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ORIGINAL RESEARCH

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The role of systemic dehydration in vocal fold healing: Preliminary findings

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Abstract

Rationale: Systemic dehydration negatively alters the expression of vocal fold inflammatory and cell junction markers. These biological changes can have downstream effects on the healing processes of injured vocal folds. In the dermis, reduced hydration prolongs inflammation and delays healing. It is unknown whether this biological effect is observed in vocal fold tissue.

Objective: To investigate the effects of systemic dehydration on vocal fold healing outcomes following acute, bilateral vocal fold injury in a rodent model.

Methods: Eighteen systemic dehydrated and 18 euhydrated adult male Sprague Dawley rats experienced bilateral vocal fold injuries or no injury ($N = 9$/group). Vocal fold gene expression levels of inflammatory mediators and epithelial cell junction markers were measured 24 h post-injury.

Results: Pro-inflammatory gene markers (*IL-1β*; *TNF-α*) were differentially expressed in response to systemic dehydration with vocal fold injury compared to non-injury. Epithelial cell junction markers (*Cadherin-3*, *Desmoglein-1*) also exhibited divergent trends following systemic dehydration, but these data were not statistically significant.

Conclusions: Systemic dehydration may affect cellular vocal fold healing processes within 24 h. These findings lay the groundwork for further investigation of how hydration status can affect vocal fold tissue recovery and influence clinical care.

KEYWORDS
dehydration, inflammation, injury, rats, vocal folds

1 | INTRODUCTION

Hydration is considered essential for voice production.[1,2] Published evidence from in vivo animal models reveals that systemic dehydration may be detrimental to vocal fold biology,[3–6] but the interplay of systemic dehydration and healing has not been studied. In dermal wounds, dehydration causes delayed re-epithelization[7–9] and prolonged inflammation.[10,11] Because of similarities between dermal and airway tissue healing processes,[12] it is possible that vocal fold dehydration also delays wound healing. Suboptimal vocal fold healing can

have devastating functional consequences on voice production.[13,14] Dehydration negatively altered recovery from vocal injury in one published human study[15]; however, the molecular details of whether dehydration interferes with vocal fold wound healing require further investigation. This study seeks to fill this gap in the literature by delineating the cellular interactions of systemic dehydration and vocal fold healing using an in vivo model.

In order to effectively induce a mild state of systemic dehydration, we employed an established water restriction protocol.[16] This physiologically-relevant dehydration protocol negatively affects the gene and protein expression of inflammatory and epithelial cell junction markers in rodent vocal fold tissue.[16] Other published studies have also found adverse dehydration-induced outcomes on inflammatory processes and vocal fold epithelial barrier integrity.[3,17] We, therefore, investigated the effects of water restriction-induced systemic dehydration on gene expression of pro- and anti-inflammatory mediators and epithelial cell junction markers 24-h post vocal injury. Euhydrated and dehydrated non-injured rats served as controls. The inflammatory mediators of interest were: interleukin 1 beta (*IL1-β*), tumor necrosis factor alpha (*TNF-α*), transforming growth factor beta (*TGF-β*). The epithelial cell junction markers of interest were Cadherin 3 (*CDH3*) and Desmoglein 1(*DSG1*). The rationale for the inclusion of these genes is provided below.

Pro-inflammatory mediators (IL1- β, TNF-α) create a chemical environment promoting tissue repair and immune cell migration.[18,19] The anti-inflammatory mediator TGF-β is responsible for modulating the inflammatory response and facilitating extracellular matrix proliferation.[20,21] In the rat model, the gene expression of pro-inflammatory mediators reaches peak expression within the first 24 h post-vocal fold injury,[22] whereas, *TGF-β* upregulation begins at 4 h, reaching peak upregulation at 72 h[22] post-vocal fold injury. Thus, we decided to investigate dehydration-altered inflammatory processes at the 24-h time point following vocal injury.

With regards to re-epithelization, a single layer of epithelial cells can cover the injury site within 3 days of vocal injury.[23] The integrity of the epithelial barrier structure is regulated by epithelial cell junctions (such as CDH3 and DSG1).[24] In recent literature, systemically dehydrated vocal fold tissue presented downregulation of these desmosomes and adherens junctions in the absence of injury.[3,16] Not only have the timelines of adherens and anchoring junction recovery been described in vocal fold healing literature,[25] but alterations in cell junctions can affect epithelial barrier integrity. Reduced epithelial barrier integrity could leave the vocal fold surface vulnerable to exogenous insults.[24]

The overarching objective of this study was to investigate the effects of systemic dehydration on vocal fold healing outcomes. Gene expression and protein level profiles of inflammatory mediators and epithelial cell junction markers were compared in dehydrated and euhydrated rats 24 h post-vocal fold injury. Rats that were not injured served as the control group for injury. We hypothesized that systemic dehydration would cause an upregulation of inflammatory mediators and a downregulation of epithelial cell junction makers at this time point. Dehydration was induced with a physiologically relevant water restriction protocol (defined as a 40%–46% reduction in average water intake compared to baseline for each animal). Vocal fold injury was induced by creating a minor incision on the bilateral vocal fold surface. This was selected over complete mucosal stripping to replicate a milder injury and a shorter healing timeline.[26]

2 | METHODS

All protocols and procedures were approved by the Purdue Animal Care and Use Committee (Protocol #1905001897).

2.1 | Experimental animals

Thirty-six male, Sprague Dawley rats (age range = 4–5 months old, baseline weight = 421.7 ± 5.1 g, [mean ± SEM]) were acclimatized for one week in a temperature and humidity-controlled room. During this period, all rats received ad libitum food and water.

2.2 | Experimental protocol

Rats were randomly assigned to one of four groups: (1) dehydrated Injury, (2) euhydrated Injury, (3) dehydrated non-injury, and (4) euhydrated non-injury. There were nine rats per group (Figure 1). Rats in the dehydrated groups had restricted access to water for a 5-day period (4 ml of water/0.1 kg body weight/day = 40%–46% reduction in baseline water intake). The euhydrated groups received water ad libitum (Figure 1). All groups received unrestricted access to food. This established protocol of water restriction induces systemic dehydration in Sprague Dawley rats.[16] On the 4th day of the experiment, rats in the injury groups received a bilateral, minor vocal fold injury, whereas those in the non-injury groups did not. All rats were euthanized on the 5th day of the experiment (i.e., 24 h post-vocal fold injury, Figure 1).

2.3 | Vocal fold injury

Rats in the injury groups were anesthetized with an intraperitoneal injection of ketamine xylazine (K, 50–100 mg/kg, X, 0.5–1 mg/kg) and suspended supine on a rodent intubation board. (Hallowell Rodent Work stand, RW-A3467, Braintree Scientific Inc., Braintree, MA). A rodent mouth speculum (11.5 cm, World Precision Instruments, Sarasota, FL) facilitated mouth opening while an apparatus housing a custom-made sharpened wire (0.63 mm × 4.5 mm, spring steel, Figure 1) and a rigid endoscope (30-degree, 2.7 mm × 187 mm, Model WA96301A, Olympus, Shinjuku, Tokyo) was inserted into the rodent airway. After laryngeal vestibular visualization, the sharpened wire was extended from the endoscope to create a superficial vocal fold incision bilaterally. Animals were then revived with a subcutaneous injection of Atipamezole (1 mg/kg) and provided with subcutaneous injections of Buprenorphine (0.003 mg/kg) every 12 h from recovery until

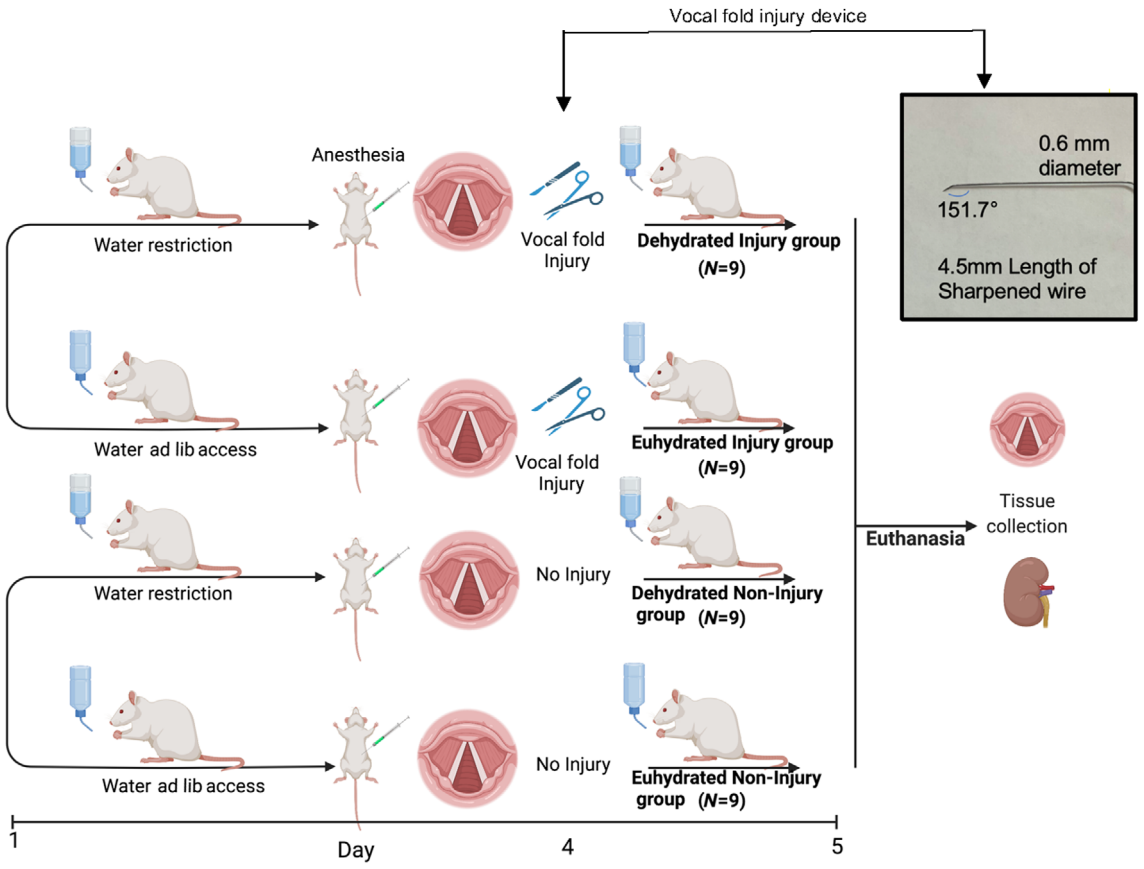

FIGURE 1 Experimental protocol

euthanasia (total injections = 2). Rats in the non-injury groups were similarly anesthetized and recovered with Atipamezole but did not receive a vocal fold injury or Buprenorphine (Figure 1).

2.4 | Euthanasia and tissue processing

At the end of 5 days, all rats were euthanized via CO_2 inhalation, and the larynges and kidneys were dissected and processed for further analysis.

2.5 | Confirming systemic dehydration

Measures of average body weight loss and the relative gene expression of *renin* in kidney tissue were used to confirm a state of dehydration. Systemic dehydration causes a decrease in body weight[4–6,16] and upregulation of *renin* gene expression in Sprague Dawley rats.[4,16]

2.6 | Gene expression analysis

Vocal fold and kidney tissue from rats in each group were collected in RNA*later*™ stabilization solution (Invitrogen™, Thermo Fisher Scientific Inc., Carlsbad, CA) and stored in −20°C for qPCR analysis. Specific primer pairs were designed using Primer-BLAST Tool[27] (Table 1). For RT-qPCR, total RNA was extracted (RNeasy Fibrous Tissue Mini Kit, QIAGEN, Hilden, Germany) and concentration and purity of RNA were assessed (Nanodrop™, Thermo Fisher Scientific, Waltham, MA, USA). Then, cDNA was synthesized with SuperScript™ IV VILO™ Master Mix (Invitrogen, Waltham, MA, USA) using 500 ng of total RNA/sample in a 20 µl final reaction. Next, qPCR was performed with the Power SYBR® Green PCR Master Mix (Applied Biosystems™ by Thermo Fisher Scientific), using 10% of 10× diluted cDNA (i.e., 2.5 µl used in 25 µl final volume) and 1 µM concentration of each primer. Each sample was run in triplicate on a 96-well qPCR plate. QuantStudio 3 Real-Time PCR System (Applied Biosystem, Waltham, MA, USA) was used to carry out the reactions with the thermal cycling parameters of AmpliTaq Gold® DNA Polymerase set as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min; and melt curve stage of 95°C for 15 s, 60°C for 1 min, and 95°C for 1 s. The presence of single amplicons was validated by melt-curve analysis. Relative gene expression of the target genes was derived from the $2^{-\Delta\Delta CT}$ method with *β-actin* used as endogenous control for all target genes.[28] Relative gene expression is expressed as fold-change relative to the euhydrated injury group.

2.7 | Statistical analysis

SPSS (Version 22, IBM SPSS Statistics) was used for all statistical analyses. Normality and variance were assessed. Grubbs' test was used to

TABLE 1 Quantitative PCR primer information

Gene	Sequence (5′–3′)	Tissue	Accession#	Product length
IL-1β	Fw: TGTCTGAAGCAGCTATGGCA Rv: ACAGGTCATTCTCCTCACTGTC	Vocal fold	#NM_031512.2	206
TNF-α	Fw: ATGGGCTCCCTCTCATCAGT Rv: GCTTGGTGGTTTGCTACGAC	Vocal fold	#NM_012675.3	170
TGF-β	Fw: CTGCTGACCCCCACTGATAC Rv: AGCCCTGTATTCCGTCTCCT	Vocal fold	#NM_021578.2	94
CDH3	Fw: TACCGGGGAGGACCTTTAGGA Rv: GTAGCCAGCAAACCTGGAGCA	Vocal fold	#NM_053938.2	106
DSG1	Fw: TTGTGCTGCAAACCAACCAG Rv: AGTGCTCGGCAGTAGATAATGA	Vocal fold	#XM_039097378.1	162
Renin	Fw: AGCCAGCTTTGGACGAATCTT Rv: GTCATGTCTACTCCCCGCTC	Kidney	#NM_012642.4	78
β-actin[a]	Fw: CCCGCGAGTACAACCTTCTTG Rv: GTCATCCATGGCGAACTGGTG	Vocal fold/kidney	#NM_031144.3	71

[a]*β-actin* was used as endogenous control when calculating the gene expression levels of target genes.

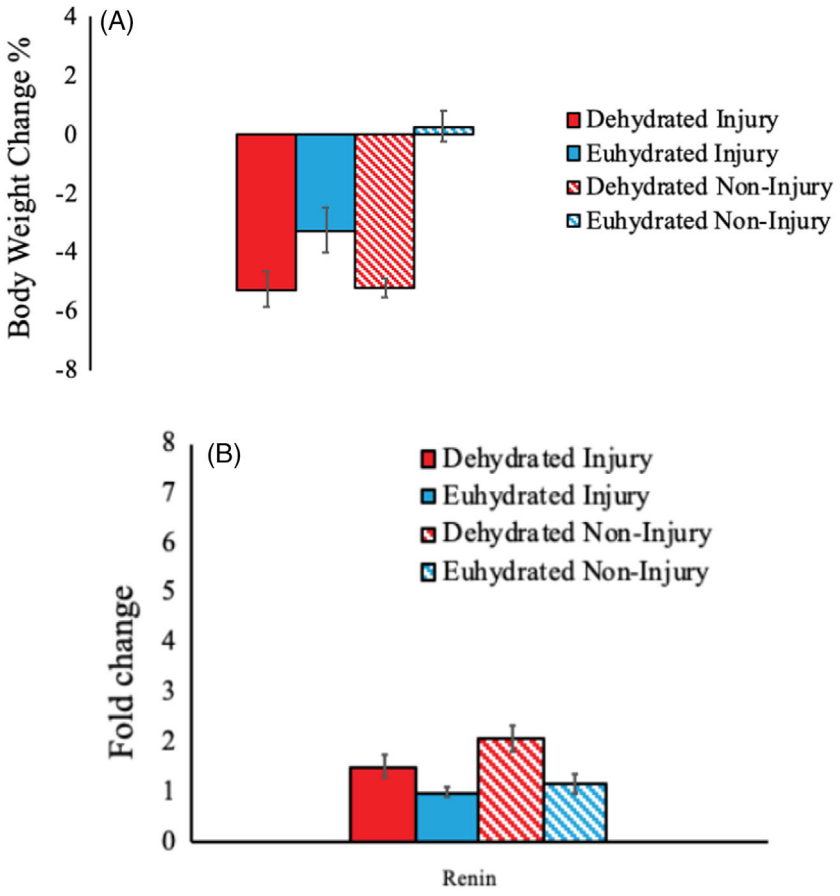

FIGURE 2 Dehydration outcomes: (A) body weight change (Mean + SEM) across groups (B) relative gene expression (Mean + SEM) of renin

assess for outliers. Two-way ANOVAs (factors: presence of injury; hydration status) and follow-up paired *t*-tests were performed for parametric data, whereas Kruskal-Wallis and Dunns'-Bonferroni comparisons were performed for non-parametric data. Alpha level was set at $p \leq .05$. Only statistically significant data are presented in the results below.

3 | RESULTS

3.1 | Confirming systemic dehydration

There was a significant main effect of dehydration on body weight change ($F(1, 32) = 17.629$, $p < .001$, $\eta_p^2 = 0.355$, Figure 2A). The

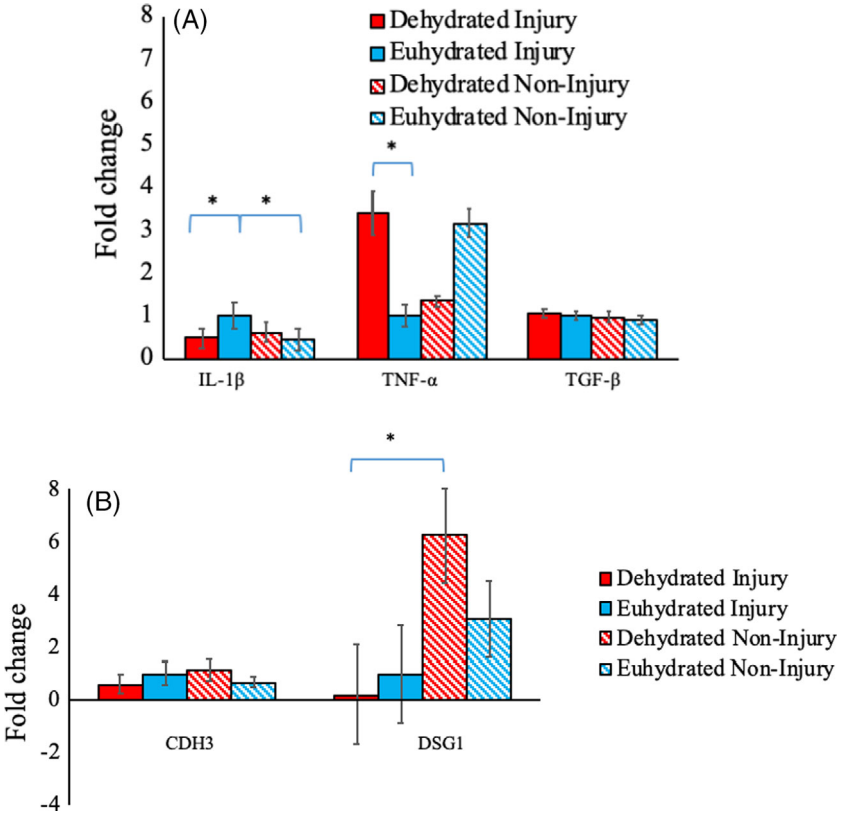

FIGURE 3 Relative target gene expression (Mean + SEM) of (A) inflammatory mediators and (B) epithelial cell junction genes.

animals in the dehydrated groups experienced greater body weight loss (−5.6% ± 0.62%) compared to animals in the euhydrated group (−1.54% ± 0.485%).

For *renin* gene expression, a significant main effect for dehydration was also observed ($F(1, 32) = 8.299$, $p = .007$, $\eta_p^2 = 0.206$, Figure 2B). A 1.24-fold upregulation of *renin* was observed in the dehydrated group compared to the euhydrated group.

3.2 | Dehydration and injury effects

The relative gene expression of target genes is depicted in Figure 3A and B, respectively. Only significant effects will be reported in this section. With regards to *TNF-α*, a significant interaction effect of dehydration and injury was observed ($F(1, 32) = 6.46$, $p = .016$, $\eta_p^2 = 0.318$, Figure 3A). Paired *t*-test post-hoc comparisons revealed a 3.4-fold upregulation of *TNF-α* in the dehydrated injury group compared to the euhydrated injury group ($p = .0088$, 95% CI = 0.15 to 0.88).

Data for *IL-1β* did not follow a normal distribution; hence, a Kruskal-Wallis test was completed and revealed significance ($H(3) = 9.616$, $p = .022$, Figure 3A). Follow-up Dunns-Bonferroni post-hoc comparisons revealed a downregulation of *IL-1β* expression in the euhydrated non-injury group compared to the euhydrated injury group ($p = .041$, 95% CI = 0.041 to 2.55) and downregulation of *IL-1β* expression in the dehydrated injury group when compared to the euhydrated injury group ($p = .031$, 95% CI = 0.1 to 2.5).

Similarly, *DSG1* data did not follow a normal distribution. Kruskal-Wallis test revealed a significant difference in *DSG1* expression across groups ($H(3) = 9.762$, $p = .021$, Figure 3B). Dunns-Bonferroni post-hoc comparisons showed downregulation of *DSG1* expression in the dehydrated injury group compared to the dehydrated non-injury group ($p = .025$, 95% CI = 0.14 to 18.6).

4 | DISCUSSION

This study compared the gene expression of inflammatory cytokines and epithelial cell junction markers in systemically dehydrated and euhydrated injured and non-injured vocal fold tissue 24 h following vocal fold injury. Systemic dehydration, induced by reduced water intake,[16] affected the gene expression of pro-inflammatory cytokines (*IL1-β* and *TNF-α*) in injured vocal fold tissue. To the best of our knowledge, this work is the first to investigate the cellular interactions of systemic dehydration and vocal fold healing in the rodent model.

Adverse dehydration-induced healing such as inadequate wound closure,[29,30] delayed re-epithelization,[7–9] and altered inflammatory response[10] have been observed in dermal tissue. In fact, an altered inflammatory response in dehydrated dermal epithelial wounds is characterized by similar pro-inflammatory cytokine patterns as those seen in the present study.[31] Specifically, in the early stages of wound healing, dehydration resulted in an upregulation of tumor necrosis factor alpha (*TNF-α*) and the downregulation of interleukin 1 beta (*IL1-β*) with injury.[31] However, the divergent direction of *TNF-α* and *IL1-β* in

dehydrated injured vocal fold tissue requires further interpretation. Whereas *TNF-α* and *IL1-β* have similar downstream gene targets, *TNF-α* uniquely regulates cell apoptosis[32] among other mechanisms[33] during inflammation. The divergent direction of *TNF-α* and *IL1-β* may be indicative of suboptimal dehydration-induced vocal fold healing; however, this hypothesis needs further investigation.

During wound healing, pro-inflammatory cytokines such as IL1-β and TNF-α are primarily responsible for the recruitment of immune cells such as neutrophils and macrophages to the site of the wound while initiating downstream signaling for extracellular matrix proliferation.[34,35] Altered expression of these cytokines may have adverse downstream effects on extracellular matrix proliferation and re-epithelization, and could negatively affect vibratory efficiency. Thus, we might also expect adverse dehydration-induced outcomes in the gene expression of anti-inflammatory marker *TGF-β*, as well as epithelial cell junction markers, *CDH3*, and *DSG1* with injury. The lack of significant gene expression differences between dehydrated injury and euhydrated injury groups for these target genes may potentially be explained by the relatively early stage of wound healing under investigation (24 h post-injury), and/or the relatively minor nature of the vocal fold injury. These hypotheses will be tested in future studies.

Although a minor incision was created on the vocal fold surface, our results confirm the reliable and consistent creation of vocal fold injury in the injured groups. Specifically, we observed the upregulation of *IL1-β* in the euhydrated injury group compared to the euhydrated non-injury group. The initial inflammatory response post-injury is characterized by the documented upregulation of *IL1-β*, followed by the upregulation of other inflammatory mediators, *TNF-α* and *TGF-β*. Interestingly, we also observed the downregulation of *DSG1* in the dehydrated injury group compared to the dehydrated non-injury group. Dehydration-induced downregulation of *DSG1* with injury may be an early indicator of delayed re-epithelization.

4.1 | Limitations and future directions

Systemic dehydration triggers inflammation and impacts epithelial barrier integrity in uninjured vocal fold tissue.[3,4,16] Across all target genes examined in this study, no significant differences were observed between dehydration and euhydration. It is possible that anesthesia may have tempered the effects of systemic dehydration. More research (with a variety of anesthetics and modalities of anesthesia) is required to confirm this hypothesis. Future research should also include protein levels (see Figures S2, S3) and markers characterizing cell proliferation in the lamina propria following injury, as the lamina propria defines tensile strength[36] and elasticity[37] in the vocal folds. It should be noted that this study investigated only one vocal fold healing timepoint (24 h post-injury). Future research should characterize later stages of dehydration-altered vocal fold healing to better understand potential downstream consequences with the inclusion of histological data. To increase the clinical translatability of these results, we studied minor vocal fold trauma (i.e., creating a vocal fold incision in the mucosa, Figure S1) instead of using the previously-studied vocal fold mucosal stripping procedure.[38,39] Future research can compare dehydration-induced healing outcomes across more substantive vocal fold injuries and resection margins and relate these cellular outcomes to functional voice changes (e.g., rodent ultrasonic vocalizations).

4.2 | Conclusions

Dehydrated vocal fold healing resulted in altered pro-inflammatory mediator gene expression 24 h post-vocal fold injury. A systemic dehydration-induced change in the immediate inflammatory response could have downstream implications on vocal fold healing post-injury. Thus, these data lay the groundwork for programmatic research (1) investigating the cellular effects of systemic dehydration on later stages of vocal fold tissue healing, (2) relating cellular outcomes of dehydration-induced changes to the restoration of vocal function.

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CONFLICT OF INTEREST

There are no conflicts of interest to report.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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