.7-mm rigid endoscope (Karl Storz Endoscopy-America, Inc, El Segundo, California) and Telecam-C camera (Karl Storz Endoscopy-America) were used to obtain video documentation of vocal fold positioning and glottic closure. Acoustic output from the animals was recorded using a Shure SM48 unidirectional dynamic microphone (Shure, Inc, Niles, Illinois) placed 10 cm from the opening of the laryngoscope and digitized using the Computerized Speech Lab (CSL Model 4500; KayPENTAX, Lincoln Park, New Jersey). Three to five 0.5- to 1.0-second samples were selected from stable portions of the acoustic waveform and extracted to determine mean phonation intensity and mean fundamental frequency. As previously described, raised intensity phonation was defined as a minimum of a 5-dB (within-rabbit) increase in phonation intensity from modal phonation. This intensity increase was maintained at or above 5 dB throughout the 30-minute phonation period.¹³ Following the 30-minute raised intensity phonation and subsequent 30-minute recovery period, all animals were euthanized and larynges harvested. The lamina propria was then dissected from the underlying thyroarytenoid muscle under microscopic visualization. Specimens did not include muscle. Tissues were stored at -80°C for later analysis.

Reverse Transcriptase

An ultrasonic dismembrator 150E (Fisher Scientific, Pittsburgh, Pennsylvania) was used to homogenize specimens. RNeasy Mini Kits (Qiagen, Valencia, California) were used to isolate total RNA. This was then treated with ribonuclease-free deoxyribonuclease I (Qiagen) to minimize genomic DNA contamination. Total RNA quantity was determined with the A260/A280 ratio using a Nanodrop ND-2000c (Thermo Scientific, Waltham, Massachusetts), and electrophoresis was used to evaluate the quality based on the appearance of the 18S and 28S ribosomal RNA bands. Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, California) using the manufacturer's recommended reaction protocol. Reactions were performed using a Veriti thermal cycler (Applied Biosystems) using the following parameters: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, and 4°C for 5 minutes.

Quantitative Polymerase Chain Reaction

Rabbit-specific primers for COX-2, IL-1 β , TGF- β 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Integrated DNA Technologies, Coralville, Iowa) were used for quantitative polymerase chain reaction (qPCR). The PCR products were verified by DNA sequencing. Quantitative PCR was performed in a final volume of 20 μ L in accordance with the manufacturer's reaction protocols. The reaction mix comprised template cDNA, 10 μ L POWER SYBR Green Master Mix (Applied Biosystems), 0.25 μ M final concentration of each primer, and ribonuclease-free water. Quantitative PCR was performed under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of denaturing at 95°C for 15 seconds and annealing at 60°C for 1 minute. Fluorescence was detected using an Applied Biosystems StepOnePlus System. The Ct method was used to determine the relative ratio of gene expression for each gene.

Statistical Analysis

Power Analysis—Statistical power was computed a priori, and the study was powered to 80% power ($\beta = .20$) for detecting differences between groups at an adjusted α level of .0125. Power was based on a number of observations for the t test of differences between 2 means for a relative effect size of 0.65.

Assessment of Treatment Effects—To assess for steroid treatment effects, independent samples Kruskal-Wallis non-parametric tests were used to investigate for overall main effects for gene expression across treatment and control groups using an adjusted P value of .0167 to control for Type I error. If the overall main effect was significant, independent samples Mann-Whitney U tests were used to investigate the following planned pairwise comparisons: control vs no treatment, no treatment vs sham treatment, no treatment vs steroid treatment, and sham treatment vs steroid treatment using an adjusted P value of .0125 to account for multiple pairwise comparisons (Bonferroni correction). All analyses were performed with the use of 2-tailed P values. Data were analyzed using PASW Statistics 18.0 (SPSS, Inc, an IBM Company, Chicago, Illinois).

Results

Treatment Effects

Log-transformed expression ratios for the following dependent variables—COX-2, IL-1 β , and TGF- β 1—were compared between treatment and control groups.¹⁵ Kruskal-Wallis testing revealed a significant main effect for COX-2 gene expression across groups ($P = .002$) (Figure 1). Post hoc testing revealed that the result in the group not receiving treatment (30 minutes of raised intensity phonation only) of 15.10 was higher than the result of 5.90 in the control group (no intervention; $P < .001$). There were no significant differences observed for COX-2 gene expression between no treatment compared with sham treatment ($P = .870$), no treatment compared with steroid treatment ($P = .096$), or sham treatment compared with steroid treatment ($P = .683$). Kruskal-Wallis testing revealed a significant main effect for IL-1 β gene expression across groups ($P < .001$) (Figure 2). Post hoc testing revealed that the result in the group not receiving treatment (30 minutes of raised intensity phonation only) of 14.70 was higher than the result of 6.30 in the control group (no intervention) ($P = .001$). There was a nonsignificant increase in IL-1 β gene expression between no treatment compared with sham treatment ($P = .014$) and a nonsignificant increase in IL-1 β gene expression between no treatment compared with steroid treatment ($P = .041$). There were no significant differences observed between sham treatment and steroid treatment ($P = .744$). Kruskal-Wallis testing revealed no significant main effect for TGF- β 1 gene expression across groups ($P = .525$) (Figure 3).

Discussion

In a recent review on steroid injections for vocal fold disorders, Campagnolo et al¹² recommended the use of steroids for acute inflammatory diseases, autoimmune laryngeal diseases, laryngeal stenosis, benign lesions of the vocal folds, and following phonosurgery. Yanagihara and colleagues¹⁶ were among the first to report the usage of corticosteroid

injections for vocal fold nodules. Subsequently, Tateya et al¹⁷ reported improvement in patient self-ratings of hoarseness from office-based steroid injections for mild Reinke edema.

Tateya et al¹⁸ also reported improved aerodynamic measures and resolution of vocal fold nodules in 17 of 27 patients treated with intracordal steroids. Similarly, Hsu et al¹⁹ reported a 91% response rate and a 59% complete remission rate with the use of corticosteroid injections for vocal fold polyps, whereas Mortensen and Woo⁹ found an 82% improvement rate in patients treated with steroids for postoperative scar, vocal fold nodules/polyps, and granulomas.

In contrast, a study by Coleman et al¹⁰ reported that triamcinolone caused a delay in wound healing, with a 12-day delay in inflammatory infiltrate and a 21-day delay in the neovascular response after microflap surgery. Despite conflicting data regarding the efficacy of triamcinolone acetonide, this steroid continues to be used as an intraoperative injection into the vocal folds to attenuate the inflammatory response.¹⁷ It is postulated that application of this steroid decreases or limits the extent of inflammation and leads to better healing and recovery of normal vocal fold function following injury.

In the present study, we performed a prophylactic evaluation of triamcinolone vocal fold injections using an in vivo animal model of acute phonotrauma. Justification for the time point used in the current study (30 minutes of phonation followed by a 30-minute recovery period) was based on the pharmacokinetics of triamcinolone, which exerts a pharmacologic effect within 1 to 2 hours, and the expression patterns of the inflammatory mediators analyzed: COX-2, which increases 30 minutes postinjury and peaks at 1 to 2 hours; IL-1 β , which increases 1 hour postinjury and peaks at 4 to 8 hours; and TGF- β 1, which increases 1 hour postinjury and peaks at 72 hours.^{15,20–22} Justification for the 400- μ g dose of triamcinolone was based on clinical usage of this medication at 40 mg/mL and volume-difference calculations between human and rabbit vocal folds.

Of the markers investigated in the present study, COX-2 is the first to peak following injury and is highly upregulated during the acute inflammatory process. In the present study, COX-2 was significantly increased in the vocal folds after acute phonotrauma (rabbits undergoing 30 minutes of raised intensity phonation without treatment) compared with control (no intervention). There was a general trend for decreased COX-2 gene expression in vocal folds receiving steroid treatment (triamcinolone 400 mcg) compared with vocal folds experiencing acute phonotrauma without treatment, although these differences were not significant. Increased COX-2 has been associated with fibroblast activation and is upregulated in the skin following injury.²³ In vocal folds, COX-2 has been shown to be elevated during the acute stages of vocal fold injury.²¹

Interleukin-1 β , which is produced by neutrophils, is an early activator of growth factor expression in macrophages, keratinocytes, and fibroblasts.²⁴ Interleukin-1 receptor (IL-1R) has been noted in multiple inflammatory responses to increase the release of prostaglandins. Neutralization of IL-1 β has been noted to decrease the inflammatory response associated with injury.²⁵ Results of the present study revealed a significant increase in IL-1 β gene

expression in the vocal folds after acute phonotrauma (rabbits undergoing 30 minutes of raised intensity phonation without treatment) compared with control (no intervention), as well as a nonsignificant increase in IL-1 β gene expression after sham treatment and steroid treatment. It is postulated that the needle insertion and/or steroid injectate may have caused the nonsignificant increase in IL-1 β gene expression by disrupting the vocal fold architecture and microvascular network. Interestingly, the finding that steroid treatment may result in an increase in IL-1 β appears to support the Coleman et al¹⁰ finding of a delay in wound healing and an increase in inflammatory infiltrate in steroid-treated microflaps at 2, 4, and 6 weeks.

Transforming growth factor- β is a well-characterized cytokine in the inflammatory pathway and is present in abundance in platelets. It is known to be an initiating and terminating factor in tissue repair and is an important mediator in tissue fibrosis and scarring.²⁶ It also has been deemed an important mediator of vocal fold inflammation and lends itself well to therapeutic manipulation.²⁰ Results of the present study revealed no significant differences in TGF- β 1 gene expression between treatment and control groups. Recent studies have revealed a possible alternative mechanism for the action of steroids in tissue. A new theory has been proposed whereby steroids prevent decreased expression and altered cellular localization of tight junction proteins, leading to decreased cell permeability.²⁷ Given the nature of the present study on acute changes in gene transcript levels, protein level changes were not examined. Based on the finding of no observable changes in the inflammatory factors investigated at the gene transcript level, it is likely that protein changes would not have been detectable at the time point investigated. Future studies will be necessary to investigate the effects of steroid treatment on inflammatory and tight junction gene and protein changes at various time points prior to and following the induction of phonotrauma, as well as the chronic effects of steroid use on vocal fold wound healing.

Conclusion

Triamcinolone is used for the treatment of myriad laryngeal disorders. However, the effects of triamcinolone have not been demonstrated at the molecular level in the larynx. Results of the present experiment revealed a significant increase in COX-2 and IL-1 β gene expression after acute phonotrauma. Prophylactic administration of triamcinolone immediately prior to the induction of acute phonotrauma resulted in no significant changes in COX-2, IL-1 β , and TGF- β 1 gene transcript levels.

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Figure 1. Log-transformed gene expression ratios for cyclooxygenase-2 (COX-2) between control, no treatment, sham treatment, and steroid treatment groups.

Figure 2. Log-transformed gene expression ratios for interleukin (IL)-1 β between control, no treatment, sham treatment, and steroid treatment groups.

Figure 3.

Log-transformed gene expression ratios for transforming growth factor (TGF)-β1 between control, no treatment, sham treatment, and steroid treatment.