Vocal Exercise May Attenuate Acute Vocal Fold Inflammation

***,†,‡Katherine Verdolini Abbott, §Nicole Y. K. Li, ‖Ryan C. Branski, †,*,‡Clark A. Rosen, ¶Elizabeth Grillo, #Kimberly Steinhauer, and *,‡,**,††Patricia A. Hebda,** *†‡#**††*Pittsburgh, Pennsylvania,* §*Madison, Wisconsin,* ‖*New York, New York, and* ¶*West Chester, Pennsylvania*

Summary: Objectives/Hypotheses. The objective was to assess the utility of selected "resonant voice" (RV) exercises for the reduction of acute vocal fold inflammation. The hypothesis was that relatively large-amplitude, low-impact vocal fold exercises associated with RV would reduce inflammation more than spontaneous speech (SS) and possibly more than voice rest.

Study Design. The study design was prospective, randomized, and double blind.

Methods. Nine vocally healthy adults underwent a 1-hour vocal loading procedure, followed by randomization to a SS condition, vocal rest condition, or RV exercise condition. Treatments were monitored in clinic for 4 hours and continued extraclinically until the next morning. At baseline (BL), immediately after loading, after the 4-hour in-clinic treatment, and 24 hours post-BL, secretions were suctioned from the vocal folds bilaterally and submitted to enzyme-linked immunosorbent assay to estimate concentrations of key markers of tissue injury and inflammation: interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor α, matrix metalloproteinase (MMP)-8, and IL-10.

Results. Complete data sets were obtained for three markers—IL-1β, IL-6, and MMP-8—for one subject in each treatment condition. For these markers, results were poorest at 24-hour follow-up in the SS condition, sharply improved in the voice rest condition, and was the best in the RV condition. Average results for all markers and responsive subjects with normal BL mediator concentrations revealed an almost identical pattern.

Conclusions. Some forms of tissue mobilization may be useful to attenuate acute vocal fold inflammation.

Key Words: Vocal fold inflammation–Wound healing–Tissue mobilization–Resonant voice.

INTRODUCTION

Traditional management of acute vocal fold injury emphasizes voice conservation. Classically, patients with acute injury are advised to restrict both amount and loudness of phonation to facilitate recovery.[1,2] The underlying physiological rationale for this approach is reasonable. Perpendicular impact stress to the vocal fold tissue is thought to be the most direct cause of phonotrauma.[3–5] Therefore, restricting phonation posttraumatically should minimize aggravating stresses, presumably enhancing the inherent tissue healing phenotype and also minimizing the likelihood of new injury during the recovery period.

This approach is imminently sensible. However, emerging data from other domains suggest the counterintuitive notion that in some cases, tissue mobilization may be anabolic, optimizing the resolution of inflammation and also the long-term outcome of injury (eg, Ref. 6). Although these principles have translated to clinical practice in other fields, they have not yet been systematically explored in the context of vocal fold injury. Recent data from our laboratory suggested that dynamic biomechanical strain limited the inflammatory phenotype in vocal fold fibroblasts *in vitro*,[7] suggesting a putative antiinflammatory role for some forms of vocal motion over voice rest. However, the clinical translation of these preliminary findings is tenuous. First, the forces placed on the fibroblasts in our *in vitro* investigations only dimly mimic the *in vivo* phonatory environment. Second, the value of mobilization or exercise after vocal fold injury in humans has not yet been reported. As such, the present study sought to systematically investigate the potential for tissue mobilization or exercise in the form of "resonant voice" (RV) exercises as a means to improve outcomes in patients with acute vocal fold injury.

Relevant background is as follows. Given their anatomic position, the vocal folds are inherently susceptible to various sources of insult, ranging from chemical to surgical injury and mechanical trauma from phonation. Regardless of the source, in most cases, tissue injury initiates a cascade of biochemical events ideally leading to the reconstitution of functional tissue. The initial stage of the wound healing response is commonly referred to as the inflammatory phase. Events in this phase control the flow of blood into the injury site, recruit inflammatory cells, neutrophils, and macrophages to ensure a sanitary and viable wound environment, and perhaps most importantly, produce growth factors and cytokines that regulate subsequent events in wound healing. In fact, processes in the acute phase of wound healing may influence the quality of the ultimate outcome of healing. Specifically, consensus exists that limiting the magnitude of the inflammatory response generally leads to improved tissue architecture and function in the long term.[8]

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From the *Department of Communication Science and Disorders, University of Pittsburgh, Pittsburgh, Pennsylvania; †Department of Otolaryngology, University of Pittsburgh Voice Center, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; ‡McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania; §Department of Surgery, University of Wisconsin-Madison, Madison, Wisconsin; ‖Department of Otolaryngology, New York University School of Medicine, New York, New York; ¶Department of Communicative Disorders, West Chester University, West Chester, Pennsylvania; #Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, Pennsylvania; **Department of Otolaryngology-Pediatric Division, Otolaryngology Wound Healing Laboratory, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; and the ††Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to Katherine Verdolini Abbott, Department of Communication Science and Disorders, 4033 Forbes Tower, Pittsburgh, PA 15260. E-mail: kittie@csd.pitt.edu

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Most relevant to laryngology is the need to limit the development of benign vocal fold lesions, such as scar. In fact, attempts are often made pharmacologically to inhibit vocal fold inflammation with steroids (systemic, peroral, or intramuscular). This practice is particularly prevalent in the management of voice problems within the performing arts community[9] but has also shown promise for problems in other patients with benign vocal fold lesions[10] and Reinke's edema.[11,12] Although clinical evidence suggests that steroids may be a satisfactory therapeutic option in the short term, the long-term negative consequences of prolonged steroid use often outweigh the therapeutic benefits.

Ideally, therapeutic intervention for vocal fold inflammation should be developed to not only attenuate the inflammatory response but also circumvent the potential negative consequences of pharmacologic treatments. Mechanical signaling paradigms appear to meet these criteria. Specifically, *in vitro* and *in vivo* data from other tissues suggest that some forms of tissue mobilization may be inherently antiinflammatory. For example, low levels of mechanical signaling reduced gene expression for many proinflammatory mediators, including cyclooxygenase-2, in cells from a number of connective tissues *in vitro*.[13–16] The anabolic effects of mechanical signaling are thought to be due to the inhibition of nuclear factor kappa B translocation into the nucleus via the inhibition of inhibitor kappa B degradation.[17,18] These processes have only recently been elucidated in the vocal folds.[7]

In other domains, *in vitro* data have translated to clinical practice. For example, historically, the primary treatment for severe ankle inversion sprains was complete immobilization. In contrast, contemporary management approaches involve tissue mobilization in these patients yielding improved outcomes, including decreased pain ratings and improved range of motion.[19] Furthermore, tissue mobilization has been associated with decreased fibrosis in the surgically injured patellar tendon.[20] These emerging data provide the primary theoretical foundation for the systematic investigation of vocal fold inflammation and the role of vocal fold mobilization tasks that may modulate postinjury vocal fold inflammation.

The fundamental challenge in this type of investigation is methodological. Until recently, no methods allowed the quantitative characterization of vocal fold inflammation in humans. Our laboratory reported on the putative utility of assaying secretions collected from the vocal fold surfaces for biochemical mediators of wound healing. In our initial report, we described the collection of secretions from a single subject before and after 1 hour of high-intensity vocal loading. Secretions were then assayed for key proinflammatory mediators, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and several key matrix metalloproteinases (MMPs). Soluble inflammatory mediators were indeed captured in the assays. As important, increased mediator levels corresponded with the clinical appearance of vocal fold injury.[21] Specifically, concentrations of IL-1β, TNF-α, and MMP-8 increased sharply after vocal loading. In contrast, levels of transforming growth factor β and prostaglandin E_2 remained constant, suggesting some degree of selectivity of this assay, in that not every mediator elevated in the underlying tissue is detectable in airway secretions. Several subsequent reports have provided evidence regarding the validity of the approach. Findings in these reports have replicated expected results for inflammatory mediators in acute and chronic vocal fold injury in both animal models and human pathologies.[22–24] Furthermore, the approach has been validated in the oral biology literature (eg, Ref. 25), and more recently, in the laryngology literature.[26] Thus, there is a peer-reviewed support for the general validity of the approach to provide insight into the inflammatory state of vocal fold tissue.

The present study uses this method to explore the potential utility of vocal fold tissue mobilization in the form of RV exercises to limit inflammation subsequent to acute phonotrauma. RV has been defined perceptually as a voicing pattern associated with anterior oral vibratory sensations in the context of "easy" phonation.[27,28] This voicing pattern has been shown to be associated with barely ad- or abducted vocal folds engaged in relatively large-amplitude and low-impact vocal fold vibrations.[27,29–31] These biomechanical features of RV make it an attractive rehabilitation approach for investigation into the potential therapeutic properties in acute phonotrauma. Specifically, the large-vibration feature of RV may help to limit the influx of inflammatory mediators into the tissue, due to cell deformation associated with cyclic tensile strain, while at the same time increasing concentrations of antiinflammatory mediators unleashed by tissue motion.[32–34] In this sense, the relatively large vocal fold vibrations associated with RV may function as something of a biological "healing" factor in acute injury. At the same time, the low-impact feature of RV may function as a biological "prevention" mechanism by limiting new injury to the tissue during the recovery period. In addition to these biological considerations, an anecdotal case report provided clinical evidence that RV may offer therapeutic benefits in patients with acute phonotrauma.[27]

Together, these considerations prompted the generation of the primary experimental hypotheses for the present study: voice rest would generally enhance resolution of acute vocal fold inflammation compared with spontaneous speech (SS) posttraumatically, but inflammation would be most improved after RV exercises, 4 hours after the initiation of treatment and 24 hours postbaseline (post-BL). Specific predictions were that concentrations of *inflammatory* mediators, IL1-β, IL-6, IL-8, MMP-8, and TNF-α, would be greatest 4 hours after treatment initiation and 24 hours post-BL in the SS condition, smallest in the RV condition, and intermediate in the voice rest condition. Conversely, concentrations of an *antiinflammatory* mediator, IL-10, should be greatest at the same time points in the RV condition and smallest in the voice rest condition, due to influx of this mediator triggered by tissue mobilization. Evidence to this effect would be consistent with results from published *in vitro* studies suggesting that some forms of tissue mobilization may have value in limiting the inflammatory response in human vocal folds.[7]

A secondary hypothesis regarded the ability of noninvasive aerodynamic and perceptual measures to quantitatively capture the time-varying inflammatory status of the vocal folds.

Specifically, we hypothesized that phonation threshold pressures (PTPs) from high-pitched, quiet phonation, and direct magnitude estimation (DME) of perceived phonatory effort (DME) would not covary tightly with inflammatory mediator concentrations, despite claims that these measures may be useful in the detection of vocal fold injury.[35,36] Our skepticism was related to the fact that both PTP and DME reflect multidimensional factors (eg, Ref. 37; Colton, personal communication), only a limited number of which would be captured by the inflammatory status of the tissue. A tertiary hypothesis, incidental to the main focus of the study but nonetheless valuable to entertain, regarded the relationship between PTP and DME values. These physiological and psychological indices of phonatory effort have long been suggested to covary.[38] However, careful scrutiny of the literature indicates that the covariance may be considerably weaker than often assumed.[38]

MATERIALS AND METHODS

General paradigm

The general experimental paradigm is displayed in Figure 1. Nine vocally healthy subjects participated in a between-subjects study. All subjects first produced vocalization samples for PTP and DME measures, as described in detail shortly. Then, after the delivery of a local anesthetic to the larynx, laryngeal secretions were suctioned from the vocal fold surfaces bilaterally. Subjects then rested for 60 minutes to allow dissipation of anesthetic effects and subsequently underwent a 60-minute vocal loading session. During loading, subjects alternated 15 minutes of loud phonation with 5 minutes of rest, for a total of three cycles over the 60-minute loading period. PTP and DME data collection occurred at the boundary between phonation and rest periods during loading, for a total of three PTP and DME collection time points during and immediately after the loading epoque. Laryngeal secretions were again collected 20–30 minutes after the completion of loading. Subjects were then randomly assigned, stratifying by gender, to either SS,[1] voice rest,[2] or RV exercise conditions,[3] described shortly. Subjects underwent treatment conditions for 4 hours in the clinic, under the supervision of a voice trainer who, for most subjects, was blinded to the experimental hypotheses. Secretion, PTP, and DME samples were collected again at the end of the 4-hour period, and subjects were sent home with instructions to continue with their respective treatments during waking hours, until their return to the clinic the next morning. Subjects were also instructed to avoid alcohol, smoke, late-night eating, and any voice use outside the prescribed treatment for the evening. Subjects returned 24 hours after initial BL for collection of final PTP, DME, and laryngeal secretion samples. The 4-hour treatment period was motivated by parallel *in vitro* studies of vocal tissue inflammation in our laboratory.[7] The 24-hour time point was motivated by an attempt to control potential time-of-day effects in the BL versus follow-up data.

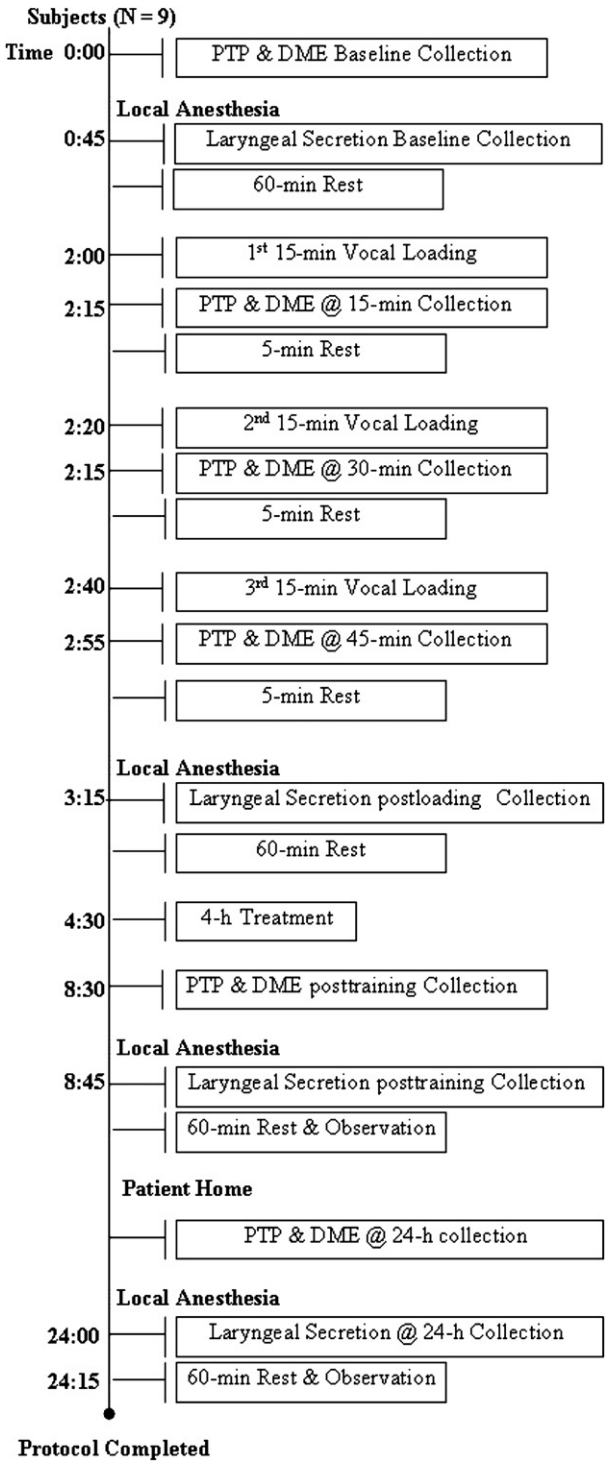

FIGURE 1. General experimental paradigm.

Participants

The study was approved by the Institutional Review Board at the University of Pittsburgh. A total of nine subjects participated in the study: six females (21–46 years) and three males (21–29 years). Subjects were compensated for their participation. Both females and males were included in the study so that the population of individuals who may have acute phonotraumatic injury would be proportionately represented in terms of gender. The ratio of females to males in our study (2:1) was guided by conservative estimates in the literature regarding the frequency of voice problems in females over males in the population (eg, Refs. 39,40).

Specific inclusion criteria were males or females between the ages of 18 and 55 years,[1] in good overall health by self-report,[2] lack of extreme gag response to light base of tongue palpation based on clinical examination[3]; nasal patency sufficient to pass a flexible scope at least unilaterally[4]; normal hearing as tested bilaterally at 20 dB to 8000 Hz[5]; ability to produce RV during initial training as determined by an examiner perceptually, despite no history of formal voice training of any type by subject report,[6] and ability to produce loud voice, between 85 and 95 dB measured 25 cm from the mouth.[7] Exclusion criteria were self-report of a current voice problem or a voice problem more than once monthly during the preceding year[1]; previously diagnosed speech and/or language deficits in adulthood (childhood disorders were not exclusionary)[2]; current use of any medications that might influence voice (eg, diuretics, decongestants, and antihistamines) or signs of currently active allergic process[3]; known or suspected allergy to any anesthetic, in particular lidocaine[4]; current smoker per subject report,[5] and report of pregnancy.[6] Also, females enrolled in the study were scheduled to participate at the midmonth mark in the menstrual cycle (ie, nonmenstruating).[7] Finally, subjects with vocal fold lesions or risk of hemorrhage were excluded, based on laryngologic examination.[8]

Experimental design

The between-subjects study used a two-way mixed model design with time (BL, postloading, 4-hour posttreatment start, and 24-hour post-BL follow-up) as the within-subjects factor and treatment condition (SS, voice rest, and RV) as the between-subjects factor. Dependent variables for the primary research question were concentrations of inflammatory mediators, IL1-β, IL-6, IL-8, MMP-8, TNF-α, and IL-10, normalized to BL values and, therefore, unitless. Dependent variables pertinent to secondary and tertiary research questions were PTP and DME data (centimeters of water and self-ratings, respectively). Subjects were blinded to experimental hypotheses, and all data analyses were conducted by individuals blinded to subjects' condition. Thus, the study was double blinded.

Equipment

PTP data were obtained using the *Aerophone II Phonatory Function Analyzer* (KayPentax, Lincoln Park, NJ) system with a Rothenberg (1973) circumferentially vented face mask (#5 Ambu). The *Aerophone II* was calibrated before each day of data collection. Target pitches for PTP and DME measures (C4, 262 Hz, males; C5, 523 Hz, females) were provided in free field using a *Dell RT 7D00 keyboard* (Dell, USA), and a target syllable production rate of 88 beats per minute for syllable utterance in the PTP task was guided by a *Sabine MT-8000 metronome* (Sabine, Alachua, FL).[41] Aerophone data were later transferred to a separate computer (*Dell Pentium 4 Prescott DT*, 3.6 GHz) with a custom software program for data analysis.

Rigid videolaryngostroboscopy was performed using the *KayPentax Model* 9016 (KayPentax, Lincoln Park, NJ). Flexible endoscopy was performed using an *Olympus 1300446 2-mm channel chip-tip flexible laryngoscope* (Olympus, Center Valley, PA).

Vocal loading trials were recorded using a *Panasonic SV 3900 professional digital audio tape deck* and *SX202 Dual Microphone Preamp Symetrix microphone converter box* (Panasonic, Japan). The microphone was an *AKG Acoustics C410 headset miniature condenser microphone* (AKG Acoustics GmbH, Vienna, Austria) with a behind-the-neck headband (fundamental frequency range, 20–2000 Hz; maximum sound pressure level [SPL], 123 dB SPL). Microphone calibration was established individually for each subject at the onset of vocal loading. For calibration, a number 33-2050 *Radio Shack Sound Level Meter* (RadioShack, Forth Worth, TX) and the microphone were positioned in parallel at a 45-degree angle three inches from the subject's mouth. A *Servox AG 51109 electrolarynx* (Servox AG, Koeln, DE) was used to generate a calibration tone delivered to the center of the subject's lips. The intensity level was noted from the sound level meter and announced and recorded on the digital audiotape.

Procedures

Approximately 30 days before their participation in the experimental component of the protocol, subjects provided informed consent and were prescreened in the clinic for gag response and nasal patency. Individuals showing heightened gag in response to light base of tongue palpation and subjects with poor nasal patency were excluded from further participation. On the initial day of the experiment proper, subjects first received pretraining in PTP and DME data collection procedures. For PTP, subjects produced repeated sets of /pi pi pi pi pi/ utterances as quietly as possible at C4 (262 Hz; males) or C5 (523 Hz; females) indicated with a keyboard and a rate of 88 beats per minute indicated by a metronome. The target pitches were chosen based on empirical observations that high pitches are the most sensitive to various experimental manipulations,[42,43] and clinically, most subjects appear able to produce those particular nonultrahigh pitches even under conditions of vocal fold inflammation. The target rate of syllable production was based on reports that this general range of rates facilitates valid estimation of subglottic pressure from oral pressure data.[41] Pitches were verified perceptually by a trained examiner to an accuracy of about one-quarter tone for each syllable. The examiner also monitored subjects' loudness for PTP trials, empirically, continually encouraging them to phonate as quietly as possible. Training for the PTP task continued until the examiner and subject considered that the subject could perform the task reliably according to criteria, typically 5 minutes or less. Subjects were then trained in DME procedures, which required subjects to rate their perceived phonatory effort for the preceding set of PTP trials on a scale on which "1" represented comfortable effort, "2" represented twice as much effort as comfortable, and so forth.[44,45] There was no upper (or lower) limit to the scale.

After subjects completed pretraining in PTP and DME procedures, the first set of formal samples of these measures was collected. For BL and all subsequent PTP and DME data collection time points, three sets of /pi pi pi pi pi/ strings were collected using the foregoing criteria, and one DME value was extracted to reflect the subject's perception of phonatory effort for the preceding set of PTP trials.

After completion of initial PTP and DME data collection, a laryngologist examined the subject's oral cavity, oropharynx, and nasal cavity and placed a cotton pledget soaked with lidocaine and decongestant into the subject's most patent nasal cavity. The oropharynx was also anesthesized via topical aerated 4% plain lidocaine. Rigid laryngoscopy with stroboscopy was performed to obtain BL images of the larynx. Then, 4% lidocaine was dripped onto the endolarynx through the working channel of a flexible laryngoscope. After about 5 minutes, subsequent to verification of anesthesia of the vocal folds to light touch, a 1-mm plastic suction catheter was passed through the working channel of the scope and guided down to the free edge and superior surface of the vocal folds, and a gentle suction was applied. This procedure allowed the collection of a small amount of vocal fold secretions, about 100 μL. Secretions were captured in a modified sinus trap and then transferred into a 0.2-mL microfuge tube via a 1 cc syringe. The tubes were labeled using codes that could not be traced to the subject or the subject's condition, except by way of a master list retained by one investigator not involved with data analysis. Tubes were placed on dry ice and stored at -80°C until analysis.

Secretion collection was followed by a 60-minute rest period to allow dissipation of the anesthetic. During the period, subjects were monitored for their compliance with instructions to be completely silent and refrain from eating or drinking. Subjects then initiated participation in a 60-minute vocal loading session. For the loading session, subjects repeated three cycles of 15 minutes of loud voice production alternated with 5 minutes of voice rest. Collection of PTP and DME data, which took less than 30 seconds, occurred at the boundary of the loading and resting phases. For vocal loading itself, subjects used theater monologues, other written material, or simply engaged in conversation with an investigator.

Although acoustic analyses of vocal loading were not planned, loading trials were audio recorded (see Equipment section) in case such analyses should become relevant. For loading, a sound level meter was positioned at a constant distance of 25 cm from the subject's mouth so that an experimenter could monitor relative intensity levels during loading. The examiner monitored the meter nearly constantly and cued subjects to maintain a target intensity range of 75–90 dB during phonatory loading.

About 20–30 minutes after loading was completed, subjects received laryngeal anesthesia and underwent secretion collection as previously. Subjects then rested again for 60 minutes to allow the dissipation of anesthetic effects. As previously, during that period, subjects were silent and refrained from eating or drinking. Subsequently, subjects were randomized to one of three treatment conditions, described in detail shortly: SS,[1] voice rest,[2] or RV.[3] Randomization was constrained by stratification by gender: two females and one male were assigned to each treatment condition. Subjects then underwent treatments for 4 hours in the clinic, as monitored by one of two voice experts. Six subjects (two subjects in each of three experimental conditions, including four females and two males distributed equally across the conditions) were monitored by a doctoral level singing voice specialist with approximately 20 years of experience, who was entirely naive to the experimental hypotheses. Three subjects (one subject in each of three experimental conditions, including two females and one male) were monitored by a doctoral level speech-language pathologist and teacher of singing, who was informed about the experimental hypotheses. Although interventionist blinding was not central to the experimental hypotheses at this stage of inquiry, a combination of blinded and unblinded examiners was used so that later explorations of the data might provide some window on whether experimental biases might influence biological results. After the 4-hour in-house treatments, subjects were dismissed from the clinic and were instructed to continue with the same general treatment procedures they had received extraclinically for the remainder of the day (evening) and next morning until they returned to the clinic. Specifics are provided shortly.

On their arrival in the clinic the next morning, all subjects were silent except during elicitation of PTP data and in phonation during laryngeal examination and secretion collection procedures. Compliance with extraclinical requirements was assessed with a checklist indicating subjects' self-reported adherence to requirements and specific times that any exercises were completed. Based on their reports, all subjects were compliant with all requirements (data available on request).

Treatments

Voice rest. For the voice rest condition, subjects were required to maintain absolute silence after vocal loading. No phonation or whispering was allowed. Subjects were encouraged to communicate with pen and paper as needed. After 4 hours of in-house monitoring, subjects were dismissed with the instruction to refrain from any voice use until they returned to clinic for repeated BL data collection the next morning.

Spontaneous speech. For the SS treatment, during the 4-hour in-house monitoring period, subjects spoke with an investigator in what they considered a normal voice about topics that interested them for alternating intervals of 16 minutes followed by 4-minute periods of complete silence. All SS trials were audio recorded as occurred for vocal loading. After 4 hours of in-house monitoring of this regimen, subjects were dismissed with the instruction to continue to use normal conversational speech until their return to the clinic the next morning for repeated BL data collection.

Resonant voice. RV exercises involved repeated prolongations of /m/, /n/, "ng," and /j/, attending to anterior oral vibrations in the context of "easy voice."[27,28] Prolongations were produced in a conversational pitch and loudness range that the clinician and subject agreed was comfortable for the subject, and also in pitch glides and scales that included notes as high as were comfortable for the subject. Following indications in the literature, the achievement of RV was determined perceptually by the clinician and the subject together based on affirmative answers to the questions: "Do you feel vibrations in the front of your face?" and "Is voice easy?" (eg, Ref. 27). Both investigators involved in subject monitoring had extensive clinical experience, 20 years or more, producing and training RV.

During the 4-hour in-house treatment period, RV exercises were produced for alternating cycles of 4 minutes followed by 16 minutes of voice rest. None of the subjects in the RV group, or in the other two groups, had any prior experience with RV.

All RV trials were recorded for later post hoc evaluations, should they become relevant. Throughout all phases of the treatment, RV training and troubleshooting by the clinician focused on the experiential dimension rather than biomechanical verbal explanations (eg, Ref. 46). That is, instructions oriented toward the subject's discovery of RV rather than biomechanical prescriptions around its production. When the 4-hour period of in-house treatment had been completed, the subject was sent home with instructions to maintain voice rest except for 4 minutes of RV exercises every 30 minutes, during waking hours, until the return to the clinic the next morning for repeated BL data collection.

The next morning, 24 hours after the acquisition of BL data, subjects returned to the clinic for final PTP, DME, and laryngeal secretion collection procedures as previously. Subjects were then monitored in-house, while they refrained from eating or drinking for 60 minutes postsecretion collection. Finally, subjects were debriefed regarding the experimental hypotheses and dismissed.

Data reduction

Secretion analysis. All secretion analyses were carried out by an investigator who was blinded to subjects' conditions (time point and treatment condition). For the analyses, a known volume of secretion was aliquoted for analysis and served as the dilution factor. The appropriate volume of sterile saline was added to the tube to bring the total volume up to 2.0 mL. Standard enzyme-linked immunosorbent assays (ELISAs) were performed for IL-1β, IL-6, IL-8, TNF-α, MMP-8, and IL-10 using the manufacturer's recommended protocol (R&D Systems, Minneapolis, MN). These particular markers were selected based on previous work in our laboratory regarding marker levels in laryngeal secretions. In addition, IL-6 and IL-8 were included as they are ubiquitous mediators of inflammation. IL-10, an antiinflammatory cytokine, was assayed to determine if antiinflammatory cytokines are measurable in secretions and if this cytokine may be a relevant indicator of tissue health. All samples were run in duplicate on the same kit to avoid interkit variability. Numeric results were generated based on the standard curve of each assay. Results were calculated as the amount of marker per milliliter of secretion; they were then normalized to the BL levels for each individual subject and combined into groups for data analysis.

PTP and DME data extraction. Custom software was used to analyze PTP data. Analysis for each /pi pi pi pi pi/ production was derived from syllables two to five.[41] Specifically, the software identified the temporal midpoint between adjacent oral pressure peaks for syllables 2–3, 3–4, and 4–5. Estimated subglottal pressures were interpolated for that time point from oral pressures, using a straight line from peak pressure in the earlier syllable to the peak pressure in the latter syllable. For each 5-syllable /pi/ utterance, average values were calculated for PTP and included in the analyses. Ten percent of the PTP data were randomly selected and reanalyzed by a second investigator to determine interinvestigator reliability, using a *Pearson r* correlation.

DME data were recorded straight from subjects' responses at the time of data collection. Reliability checks for DME data were not possible because of the nature of the data collection. That is, we would have had to ask subjects to repeat, within a few moments, their DME estimates for an immediately preceding PTP trial, and they would clearly have remembered what they just said, or alternately we would have had to ask them to recall, at a later time, their sense of phonatory effort for earlier trials. Neither approach was appealing, and thus reliability for DME measures was not evaluated.

Statistical analyses

Inflammatory biomarkers. First, assumptions required for analysis of variance in this two-factor mixed-model design were evaluated. Results revealed that assumptions of normality and homogeneity of variance were patently violated in these biological data. In fact, inspection of the full data set (Table 1) revealed that considerable variability was seen in the data across subjects.

Inspection of the data further revealed that the data sorted into three main types across subjects and inflammatory markers (Figure 2): data showing high BL concentrations of proinflammatory markers ($\geq$1 standard deviation [SD] in the total data set, designated as "preinflamed" data)[1]; data showing normal BL concentrations of markers (<1 SD in the data set), but paradoxically decreasing postloading (nonresponsive data)[2]; and data showing normal BL concentrations of markers (<1 SD in the data set)[3] and numeric increase after loading (responsive). Also, Table 1 shows that a limited number of data points were invalid due to thick secretions that precluded ELISA analysis. Data in each of these categories were relatively evenly distributed across subjects and treatment groups.

In light of these findings, the most straightforward approach to our primary experimental question was to focus the main analyses on the "responsive" data set for subjects showing normal BL mediator concentrations. Completely fortuitously, it turned out that one subject in each of the treatment conditions provided the optimal data set, all having valid responsive data for IL1-β, IL-6, and MMP-8 (subjects 1, 2, and 3, shown among unshadowed data, Table 1). Of note, those subjects were among those who received their respective treatments by a blinded clinician. We proceeded to normalize those subjects' data to their own BL values for the noted markers and evaluated the normalized findings relative to the predicted pattern of results using nonparametric binomial tests, one for the 4-hour and one for the 24-hour time point. For each test, the question was asked whether the data were positioned in the predicted position for the particular marker in question. Specifically, were normalized mediator concentrations for the *inflammatory* mediators (IL-1β, IL-6, IL-8, TNF-α, MMP-8) *greatest* for SS, *lower* for voice rest, and *lowest* for RV conditions post loading? For the *antiinflammatory* mediator, IL-10, which is reportedly triggered by tissue motion,[32] were normalized concentrations *lowest* for

TABLE 1.
Complete Data Set

Treatment Group	Time Point	Subject	Sex	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	TNF-α (pg/mL)	MMP-8 (ng/mL)	IL-10 (pg/mL)
SS	BL	3	F	83	20	1500	3	100	42
	Post			103	27	7200	4	303	83
	4 h			266	116	6600	3	333	75
	24 h			1066	1333	23 000	18	1300	175
	BL	6	F	***128***	*632*	***2151***	**33**	***178***	187
	Post			***28***	*314*	***300***	**36**	***30***	204
	4 h			***88***	*546*	***835***	**119**	***80***	732
	24 h			***466***	*98*	***1443***	**32**	***276***	202
	BL	8	M	**1333**	167	**29 180**	**54**	**1667**	**166**
	Post			**405**	664	**3564**	**73**	**1297**	**140**
	4 h			**15**	220	**420**	**52**	**37**	**265**
	24 h			**57**	107	**440**	**46**	**32**	**346**
Voice rest	BL	1	M	115	4	*4800*	*217*	52	***183***
	Post			222	43	*4100*	*7*	188	***30***
	4 h			408	85	*15 000*	*8*	719	***117***
	24 h			215	37	*7900*	*13*	104	***120***
	BL	5	F	*691*	*488*	***2183***	35	*254*	223
	Post			*98*	*37*	***512***	41	*28*	550
	4 h			*79*	*198*	***733***	43	*45*	120
	24 h			*39*	*236*	***411***	39	*32*	300
	BL	7	F	*770*	*26*	*3187*	*77*	*82*	*151*
	Post			*45*	*24*	*1889*	*35*	*36*	*143*
	4 h			*179*	*20*	*2038*	*40*	*95*	*124*
	24 h			*30*	*15*	*744*	*49*	*28*	*120*
RV	BL	2	F	80	6	620	***13***	80	6
	Post			121	60	3900	***8***	121	60
	4 h			296	77	2600	***10***	296	77
	24 h			36	0	1300	***1***	36	0
	BL	4	M	*34*	**154**	*2307*	*77*	*43*	**667**
	Post			*17*	**257**	*503*	*33*	*26*	**236**
	4 h			*17*	**500**	*252*	*35*	*16*	**2635**
	24 h			*34*	**371**	*701*	*34*	*18*	**213**
	BL	9	F	**18**	75	**8494**	48	**28**	256
	Post			**710**	536	**33 333**	60	**1188**	423
	4 h			**13**	26	**188**	62	**48**	257
	24 h			**1333**	412	**22 206**	55	**1667**	374

Abbreviations: Post, 20–30 minutes postloading; 4 h, 4 h after initiation of treatment; 24 h, 24 h post-BL; M, male; F, female.
Raw data values for marker concentrations across time points, for all subjects.
"Invalid data" are shown in bold. "Preinflamed (only) data" are underlined. "Nonresponsive data" are in bold italics. "Preinflamed-plus-nonresponsive data" are in italics (see text for explanation).

voice rest (no mobilization), *intermediate* for SS (some mobilization), and *greatest* for RV post loading, due to the relatively large-amplitude nature of this latter voicing modality? For both 4- and 24-hour data, each occurrence of a marker in the predicted position within the panel of markers was considered a "success." Separate binomial tests were then used to evaluate the likelihood of the number of "successes" for the total number of "trials" (three markers × three conditions = nine "trials"), for each time point. To evaluate the fuller data set, averages for all valid, BL-normal, and responsive data from all subjects were then evaluated with similar binomial tests to assess results compared with protection. To control alpha inflation, we applied a Bonferroni correction to an overall alpha level of .05, dividing by four tests, such that the alpha level for each test was .0125.

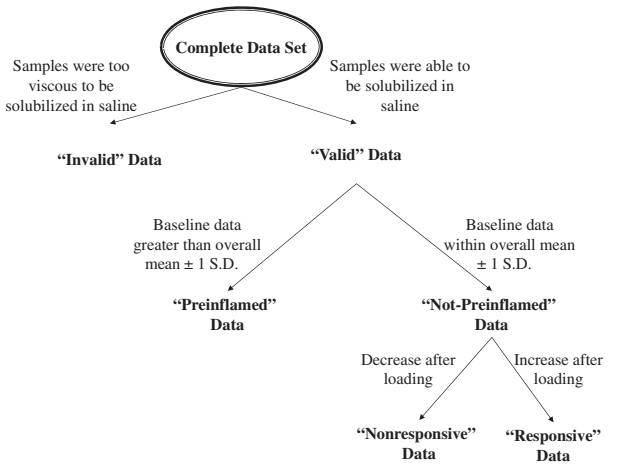

FIGURE 2. Samples with high viscosity could not be solubilized in saline during the dilution procedure and were thus considered "invalid." Remaining data were then sorted into three main categories: "preinflamed data," "nonresponsive data," and "responsive data," as described in the text.

Phonation threshold pressure and direct magnitude estimation. The experimental questions surrounding PTP and DME regarded the extent to which these measures might capture biological variations in the tissue as determined by correlation with inflammatory marker concentrations, and secondarily,[1] the extent to which PTP and DME might covary with each other.[2] For both analyses, we used curve estimation to identify linear, quadratic, and curvilinear relations between PTP and normalized inflammatory concentrations, and between DME and normalized inflammatory concentrations, for the focus data set (subjects 1, 2, and 3 for IL1-β, IL-6, and MMP-8). To address the relation between PTP and DME data, data were used from all subjects and all time points to identify linear, quadratic, and curvilinear relations. Again, Bonferroni corrections were used in statistical testing (see Results section).

RESULTS

Inflammatory mediators

Full data set. Raw values for the complete data set are shown in Table 1. As noted, variability in the data was shown with respect to BL and immediate postloading time points, before subjects were assigned to experimental condition. Table 1 highlights markers showing "preinflamed," "nonresponsive," "responsive," and "invalid" states, as defined in the Methods section.

Focus data set. Results for the nonpreinflamed, responsive data set—which we designate as the "focus data set"—are summarized in Table 2 and Figure 3 (subjects 3, 1, and 2). Those displays show normalized BL concentrations of IL-1β, IL-6, and MMP-8 for the identified subjects as a function of treatment condition and time point. At the 4-hour time point, no clear pattern of results was observed. In contrast, the 24-hour data revealed that the normalized inflammatory concentrations for the identified markers were 100% aligned with predictions. Values for inflammatory mediators, IL-1β, IL-6, and MMP-8, were greatest for SS, lower for voice rest, and lowest for RV. Values for the antiinflammatory mediator, IL-10, were greatest for RV, intermediate for SS, and lowest for voice rest. The probability of that result, which involved nine/nine prediction "successes" (three markers × three subjects), was statistically significant at $P = 0.004$.

TABLE 2.
Normalized (Unitless Ratio) Data Values for Concentrations of IL-1β, IL-6, and MMP-8 Across Time Points, for Subjects 1, 2, and 3 (See Text)

Treatment Group	Time Point	Subject	Sex	IL-1β	IL-6	MMP-8
SS	BL	3	F	1.00	1.00	1.00
	Post			1.21	1.32	3.04
	4 h			3.13	5.56	3.33
	24 h			12.52	63.86	13.34
Voice rest	BL	1	M	1.00	1.00	1.00
	Post			1.93	10.62	3.62
	4 h			3.54	20.94	13.82
	24 h			1.87	9.16	2.00
RV	BL	2	F	1.00	1.00	1.00
	Post			1.51	9.54	1.21
	4 h			3.68	12.25	1.18
	24 h			0.45	0.00	0.38

Abbreviations: Post, 20–30 minutes postloading; 4 h, 4 h after initiation of treatment; 24 h, 24 h post-BL; F, female; M, male.

Fuller data set. A binomial test using average data after data reduction, including all valid, nonpreinflamed, responsive, normalized data for all subjects similarly revealed a benefit of voice rest over SS, and of RV over voice rest, at the 24- (but not the 4-) hour time point. Table 3 shows the results. Specifically, again, for the 4-hour time point, no discernible pattern was detected. However, at the 24-hour time point, normalized data for IL-1β, IL-6, and MMP-8 showed the identical pattern of results as for the focus data set. Moreover, including additional markers not available in the focus data set, a conceptually similar pattern was seen. Average concentrations for the inflammatory mediator, IL-8, were worst (greatest) for SS and best (lowest) for RV at 24 hours (data were not available for this marker in the rest condition). For TNF-α, values were marginally greater for the RV as compared with the voice rest condition, but as predicted, worst for SS. Moreover, average concentrations of IL-10—the *antiinflammatory* mediator we evaluated—were best (greatest) for RV, intermediate for SS, and worst (lowest) for voice rest. The overall P value for this data set was statistically significant at 0.002, using a binomial test (15/17 mediators in predicted position).

Relations across PTP, DME, and inflammatory mediator data

Interinvestigator reliability for PTP was 0.997 ($P < 0.05$; Pearson correlation) for 10% of randomly selected utterances

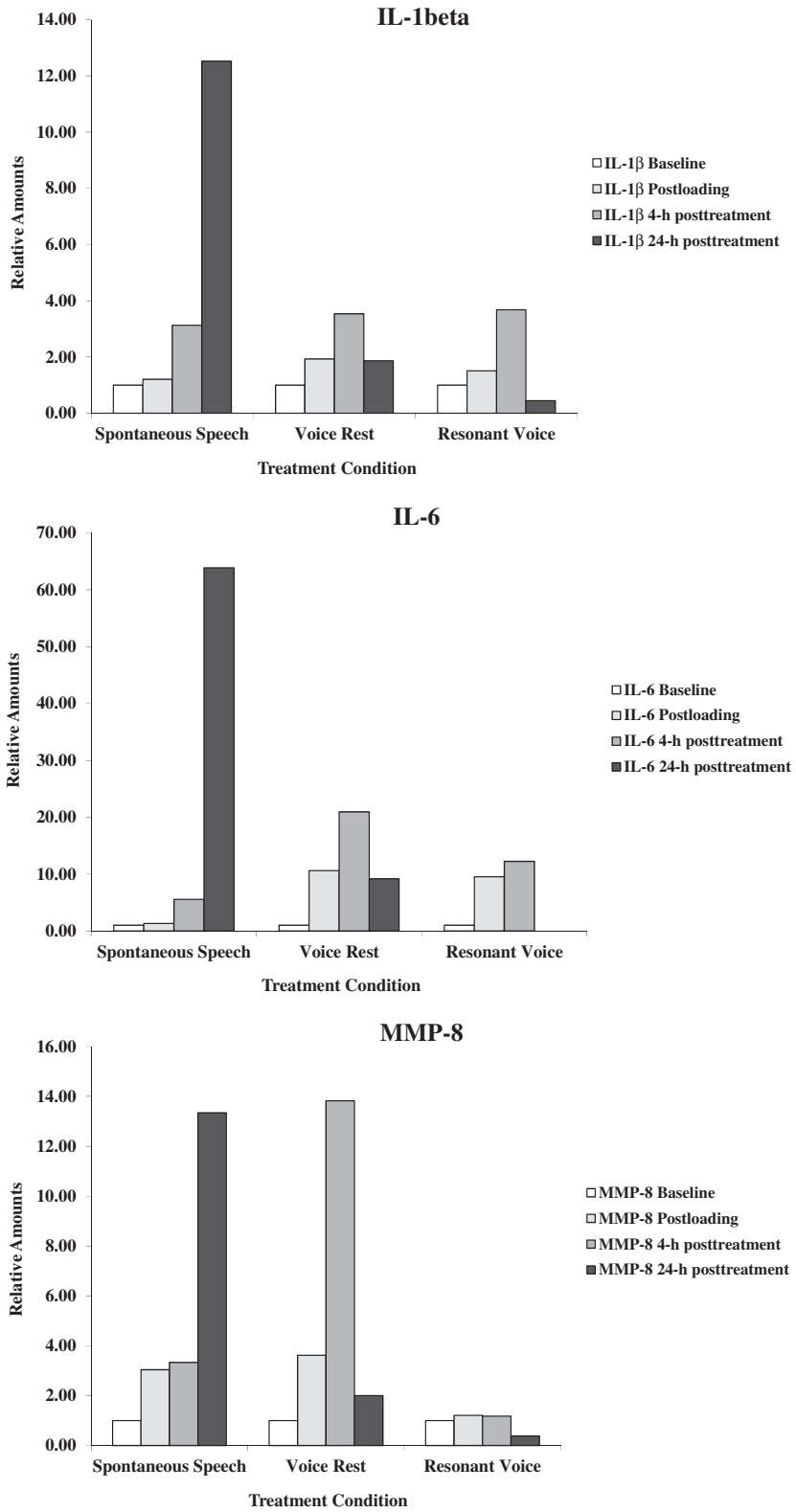

FIGURE 3. Normalized (unitless ratio) values for three markers, IL1-β, IL-6, and MMP-8 for subjects 3 (SS), 1 (voice rest), and 2 (RV) at BL, immediate postloading, 4 hours posttreatment onset, and 24 hours post-BL time points (see text).

(reliability for DME values was not assessed; see the Methods section). Raw PTP and DME data are shown in Table 4. For the focus data set across all time points, all linear, quadratic, and cubic slopes were about as close to 0.00 as they could get, ranging from −0.001 to −0.045 (Table 5). Oddly, all slopes were also numerically *negative*. R^2s—which were necessarily positive due to squaring of values—ranged between 0.127 and 0.652 (Table 5). Using Bonferroni protection for multiple

TABLE 3.
Full Data Set

Time Point	IL-1β SS	IL-1β Voice Rest	IL-1β RV	IL-6 SS	IL-6 Voice Rest	IL-6 RV
N	1	1	1	2	1	2
Post	1.21	1.93	1.51	2.65 (1.33)	10.62	8.31 (1.24)
4 h post	3.13	3.54	3.68	3.44 (2.12)	20.94	6.30 (5.96)
24 h post	12.52	1.87	0.45	32.25 (31.61)	9.16	2.72 (2.72)

Time Point	IL-8 SS	IL-8 Voice Rest	IL-8 RV	TNF-α SS	TNF-α Voice Rest	TNF-α RV
N	1	0	1	1	1	1
Post	4.57	Nil	6.22	1.25	1.18	1.26
4 h post	4.18	Nil	4.23	0.96	1.22	1.30
24 h post	14.81	Nil	2.08	4.69	1.11	1.14

Time Point	MMP-8 SS	MMP-8 Voice Rest	MMP-8 RV	IL-10 SS	IL-10 Voice Rest	IL-10 RV
N	1	1	1	2	1	1
Post	3.04	3.62	1.21	1.53 (0.44)	2	1.16
4 h post	3.33	13.82	1.18	2.85 (1.07)	0.56	1.59
24 h post	13.34	2.00	0.38	2.62 (1.54)	1.38	4.09

Individual normalized (unitless) values (for data from one subject only) and means (for data from more than one subject; standard error of means in parentheses) for each marker at each time point after data reduction (removal of invalid, preinflamed, and unresponsive markers) and normalization to BL values.

TABLE 4.
Raw Data Values for PTP (in cm H20) and DME of Phonatory Effort (Unitless) Across Time Points for Each Subject

Treatment Group	Time Point	Subject	PTP (cm/H_20)	DME
SS	BL	3	5.36	3
	Post		3.19	1
	4 h		2.28	2
	24 h		3.28	1
	BL	6	11.34	2
	Post		5.28	2.5
	4 h		4.04	2
	24 h		5.18	2
	BL	8	3.97	2
	Post		6.79	3
	4 h		6.91	3
	24 h		5.44	2
Voice rest	BL	1	8.55	2
	Post		5.63	2
	4 h		4.33	1
	24 h		7.08	1
	BL	5	3.98	1
	Post		8.22	2
	4 h		6.51	1
	24 h		5.74	1
	BL	7	4.45	1
	Post		7.51	3
	4 h		7.49	2
	24 h		6.73	1
RV	BL	2	6.94	3
	Post		4.45	3
	4 h		2.97	2
	24 h		6.84	3
	BL	4	6.32	1
	Post		4.04	1.6
	4 h		3.23	1
	24 h		4.86	1.1
	BL	9	7.65	5
	Post		6.33	2
	4 h		7.42	2
	24 h		6.01	3

comparisons (.05 alpha level/18 tests for three markers × two dependent variables × three levels of relation, $P = .0028$), none of the relations achieved statistical significance. Stated differently, there was no evidence that either PTP or DME might function as a noninvasive proxy for inflammatory mediator concentrations.

Similarly, no clear evidence was seen of any linear, quadratic, or cubic relations between PTP and DME as sometimes suggested in the literature[38,47–49] (slopes ranged from −0.216 to 0.537; P values were 0.10–0.25).

DISCUSSION

In extralaryngeal tissues, mobilization has been shown to be anabolic and to facilitate a regenerative tissue healing response. To date, these phenomena have not been formally reported in the context of vocal fold injury. In fact, traditional practice in rehabilitation of acutely injured vocal folds mandates limited voice use and limited vocal intensity, and even voice rest. Preliminary *in vitro* data from our laboratory suggest that the cellular response to mechanical signaling in the vocal folds may, in fact, mimic other connective tissues, pointing to the possibility that voice rest may not be the ideal approach to optimize healing outcomes in all patients with acute injury.[7] One potential limitation to this type of investigation in humans is methodological: traditional methods to investigate the inflammatory phase of healing are not feasible in the human vocal folds, *in vivo*. As such, our laboratory described the immunoassay of soluble mediators of wound healing present in secretions collected from the surface of the vocal folds as a means to quantify these wound healing events.[21]

Using this approach, we sought to determine the effects of tissue mobilization in the resolution of vocal fold

TABLE 5.
Linear, Quadratic, and Cubic Slopes (b), R^2, and *P* Values for PTP (in cm H20) and DME of Phonatory Effort (Unitless) Versus IL-1β, IL-6, and MMP-8

Curve Fitting	PTP–IL-1β	PTP–IL-6	PTP–MMP-8
Linear			
b (slope)	−0.003	−0.002	−0.003
R^2	0.193	0.127	0.228
P	0.153	0.255	0.116
Quadratic			
b (slope)	−0.012	−0.045	−0.010
R^2	0.293	0.652	0.370
P	0.210	0.009	0.125
Cubic			
b (slope)	−0.015	−0.045	−0.028
R^2	0.285	0.652	0.513
P	0.399	0.009	0.108
Curve Fitting	**DME–IL-1β**	**DME–IL-6**	**DME–MMP-8**
Linear			
b (slope)	−0.002	−0.001	−0.001
R^2	0.317	0.158	0.374
P	0.057	0.200	0.035
Quadratic			
b (slope)	−0.006	−0.008	−0.004
R^2	0.473	0.240	0.495
P	0.056	0.290	0.046
Cubic			
b (slope)	−0.010	−0.008	−0.007
R^2	0.480	0.240	0.510
P	0.137	0.290	0.110

Referent data are from focus data set (subjects 1, 2, and 3) collapsed over four time points (BL, postloading, 4 hours posttreatment onset, and 24 hours post-BL). (With Bonferroni correction for overall α set at .05, for 18 tests, criterion for each test is .003. None of the tests achieved statistical significance.)

inflammation after acute phonotrauma induced in the laboratory. Biological data from our study suggest that voice rest and RV exercises yielded improved posttraumatic inflammatory profiles in subjects with interpretable data at 24-hour post-BL, compared with SS, for which posttraumatic profiles were generally worse than BL. Specifically, although no discernible pattern of results was evident after 4 hours of in-clinic interventions, for subjects with interpretable data in both focus and fuller data sets, with one minor exception normalized concentrations of *inflammatory* mediators, IL-1β, IL-6, IL-8, TNF-α, and MMP-8, were greatest at 24 hours post-BL for the SS condition, improved after voice rest, and lowest after RV exercises (the exception was that average concentration of TNF-α was about the same for voice rest and RV at 24 hours). Moreover, average normalized concentrations of the *antiinflammatory* mediator, IL-10, were greatest at 24 hours after RV exercises, somewhat lower for SS, and lowest after absolute voice rest, the only condition involving no vocal fold vibrations whatsoever posttraumatically.

If tissue mobilization improves the acute inflammatory profile in vocal fold tissue compared with rest, the question can be asked why RV appeared to reduce inflammation but SS actually increased it. Potentially, as previously discussed, the combination of relatively large-amplitude and low-impact vibrations associated with RV is critical for its benefit. However, it is likely that dose dependency is also in play. The SS condition involved 16 minutes of phonation followed by 4 minutes of voice rest during the in-clinic treatment, as compared with the reverse pattern that was used for RV. It is reasonable to posit that there is an ideal phonation dose to improve acute inflammation, as shown in our *in vitro* studies.[7,50] We are currently working with computational modeling ultimately to address dose dependency in inflammation.[51,52] However, in the meantime, the present data clearly show that a limitation of phonation time is not the only factor in inflammation control. If it were, results would have been best for voice rest, and they were not.

One issue that is critical to address is the variability in the biological data reported here. In recruiting vocally healthy individuals, as clinically assessed, we assumed that participants would have low concentrations of inflammatory markers at BL, and that concentrations would increase postloading, consistent with findings from our previous work.[21] Although all subjects had normal values for at least one inflammatory mediator at BL, seven/nine subjects also had abnormally high BL values for one or more mediators. Several factors may have been contributory in BL data. For example, asymptomatic episodic laryngopharyngeal reflux or casual environmental exposures to pollutants could have elevated certain inflammatory concentrations at BL for some subjects. Moreover, perhaps clinical examination and self-report are insufficient to detect vocal fold inflammation, which instead requires more sophisticated technology such as the technology used here. A similar question can be posed regarding some subjects' apparent nonresponse to the vocal loading protocol, based on inflammatory mediatory concentrations. Again, although all subjects showed responsiveness in at least one mediator postloading, six/nine subjects did not show any increase in one or more mediators postload. A plausible speculation is that the loading protocol did not exceed the threshold required for injury for those subjects, for those particular markers. In fact, it is clear that the wound healing response is complex, involving many cell types and soluble mediators,[53–57] for which patterns may vary across individuals. One component in biological responses may be a genetically predetermined balance between inflammatory and antiinflammatory mediators in wound healing.[58–60] Moreover, in our study, we did not control for voicing modality during loading. A fundamental premise in voice therapy is that some modalities—for example pressed voice, associated with high impact stresses—are more damaging to the tissue than others.[61] Therefore, we expect variability in individuals' response to vocal loading and injury based on the interaction of genetic and phonatory variables, at minimum.

Having said as much, one potentially disconcerting aspect of the data is seen in PTP measures, for which values actually *decreased* within the immediate postloading window in six/nine subjects. This finding contrasts with general clinical

expectations that PTP should increase with laryngeal injury.[62] Stated differently, although biological data indicated that vocal loading was inflammatory to some degree for all subjects, the PTP data pose the question of whether the loading protocol constituted actual loading for fully two-thirds of subjects. This possibility is interesting to entertain. Visual observations of physical changes to the larynx provide no help to address this issue, as such observations were not made systematically, and no spontaneous observations emerged along these lines. However, recent data suggest that PTP fluctuates with training alone.[63] Thus, perhaps some of the fluctuations seen in PTP were due to learning rather than biological factors. Moreover, as for biological data, several factors regulate PTP, one of them being glottal gap between the vocal folds width, which is directly related to threshold pressure.[64,65] As laryngeal tissue becomes engorged with the initial results of inflammation, perhaps the glottal gap is decreased thus paradoxically reducing PTP. In sum, comparing results for the biological mediators in the present study versus PTP, we may need to entertain the notion that perhaps clinical concepts around PTP as an indicator of laryngeal injury may be misguided, at least where acute injury is concerned.

Although we are cautiously optimistic about the interpretable results that emerged in the data above the noise, we would be remiss to exclude discussion regarding the validity of the secretion analysis technique. We acknowledge the limitations of the assays, as seen in some of the noted challenges encountered in data collection. However, independent data do indicate that there is covariation between mediator concentrations in secretions and underlying tissue. Using an animal model of controlled subglottic mucosal injury, Hebda's laboratory demonstrated that IL-1β and other soluble inflammatory mediators became elevated in secretions within 24 hours postinjury, the response reflected degree of injury, and a positive correlation was shown between mediator increase in the secretions and localized upregulation of expression of the mediator in the subglottic tissue.[66–69] Although we recognize that the specific cell source of these markers is not known, Hebda's findings appear to support the utilization of select inflammatory mediators in secretions as surrogate markers for phonotrauma. Outstanding questions invite further investigation.

Turning to results for PTP and DME, as we hypothesized, there was no evidence of any links between these variables and mediator concentrations. Slopes for linear, quadratic, and cubic relations were essentially nil, failing to approach statistical significance. As such, results from our study do not provide optimism that either PTP or DME may be reasonable surrogates for more invasive estimates of acute vocal fold injury.

A further question regards the relation between PTP and DME. Logically, it would seem that these physiological and perceptual estimates of phonation effort should covary. Our data showed no indication of any such relation. In fact, other recent data show that these variables may not be as tightly related as sometimes assumed.[38,47–49] One possibility is that PTP and DME may capture different biological processes in vocal fatigue or injury, as PTP has been reported to return to BL within about an hour of a vocal loading task in comparison to DME, which according to one report requires a full day.[47] In that case, the measures would not be expected to covary tightly.

In summary, despite its limitations, the present study is the first to address systematically biological mechanisms in behavioral voice therapy for patients with acute vocal fold injury. Our data suggest that some forms of tissue mobilization may represent a rational treatment approach for acute mucosal injury, in some individuals. Although the direct translation to clinical practice is not yet straightforward, the present study certainly suggests that some clinical value may be found in controlled vocal exercise in the context of acute phonotrauma.

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