Assessing Structural and Physiologic Laryngeal Changes in Response to Systemic Dehydration in a Rabbit Model

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CHAPTER 1

INTRODUCTION TO VOICE DISORDERS

Voice Disorders Within the Context of Speech Pathology

Voice disorders, although sometimes underemphasized in the broader context of speech pathology, are very much under the purview of "communication disorders." A communication disorder is defined as "an impairment in the ability to receive, send, process, and comprehend concepts or verbal, nonverbal and graphic symbol systems. A communication disorder may be evident in the processes of hearing, language, and/or speech."[1] In the overall scope of speech language pathology there is a considerable focus on people who have trouble communicating at a higher level of the neuro-motor process. Language disorders often involve the cortical centers within the frontal and parietal lobes of the brain, and speech and articulation disorders are often associated with the frontal lobe in the premotor and motor cortices. In contrast, voice disorders tend to be related to anatomical mechanisms used to produce sound, which may be altered in some way. The nature of voice disorders differs fundamentally from other types of communication disorders, primarily in presentation and etiology. Different and unique research methods are needed to effectively understand their development, and to aid in optimizing treatments.

Compared with other areas of communication disorders, voice disorders have tended to receive less emphasis in terms of both clinical training and basic research. While nearly 30% of people report having a voice disorder at some point in their lives, less than 5% of speech pathologists consider themselves experts in the treatment of voice.[2,3] These patients often have no difficulty with language or motor processing; instead their anatomical structures associated with

speech production, especially vocal fold vibration, are functionally impaired. In extreme cases it may be difficult or impossible for them to express their wants and needs to others. Although the location of the disruption in the communication cascade is unique for these patients, they suffer similar quality of life changes as those with other kinds of communication disorders. Indeed, it has been argued by Cohen et al. that voice disorders reduce the quality of life "to levels comparable to those in patients with other chronic illnesses of public health concern."[4] Research on the causes and treatment of voice disorders should have priority on a par with other kinds of communication disorders.

At any given time about 6% of people have a voice disorder. With this prevalence the treatment of voice disorders accrues more than 175 million dollars per year in direct healthcare costs.[5,6] While the impact of voice disorders on patient quality of life has been well characterized [6], the underlying mechanisms are not fully understood. Given this uncertainty about the etiologies and risk factors associated with vocal fold lesions and laryngeal disorders, it can be challenging to offer effective treatment. The current practice guidelines for many voice disorders stipulate that behavioral modification through voice therapy is almost always indicated prior to surgical intervention.[7,8] This is a safe guideline given the relative risks associated with surgery compared with less invasive treatment, but there is little empirical evidence to support the purported anatomical benefit of these therapies on behaviorally based voice treatment. The dichotomy of surgical intervention vs. behavioral treatment is relatively unique to voice disorders, causing these patients to engage with professionals from the disciplines of both speech pathology and laryngology, sometimes with inconsistent advice. Fortunately, increasing numbers of large voice clinics around the United States are adopting a cohesive and collaborative multidisciplinary approach to voice disorder diagnosis and treatment.

What We Need to Know

The authors of the Clinical Practice Guidelines acknowledge that more work needs to be done "to better understand what factors increase the likelihood of developing voice disorders such that [patients] can be targeted for educational and preventive interventions."[7,8] While it is widely accepted and promoted that being well hydrated is better for voice function, it has not yet been established unequivocally that dehydration puts patients at higher risk of vocal pathology. Instead, clinicians rely on an assurance that intervention focused on adequate hydration has a "preponderance of benefit over harm." While the recommendations related to hydration are reasonable and widely utilized clinically, the development of pathology would be better understood if the mechanisms that change vocal function as a result of dehydration were empirically investigated.

Based on the research literature that exists, we can relatively safely conclude that good overall hydration does not negatively affect vocal function, and even that poor hydration can lead to poorer vocal function.[9–11] However, in order to provide effective recommendations as part of the behavioral therapy approach, there is a need to better understand how changes in vocal fold hydration lead to structural and molecular alterations in the tissue. These changes may then result in a tissue system less able to adequately repair following injury, as is the case in the development of phonotraumatic vocal fold lesions.[12]

Types of Voice Disorders and Treatments

Voice disorders fall into several categories, and in order to provide the best possible treatment for voice disorders (including recommendations related to vocal hygiene and hydration), it is important to be able to distinguish these categories based not only on presentation, but also on

etiology. The different categories include phonotraumatic vocal fold lesions, functional disorders, and other structural and anatomical disorders which fall into the category of cancer, paralysis or neurologic. Phonotraumatic lesions and functional disorders are both understood to arise from vocal overuse, making differentiation of etiology and risk very important in further contrasting these two populations. This understanding of etiology is of greatest importance in the context of treatment, under the theory that understanding the cause of the lesion (including additional risk factors such as dehydration) will allow us to better affect change and ultimately, resolution of the voice disorder.

General Treatment Options

Functional disorders have the most straightforward treatment paradigm, as there is no anatomical pathology to be addressed. As such, treatment falls squarely under the purview of behavioral voice therapy provided by a speech pathologist. On the other end of the spectrum are the cancer/paralysis/neurological cases, for which structural/surgical management is often the primary indication, and behavioral therapy does not often provide benefit. The middle ground is held by a category of functionally derived anatomical changes to the laryngeal anatomy, phonotraumatic lesions. These cases are often managed by an otolaryngologist and a speech pathologist in conjunction, as they are defined by a structural anomaly (sometimes requiring surgical intervention) caused by a behavior (which typically requires voice therapy to address).

Regardless of the type of voice disorder, there is an underlying treatment paradigm, called vocal hygiene, which is recommended to all voice disorder patients, regardless of which category they fall into. The idea is that there are a number of behavioral modifications that a patient can make to maximize the health of the vocal fold tissue itself in the presence or absence of anatomical

pathology. These modifications include hydration of the vocal fold tissue (both through external humidification and water intake), managing possible laryngopharyngeal reflux symptoms, and decreasing intake of caffeine and alcohol.

Phonotrauma and Vocal Fold Injury

The vocal folds are membranes stretching across the larynx that modulate the flow of air during production of speech or other vocal sounds. Phonotrauma is the term for damage to the laryngeal mechanism, especially the vocal folds, from repeated, excessive actions such as yelling. Phonotraumatic vocal fold lesions are unique from an anatomical and pathological perspective. These unilateral or bilateral lesions ultimately create stiffness and/or increased mass to the vibratory edge of the vocal fold, usually leading to a pressed or rough vocal quality and patient perceived increase in phonatory effort.[12] These lesions (polyps, cysts, and nodules) are thought to be the result of repeated and chronic injury and re-injury of the vocal folds.[13] The forces (shear, stretch, compression, etc.) that the vocal folds are subjected to during vibration create acute microscopic changes to the vocal fold epithelium and lamina propria.[14–16] These changes then need to be repaired in order to restore the vocal fold to its baseline level of resiliency and function. When these injuries are more severe, which can happen when the voice is used too often or too aggressively, the vocal fold tissue does not have the opportunity to repair completely before the next injurious vibratory dose inflicts new damage to the tissue.[17] The forces imposed by vocal fold vibration then cause greater damage to the unrepaired tissue. This pattern of incomplete repair and repeated trauma is perpetuated until a lesion develops. The presence of the lesion then further potentiates continued damage, as the vocal fold's vibratory properties become altered by the presence of an often stiff, massive lesion at the glottal edge (the glottis is the space between the

vocal folds). This asymmetry and increased viscosity of the vocal fold edge perturbs the vibratory characteristics and subsequently increases the damage associated with the forces of vibration, ultimately exacerbating the injury. This process is delineated in Figure 1.

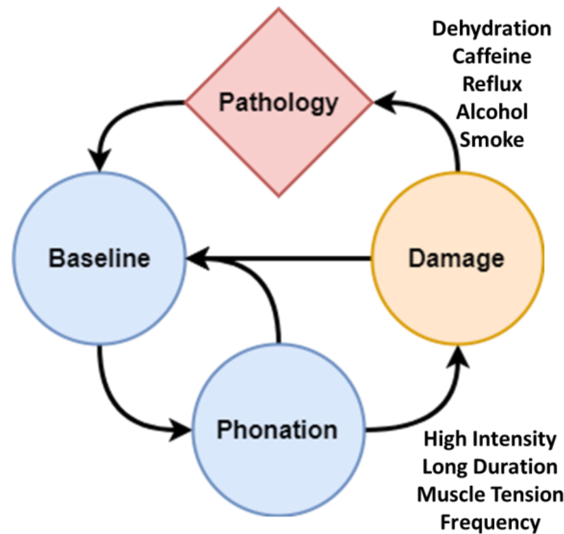

Figure 1. Phonotrauma Etiology Paradigm

There may be identifiable risk factors that make someone more likely to develop vocal fold lesions. Although the evidence for this is mixed, many behavioral treatment strategies in voice therapy are based on these putative risks.[18–24] The risk factors and associated treatment strategies are in the categories of vocal fold vibratory behaviors and vocal hygiene parameters. Vocal fold vibratory patterns can affect the forces (shear, stretch, strain, compression) that the vocal folds undergo. By altering the vibratory characteristics (through resonant voice therapy or vocal function exercises), the amount of force and thus the amount of damage the vocal folds withstand can be mitigated.[14,25–27] Vocal hygiene is the more passive therapeutic approach. The premise is to optimize the laryngeal environment through lifestyle changes, including moderation of voice use,

hydration, decreased caffeine and alcohol, and reflux management. These two treatment modalities are most often used in parallel.

Functional Voice Disorders

The symptoms of muscle tension dysphonia are often similar to those associated with phonotraumatic lesions. In fact, the etiology of these disorders also often looks similar in terms of patient report and history. The difference is that in the case of a functional disorder, there is not (yet) a detectable anatomical abnormality that is causing the dysphonia. Functional disorders arise when the *muscular framework* utilized in the motor process of voicing becomes discoordinated. This often presents as palpable extrinsic laryngeal muscle tension, ventricular fold hyperfunction and anterior-posterior laryngeal squeeze, and decreased airflow through the glottis during phonation. These changes in concert ultimately lead to a more rigid and pressed laryngeal motor pattern that translates to a pressed, hoarse, effortful voice quality.

There is little evidence to suggest why some patients develop a functional disorder and others a phonotraumatic lesion, as both typically arise from an increase in vocal load.[13,28–30] A further complication is that muscle tension, the primary symptom and diagnostic criterion of a functional disorder, can be a secondary presentation in the presence of a phonotraumatic vocal fold lesion. Likewise, the vibratory patterns associated with muscle tension dysphonia may contribute to the development of phonotraumatic vocal fold lesions.

Despite the similarities in clinical presentation, phonotraumatic lesions and muscular tension disorders are easily distinguished and differentially diagnosed upon laryngeal stroboscopic exam. Functional disorders are defined by a lack of anatomical changes to the vocal folds, and phonotraumatic lesions are defined by the presence of an anatomical abnormality of the vocal fold

edge that inhibits vibration. In the case where a lesion is identified in addition to added laryngeal tension and hyperfunction, the patient is diagnosed with a primary phonotraumatic lesion and secondary muscle tension dysphonia.

The prognosis for muscle tension dysphonia treated with behavioral therapy is very good, whereas outcomes for behavioral therapy in vocal fold lesions are more varied.[20,30–33] In both cases, behavioral therapy is almost always tried before surgical intervention is considered, but in a large portion of patients with phonotraumatic lesions, surgery is often ultimately required despite undergoing a course of voice therapy first. As the two disorder categories likely arise from similar behaviors, it is of great value to better understand the nature of this difference in presentation. In identifying the molecular underpinnings of the development of lesions, better treatment options may be elucidated that ultimately allows for resolution of these lesions without surgical intervention. Further better understanding of the additional factors that play into the development of lesions in some patients that may not be present in patients with muscle tension dysphonia alone is critical. These risks could include the same factors discussed above, including vocal overuse, and poor vocal hygiene.

Voice Therapy Treatment for Phonotraumatic Lesions

Vocal Hygiene

Vocal hygiene is the overarching umbrella that encompasses everything related to the baseline health of the vocal fold tissue.[18,19,22] It includes recommendations related to hydration, caffeine, alcohol, cigarette smoke, reflux, and allergies. The underlying theory is that, by managing

these factors, the health of the vocal fold tissue and its consequent resilience to injury can be maximized.

The present dissertation project focuses on the role of hydration in vocal fold function and injury. Dehydration may play a direct causal role in adverse vocal fold function. It may also potentiate the effects of other factors such as excessive vocal use and exposure to cigarette smoke. Dehydration has been explored in two ways within the voice outcomes literature: systemic dehydration where the whole system has decreased water content (as is the case when someone doesn't drink enough water), and local/surface dehydration where the local superficial tissues of the upper airway become desiccated (such as when someone breathes dry air). There is anecdotal clinical evidence that systemic dehydration is associated with an increased risk of vocal pathology, and empirical evidence that dehydration increases phonation threshold pressure and perceived phonatory effort.[9,34–39] As a result of general professional consensus and the support of a limited literature base, standard practice for the treatment of voice disorders includes the recommendation for increased systemic hydration. However, the systemic hydration-induced structural and molecular changes within the vocal fold tissue and subsequent long-term consequences have not been well investigated.

CHAPTER 2

DEHYDRATION AND THE VOCAL FOLDS

Current Status of Vocal Fold Dehydration Research

The current body of literature on dehydration and vocal function, summarized in Chapter 2, is of good quality, although its scope and diversity are limited. Based on small sample human prospective studies, *ex vivo* animal studies, and computational modeling, it is reasonable to conclude that recommending increased hydration to voice patients is not harmful, and could help with acute vocal function complaints such as fatigue and increased phonatory effort.[7,8] However, there is much more related to vocal fold hydration that we do not yet fully understand. Disruption of vocal fold tissue homeostasis has negative implications acutely following phonotrauma, as well as over time as the tissue of the vocal fold is damaged and repaired continuously. In the present study, the goal was to identify the specific tissue changes associated with systemic dehydration. In better understanding the changes at a tissue and cell level, more insight can be gained about the interaction between dehydration and phonotraumatic injury and repair.

Human Studies

There have been several prospective small-cohort human subject studies assessing the effects of hydration on vocal function. In these studies, variables such as phonation threshold pressure (PTP), perceived phonatory effort, acoustic outcome measures, and laryngeal imaging measures were analyzed and found to be largely negatively affected by dehydration.[10,11] (Phonation threshold pressure is a widely used measure, especially in research studies. It is the

minimum pressure from airflow through the glottis that will induce vocal fold vibration. When vocal fold function is impaired, stronger airflow is required.) The studies vary considerably in their methods of inducing a dehydrated condition. The methods include reduced water intake prior to the experimental session, ingestion of a decongestant or diuretic, and exposure to a dry (low humidity) environment. A review by Hartley et al. synthesized these studies into tables that make it easy to compare approaches.[40] Based on their review, the consensus was that dehydration can cause an increase in perceived phonatory effort as well as in phonation threshold pressure, sometimes also negatively affecting acoustic outcome measures such as jitter/shimmer. However, a statistical meta-analysis by Leydon et al. was unable to synthesize the phonation threshold pressure data from existing studies to find a significant effect of dehydration.[41] They concluded that while some individual studies showed effects of dehydration, the range of methods used across this research area makes generalizations difficult. Further, the level of dehydration of subjects across studies was not consistent or well documented, so comparing effect sizes across studies was challenging. Despite these shortcomings within the literature, there is consensus that dehydration, whether surface or systemic, causes an increase in both perceived effort and phonation threshold pressure.

Ex-Vivo Animal Studies

One supplement to the limitations of human studies is the use of excised whole laryngeal models to better quantify the changes in tissue properties induced by dehydration. Unlike *in vitro* studies where individual live cells are studied in a controlled physiologic condition, an *ex vivo* approach maintains the whole organ structure of the tissue, such that anatomic conditions can be assessed. However, in excising the tissue in this manner, information about the real-time

physiologic response of live tissue is sacrificed. This approach has been reported in canine, porcine, and several other model animal species. In these studies, summarized well by a review from Sivasankar and Leydon (2010), dehydration of tissue can be achieved through submersion in hypertonic solution where osmotic pressure drives water out of cells and extracellular spaces. Once the experimental tissue dehydration has been induced, vibration can be achieved in an excised larynx cabinet, and biomechanical laryngeal data can be collected. These studies investigate the effects of overall tissue hydration by submerging the tissue in different solutions[42], changes to tissue biomechanics following surface drying challenges[43], and the effects of covering the vocal folds with viscous solutions that mimic thick mucus.[44]

These excised larynx studies have shown that water content within the vocal folds plays a strong role in determining their biomechanical properties. However, the extent of dehydration (as measured by percentage of cell volume change) assessed in these experiments is far beyond what is physiologically tolerable in a living system. While the measured biomechanical changes in these *ex vivo* models are informative about underlying processes, they may not be easily generalizable to an *in vivo* system where the body system is actively working within the tissue to maintain homeostasis. These studies together, despite uncertainty of generalizability, suggest that changes in vocal fold viscosity may be in part contributing to the changes in perceived effort and phonation threshold pressure identified in the human studies described above, as dehydrated tissue is more viscous.

Computational Modeling

Computational modeling provides a different perspective on the questions associated with dehydration vocal function. By combining known information about the viscoelastic properties of

the vocal folds (found through *ex vivo* experimentation) with computational modeling and algorithm-based prediction, investigators can estimate the amount of shear and strain that the vocal folds undergo. Areas of greatest strain can be identified, and subsequent consequences of viscoelastic changes (i.e. decreased water content) can be modeled, thus elucidating potential mechanical processes associated with lesion development.

A study by Hanson et al. (2010) used an excised canine model to confirm the biphasic theory of vocal fold physiology.[45] The biphasic model suggests that the viscoelastic properties of vocal fold tissue are comprised of two main components: a relatively rigid extracellular matrix mesh, and a more mobile interstitial fluid that fills in the space between cells. By confirming the validity of this simplification, Hanson et al. (2010) significantly decreased the computational complexity of running simulations in the vocal fold model system. Using the biphasic model of vocal fold tissue, the vibratory stresses and strains of vibration can be quantified with spatial and time resolution in varying conditions of interstitial fluid volume. Thus, the implications of water loss on the overall damage incurred by the vocal fold during vibration can be elucidated and eventually can inform clinical recommendations.

Bhattacharya and Siegmund (2015) took this model one step further and attempted to integrate fluid flux within the vocal fold during vibration into the biphasic model. Because the fluid in the vocal fold is relatively free moving in relation to the extracellular protein matrix, it follows that the stresses and forces induced by vibration could cause flow of this fluid across regions of the vocal fold during each vibratory cycle. This could induce short term, local changes in hydration status of the vocal fold tissue. The study confirms the use of a more complex modeling scheme that can effectively account for these fluid flux changes, but it has yet to be applied to different starting hydration conditions.

In-Vivo Animal Studies

Two *in vivo* animal studies of vocal fold properties in dehydration have been reported, both by the same research group. Oleson et al. first utilized a rat model to investigate vocal fold changes related to dehydration.[46] This was a preliminary study in which the feasibility of using proton density weighted magnetic resonance imaging (MRI) to detect hydration within the vocal folds was assessed. Following a 10% reduction in rat total body weight resultant from water deprivation, there was a statistically significant reduction in detected water content of the vocal folds. Unfortunately, as this was a preliminary study, no functional outcomes were investigated. A second study completed by the same group then investigated the same measures in rehydration following acute dehydration.[47] While these studies achieve a step in the right direction toward understanding physiologic changes to the vocal fold, there are questions still left unanswered, as this study still only focused on the presence of water itself within the tissue.

Summary of Current Knowledge

All of these models are useful tools in investigating different aspects of the acute functional effects of dehydration on vocal function, but they fall short of providing evidence that dehydration is linked to chronic pathology. To that end, there is no indication of the mechanism behind changes to perceived phonatory effort or phonation threshold pressure quantified in human studies. However, there are several important common findings: computational modeling and *ex vivo* studies show that decreased water in the vocal fold increases viscosity of the vocal fold cover, and thus the vocal folds require greater pressures to induce and maintain periodic vibration. Additionally, *ex vivo* and human studies show that increased thickness of secretions (which may be caused by dehydration) can change the vibratory cycle significantly and alter acoustic

parameters. Together, these changes in vibratory function lead to predictable changes (through computational modeling) in the shear and strain profile of the vocal fold tissue system during vibration, creating potential for physiologic and anatomic pathology.

Broader Literature in Dehydration

Systemic Dehydration and its Effects

The systemic effects of dehydration are far-reaching. Many systems within the body are affected by even mild levels of systemic dehydration. Among the different tissue structures affected by dehydration are extracellular interstitial fluid spaces, blood plasma, skeletal muscle, glands, and vascular structures. These changes each have their own distinct implications for vocal fold tissue structure and function.

Decreased Lamina Propria Water Content – Viscoelastic Changes: The best-understood change to vocal fold structure following systemic dehydration is the decrease in water volume of the lamina propria, as has been studied through excised larynx experiments and computational modeling. Decreased water intake causes an increase in systemic plasma osmolality and viscosity, causing a corresponding change in the osmotic gradient between blood vessels, interstitial fluid (such as that found in the extracellular matrix of the lamina propria), and the interior of cells. The osmotic gradient drives water into the blood plasma, reducing water volume within the interstitial space, such as the extracellular matrix of the lamina propria.

While not a direct physiologic replicate of dehydration *in vivo,* excised tissue studies have demonstrated changes to the viscoelastic properties of the vocal fold in response to local tissue dehydration. The water content within the vocal folds is a large factor in determining

biomechanical properties of the vocal folds, and these studies allow for systematic measurement of the level of dehydration achieved in their conditions.

Decreased Lamina Propria Water Content – Cellular and Molecular Changes: A decrease in vocal fold fluid volume has direct implications for tissue viscosity and structure [48], but it can also potentially affect cellular and molecular function within the vocal fold. An inherent limitation of *ex vivo* studies is that the tissue is removed from its host. As soon as this occurs, the tissue loses its dynamic ability to compensate for environmental changes and respond to signaling within the organism.

As free-fluid space decreases, enzymes have less space for activation and degradation. Additionally, less fluid makes it more difficult to maintain optimum pH within the interstitial fluid, which can further inhibit enzyme and protein activity. It is possible that tissue dehydration inhibits enzyme and protein activity such that inter- and intra-cellular signals cannot be transmitted and terminated in a timely manner. This dysregulation could ultimately lead to buildup of unfavorable signaling molecules such as those that regulate inflammation or cell division. These changes could quickly cause a chain reaction of adverse consequences that would otherwise be quenched within a well-hydrated tissue system. While the severity of tissue dehydration would likely need to be very high for an effect of this magnitude to result, this cascade demonstrates the effect that dehydration can have on molecular mechanics and interactions, and vocal fold tissue is susceptible to these consequences in the face of repeated phonotrauma.

Decreased Vascularity: Further fuel for the inflammatory cascade is the constriction of blood vessels. To maintain systemic blood pressure when blood volume is low, the capillaries and small vessels that feed non-vital organs contract. All small vessels are vulnerable to constriction in dehydration, including the vessels that feed the vocal fold tissue. Subsequent decreased blood

supply can lead to diminished nutrient availability as well as reduced capacity to flush out waste. Especially in the case where phonotrauma has occurred, and lots of tissue remodeling is underway, the removal of waste is critical to the maintenance of a homeostatic and functional vocal fold system. The vocal folds are nearly continuously undergoing damage and remodeling, therefore limited vascularity in dehydrated vocal fold tissue is an especially critical problem.

Changes in Mucosal Blanket: Dehydration also causes decreased hydration of mucosa and mucous lining. In the vocal fold this not only has negative implications for the epithelium and mucosal blanket as a physical barrier[49], but it also has negative implications for vibratory efficiency.[44] Thicker mucous on the surface of the vocal fold is stickier and more viscous, which prolongs the closed phase of the glottal cycle artificially. This change in glottal cycle contributes to decreased vibratory efficiency. Due to functional vibratory changes, associated vibratory forces also change and create a greater propensity for phonotraumatic damage.

Skeletal Muscle Fatigue: Finally, efficient vocal fold vibration requires activation of several different muscle groups within and around the larynx. In dehydration, there is evidence that muscles have less strength and endurance.[50] Phonatory function would certainly be impacted by decreased muscle efficiency of both intrinsic and extrinsic laryngeal muscles, and a dehydration-related decrease in laryngeal muscle strength and endurance could be perceived as increased vocal effort and faster vocal fatigue, as has been reported in human studies describing exercise fatigue in dehydration.[51–53] The larger abdominal muscles required for respiration would be similarly affected by dehydration. As the source for airflow in phonation, changes to this muscle group could also contribute to patients' report of increased vocal fatigue.

The Proposed Model

Preliminary studies have demonstrated the usefulness of the rabbit model for systematic investigations in vocal fold biology.[15,54–57] Although no perfect animal model exists for the complete assessment of vocal fold function and physiology, the rabbit model is well-established in the investigation of cellular changes to the vocal fold tissue, and provides a robust system for the investigation of dehydration-induced laryngeal changes in a laryngeal tissue structure similar to humans.[58–60] The rabbit vocal fold shares similarities with the human vocal fold with respect to vocal fold extracellular matrix components, and direct, accurate measurement of vocal fold tissue changes and gene expression is possible.

Achieving Dehydration

There are several ways to model dehydration for research, and these methods have been used across many different species. Among these methods are: water deprivation, sweating with heat or exercise without water replenishment, the administration of a diuretic such as furosemide, or a combination of the above [51,61–65]. In humans, certain medications can also place people (especially older adults) at higher risk of dehydration including diuretics, vasodilators, β-Blockers, aldosterone inhibitors, ACE inhibitors and Angiotensin II inhibitors.[66] Literature suggests that in humans, as little as a 2% loss in body mass (in water) can cause clinically significant physiologic change within the body.[51]

Intracellular dehydration (hyper-osmotic hypovolemia) is defined when the water lost is hypo-osmotic, meaning that the salt content of the water lost is less than the salt content of the blood plasma, and is most commonly induced with water deprivation. As solute concentrations in the blood plasma increase, so does the osmotic pressure between the capillaries and the

extracellular fluid. As this osmotic pressure equilibrates through water leaving the extracellular fluid and entering the blood plasma, this osmotic pressure gradient continues, and water is directed out of cells and into the extracellular fluid to equilibrate their solute concentrations. Overall, this cascade creates a decrease in water content across all three water compartments: blood plasma, extracellular fluid, and intracellular fluid. Subsequent changes to the vascular system attempt to compensate for the acute changes to blood plasma volume, involving peripheral and renal vasoconstriction, as well as renal water and sodium retention via arginine vasopressin and rennin-angiotensin-aldosterone.[51]

Acute systemic water deprivation is the dehydration method selected for this study, as it induces dehydration of all three water compartments, including cells, extracellular fluid, and blood plasma. Additionally, use of water deprivation does not create additional confounding factors, as would be inherent in a drug administration model (i.e. diuretics). A dehydration level of 10% is the target for this study, as rabbits have been found to tolerate this level of dehydration, and a robust response is desirable for an acute dehydration condition such as what is proposed in this study.

Measuring Structural Tissue Changes

Histological tissue analysis has been a gold standard in the field of laryngology and voice research [12,67–71]. The vocal fold tissue is extremely specialized in function, and as such has a very distinct anatomical structure with unique and important cellular mechanisms by which that structure is maintained. Thus, anatomical structure, both in gross morphology as well as cell localization, has been well documented across the literature in many species including rabbit and human.

Use of histological analysis to investigate potential changes in the physiologically relevant anatomical features of the vocal fold will be helpful in understanding the impact of dehydration on vocal fold function and wound healing. This is particularly true in light of the changes associated with dehydration in other tissue types. Measures of vocal fold volume, vascularity, immune cell infiltration, and fiber organization can all be assessed within histological analysis. One aim of the present study was to measure the effect of systemic dehydration on vocal fold structure and protein localization in the *in vivo* rabbit model of acute systemic dehydration using histological analysis.

Measuring Changes to Tissue Homeostasis and Cellular Processes

Another methodology that is well established in basic science literature is the assessment of gene expression within a tissue sample of. Theoretically, within the framework of biology at the molecular and cellular level, gene expression is one step prior to the manifestation of structural changes visible in histological analysis. Gene expression changes, or changes in gene transcription, can and do occur in response to different stimuli.[72] Within the DNA of each cell are genes that encode for every possible function within the cell. In response to a change in environment or stimulus, the cell will alter the level at which different genes are expressed, ultimately affecting downstream the proteins that are produced and expressed within the cell. In the case of dehydration, there are upstream messages sent within the cell, in the form of mRNA, that signal the cell to alter protein production. For example, the cell might start to produce more factors that signal changes to the inflammatory cascade, or changes in endothelial cell size or configuration. The quantity of mRNA (coding for a particular gene of interest) that is being produced within the cell can be quantitatively assessed using a methodology called real-time quantitative polymerase

chain reaction, or QRT-PCR.[73,74] Tissue is collected and immediately preserved in RNAlater, such that the mRNA within the tissue can be extracted and quantified in a very directed manner. The second aim of the present study was to measure the effect of systemic dehydration on laryngeal regulatory and inflammatory gene expression. This was achieved in an *in vivo* rabbit model of acute systemic dehydration using quantitative real-time polymerase chain reaction methodologies.

CHAPTER 3

MATERIALS AND METHODS

Animals and Protocols

All procedures were completed in accordance with a protocol approved by the Vanderbilt Institutional Animal Care and Use Committee, under protocol number M1900028: Safety and Efficacy of Treatments for Voice Disorders - Dehydration. A total of 16 New Zealand White rabbits were used in this study, 4 for protocol development and 6 for each aim of the completed study according to Table 1 for a total of 12 animals used for data collection.

In order to minimize inter-subject variability in this pilot study, only male adult rabbits were utilized. All animals were ordered through Charles River, and weighed between 3.79 and 4.08 kg (mean=3.94kg, SD=0.092kg) at the start of the study. All rabbits were allowed a 7-day acclimation period upon arrival to the Vanderbilt animal care facility and prior to start of the 72-hour experimental protocol. Part of this acclimation period included exposure to the canvas restraint bag and handling. Additionally, enrichments were limited such that the transition to the experimental protocol would be as smooth as possible. These adjustments included providing water only via bowls and bottles such that water consumption could be tracked, and the only edible treats provided were yogurt drops. One cup of standard chow was available to each rabbit per day, per the standard operating procedures for rabbits in the Vanderbilt animal care facility. Toy enrichments remained unchanged and included chew toys, noisemakers, and balls.

In preparation for the experimental protocol, all rabbits' ear fur was clipped prior to experimental start.

Dehydration Procedure

During the experiment, animals had access to 1 cup of standard chow per day and were rewarded following each handling event with 3 yogurt drops (Vitakraft Drops with Wild Berry, 3.5% moisture max). For animals undergoing water deprivation, water was withheld for the predetermined 72 hours (achieving approximately 10% body weight loss).[75–77] Free water control group animals were treated in an identical manner, except water consumption was tracked and not withheld.

Every 6-10 hours over the course of the study (3 times per day), each animal was weighed, and a blood sample (<700µl) was collected for analysis according to Figure 2. Blood samples were collected via peripheral ear vein using a 24-gauge ¾ inch catheter and 1ml syringe. Prior to catheterization, the animal was placed in a canvas animal restraint bag (Medivet Feline Restraint Bag, 5-10lbs), and the rabbit's ear was sterilized with an alcohol wipe and anesthetized using a 5% lidocaine topical cream (RectiCare Anorectal Lidocaine 5% Cream). The lidocaine was allowed 5 minutes to achieve effectiveness before catheterization.

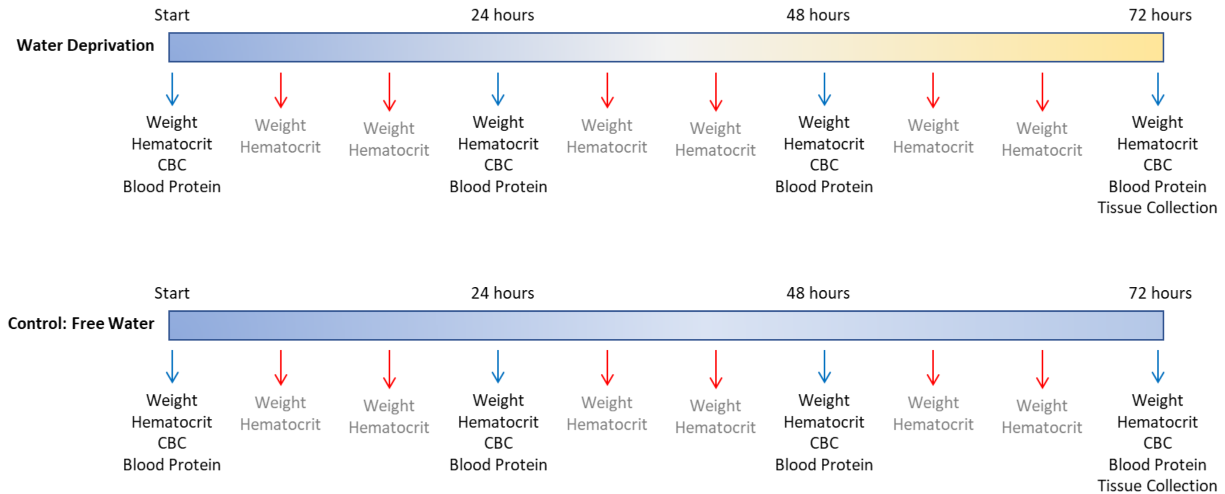

Figure 2. Study Timeline

The morning of each study day, blood was utilized for hematocrit analysis, a CBC panel, and additional blood composition analysis (700µl). At the other two time points each day, blood was collected for hematocrit only (100µl). For hematocrit analysis, blood was drawn directly from the catheter into a blue-tip capillary tube, spun in a centrifuge at 5000rpm for 3 minutes (Unico Hematocrit Centrifuge), and immediately assessed for hematocrit percentage by measuring the ratio of red blood cells to total volume (reported as a percentage). For CBC analysis, approximately 200µl of whole blood was transferred to a 0.5ml EDTA pre-treated tube and subsequently analyzed using the TPSR core facilities (Forcyte CBC Analyzer, Oxford Science Inc.). Serum from the remaining whole blood collected each morning (approximately 400µl) was spun out and processed by TPSR core facilities (Vet Axcel Chemistry Analyzer, Alfa Wasserman) for protein and ion concentrations.

At the end of the experimental protocol, a final blood sample (700µl) was collected via the same method described above, but prior to catheter removal, 2ml of Euthasol was administered

intravenously as a means of humane euthanasia. Immediately following euthanasia, the larynx was harvested and processed for subsequent structural and gene expression tissue analysis.

Data Collection

Dehydration Measures

Body weight change in each animal served as a proxy in this study for gross water loss, as there is other literature to suggest that 10% body weight loss is a reasonable level of dehydration in New Zealand white rabbits.[75,77–79] However, because hematocrit is a better established and more robust measure of dehydration overall compared to body weight change, hematocrit was also collected in this study to provide additional information and to build a better foundational baseline in a rabbit model of dehydration for future studies.[78,80,81] Blood samples were also processed for CBC (complete blood count) panel and total protein analysis. These measures, including hematocrit and blood osmolality, provided within-subject information about changes to overall health and hydration during the study period.

Slide Processing and Analysis

Among the 6 animals in each group, (water deprivation and free water control) each harvested larynx was bisected such that at least 3 half-larynges (from distinct subjects) were available for each type of tissue analysis in each group. These three types of tissue analysis included: formalin fixation, flash freezing in OCT, and sub-dissection of vocal fold tissue for storage in RNA later. A schematic of sample distribution can be found in Figure 3.

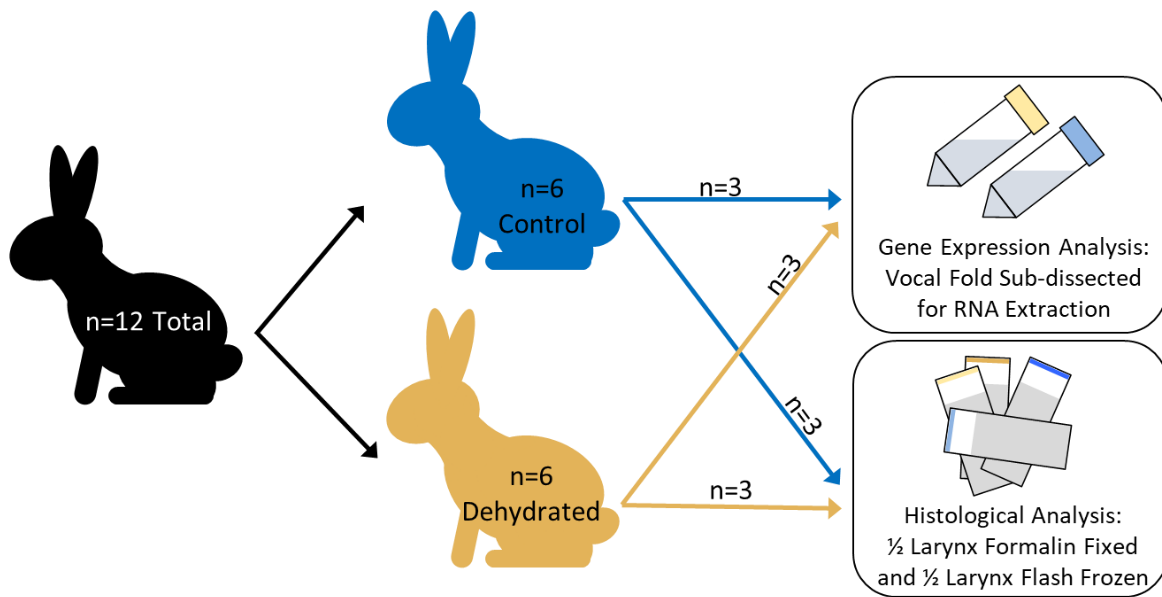

Figure 3. Schematic of Subjects and Samples

Formalin fixed tissue specimens were embedded in paraffin and sectioned at 5 or 10µm thickness, such that sections representing the middle third portion of the vocal fold were utilized for analysis. Paraffin tissue sections were deparaffinized with xylene solution and stained with the following standard stains per manufacturer specification: Hematoxylin and Eosin (H&E) – gross structure of the layers of the lamina propria of the vocal fold as well as muscle fiber organization and thickness and muscle fiber density; Elastica van Gieson (EVG) – fibrous organization of the vocal fold lamina propria; Grocott's Methenamine Silver (GMS) – mucous blanket and sub-glottic laryngeal glands. Due to variability in section thickness for the formalin fixed tissue slides, quantitative image analysis could not be completed. Future blinded qualitative assessment of these samples is planned, but was not included in the scope of the present study.

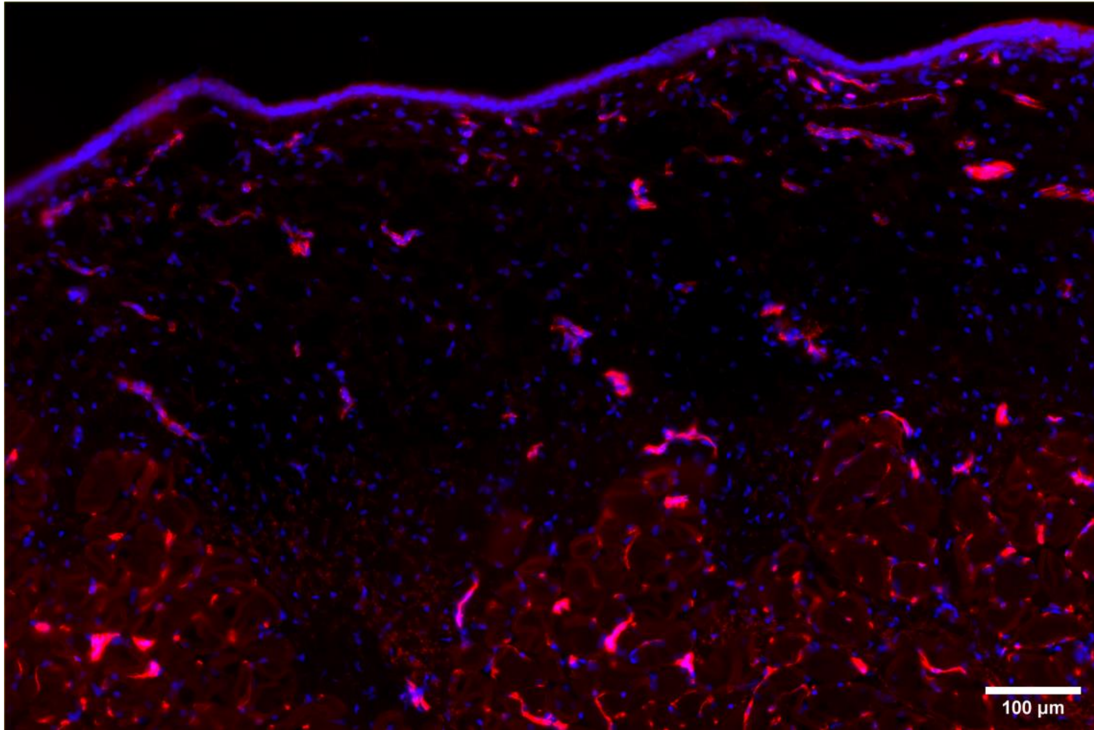

Figure 4. Lamina Propria Vascularity. Fluorescence microscopy image of vocal fold lamina propria labeled for CD-31 (red) and counterstained with DAPI for nuclei identification (blue). Scale bar represents 100µm.

For immunofluorescence labeling, tissue flash-frozen in Optimal Cutting Temperature (OCT) were sectioned at 12µm thickness. Slides were fluorescently immunolabeled using antibodies specific to: E-cadherin (epithelial marker); CD31 (endothelial cell marker of vascularity); CD11b (inflammatory cell marker); TUNEL (apoptosis[60]). An example of CD-31 lamina propria labeling can be found in Figure 4, and an example of E-cadherin gland labeling can be found in Figure 5.

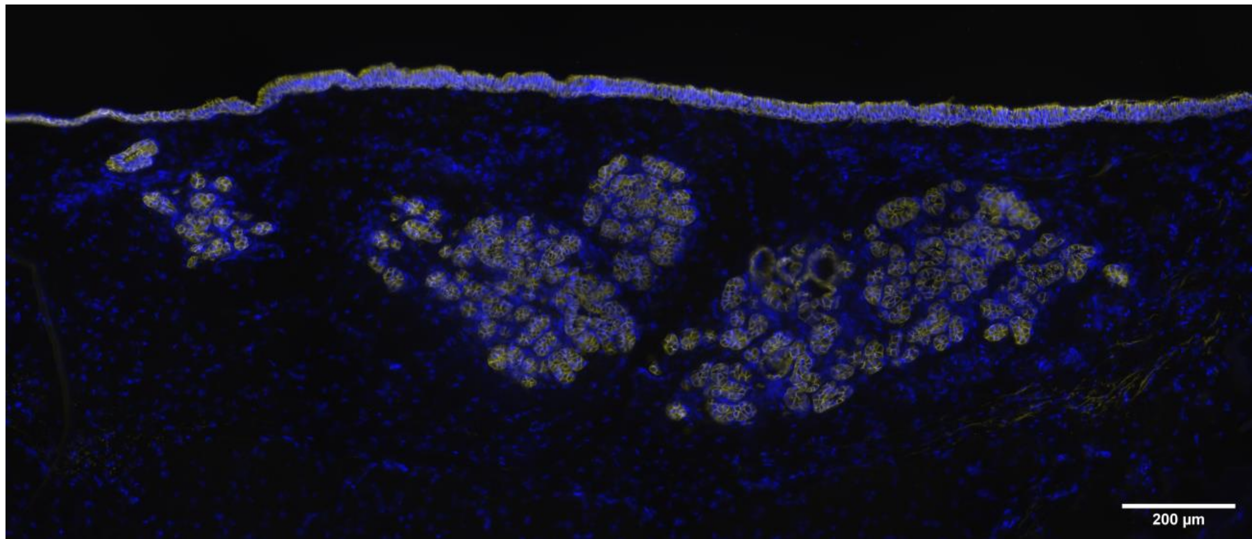

Figure 5. Laryngeal Gland. Fluorescence microscopy image of subglottic laryngeal gland labeled for E-cadherin (yellow) and counterstained with DAPI for nuclei identification (blue). Scale bar represents 200µm.

Each stained/labeled slide was captured digitally using a Nikon confocal microscope at 20 or 40X magnification. Stained slides were imaged at the superior superficial edge of the vocal fold for all samples.

For all immunolabels, images were first randomized and deidentified to avoid biased results. Fluorescence labeling of CD-31 and E-cadherin was subject to quantitative analysis in ImageJ. CD-11b and TUNEL assays yielded labeling levels too low for reliable and accurate assessment between groups, as the amount of positive staining fell below the reliable range for this methodology (in some cases zero). Using the randomized images of CD-31 and E-cadherin, first a region of interest containing the lamina propria or glands was selected. The percent area of positive labeling within the region of interest was then quantified using ImageJ's "color threshold" function using the thresholding parameters dictated by threshold presets. The "analyze particles" function was used to quantify the percent area within the region of interest that was positively labeled for the protein of interest.

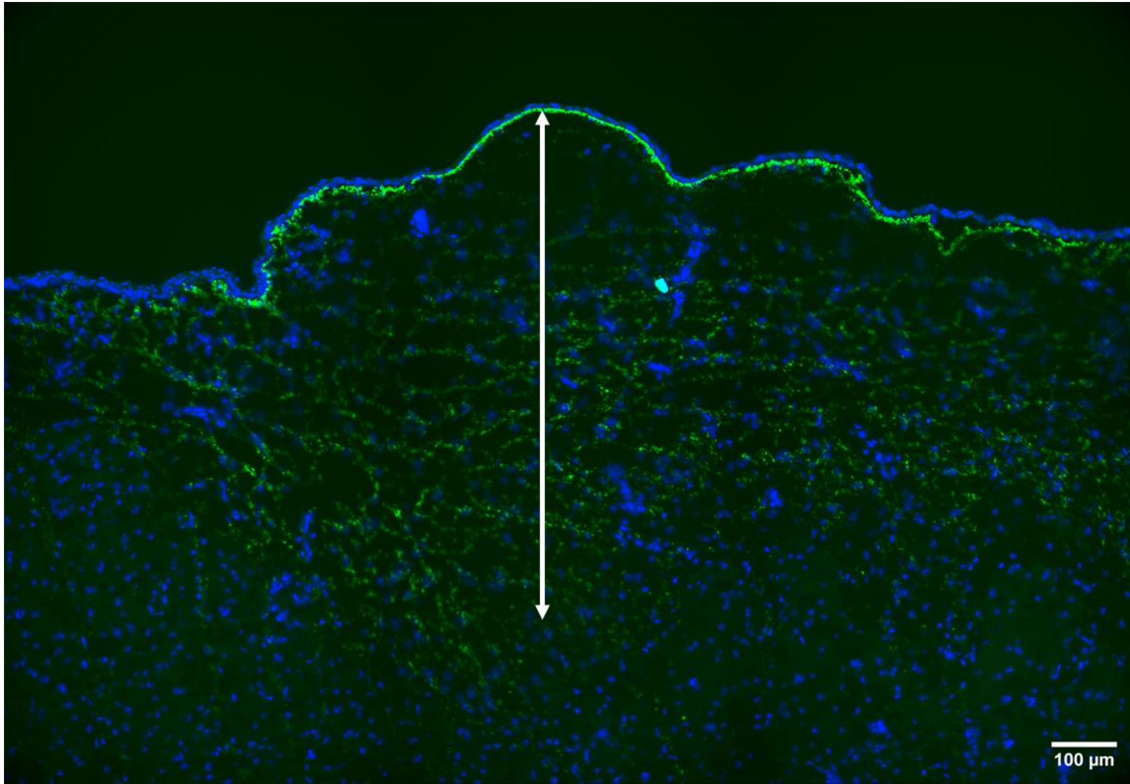

Figure 6. Lamina Propria Measurement. Fluorescence microscopy image of vocal fold lamina propria, where autofluorescence of extracellular matrix fibers appears in green, and DAPI counterstaining highlights cell nuclei (blue). White arrow represents an example of the length measurements collected along the true vocal fold length. Scale bar represents 100µm.

CD-31 images were additionally analyzed to measure lamina propria depth, as flash freezing tissue best preserves tissue volume compared to formalin fixation and paraffin embedding. These fluorescence images were used to visualize the autofluorescence of mature elastin fibers within the lamina propria. The 'Grid' plugin was used to manually measure the depth of the lamina propria (perpendicular distance from basement membrane to muscle fibers) every 63.2µm (√4000µm) along the length of the vocal fold edge and averaged across the image (Figure 6).

Gene Expression Quantification and Analysis

The vocal fold tissue preserved in RNAlater was the placed in a 2ml Eppendorf tube with approximately 15 2.0mm zirconium oxide beads (Next Advance) and 700µl TRIzol. The samples were homogenized in an Omni International BeadRuptor Elite for 20 second cycles at 7m/s, cooled on ice for 1 minute and 40 seconds, and homogenized for an additional 20 seconds at 7m/s. All samples were centrifuged to remove debris, and supernatant was transferred to new clean tubes for RNA extraction according to the Zymo Direct-zol RNA MiniPrep Kit. Following RNA extraction, cDNA was synthesized using the Applied Biosystems High Capacity cDNA Kit. Transcript levels were detected using quantitative real-time PCR (BioRad). Custom probes and primers were designed to target transcripts of the following genes: Endothelin 1 (EDN1) which is a marker of vasoconstriction[82], Endothelial nitric oxide synthase (eNOS) which is a marker of increased blood pressure and vasodilation[83], and IL-17 which is a pro-inflammatory cytokine implicated in fibrosis.[84]

Transcription of these three genes was normalized by use of the housekeeping gene SDHA (succinate dehydrogenase subunit A), which plays an important role in the mitochondrial Krebs cycle, and was hypothesized and assumed to be unchanged between the experimental conditions. Relative gene expression levels were detected and measured using probes featuring a 5'FAM reporter and 3'TAMRA quencher in a BioRad. These data were captured and analyzed using a Bio-Rad CFX Connect Real-Time PCR Detect System and accompanying CFX Maestro software. Primer and probe sequences for these 4 gene transcripts are listed in Table 1.

Table 1. Primer and Probe Sequences for Gene Expression Analysis

Gene	Forward Primer	Reverse Primer	Dual-Labeled Probe
Rabbit SDHA	GCTGCATTTGGCCTCTCGGA	CAGAGCCCTTCACGGTGTCG	TGCCTCCCTGTGCCGCCACA
Rabbit IL-17	CACCGCAACGAGGATCGTGA	GGACTCCCTGCGTAGGACCA	TGGCGGCACTTGGCCTCCCA
Rabbit EDN1	CGGGCACATCGTCCCGTATG	TGTGGTACGCTGCCCTGGTA	AGCCCGTCCAGGTCCAAGCG
Rabbit eNOS	CTGCGGGATCAGCAACGCTA	ATGCGGCTTGTCACCTCCTG	TTCGGGCTCACGCTGCGCAC

Statistical Analysis

All data were analyzed using Graphpad Prism 8.

For all measures collected *during* the dehydration experiment, sample sizes were larger due to the pooling of subjects between the two tissue processing methods discussed above. A mixed-effects repeated measures regression model was used, with significance set at $p>0.05$, with time and condition as the independent variables. In some cases, post-hoc two-tailed Sidak's Multiple Comparisons testing was completed using adjusted p-values to further identify the sources of variance. For each of these measures, n=6 per group. The measures analyzed in this way included body weight, hematocrit, and blood concentrations of creatinine, albumin, calcium, chloride, potassium, and sodium. Peripheral blood counts of basophils, eosinophils, monocytes, lymphocytes, neutrophils, and total white blood cell counts were also analyzed using a mixed-effects repeated measures regression.

For each of the quantitative tissue analysis measures completed *following* euthanasia and tissue harvest (percent positive immunolabeling within the region of interest, or lamina propria depth), sample sizes were smaller due to the division between the two tissue processing methods. Therefore, main effects between experimental (water deprivation) and control (free water) groups were tested using a non-parametric two-tailed Mann-Whitney U, with significance at $p<0.05$, and condition as the independent variable. Each subject (n=3-4 per condition) had a maximum of 3

slides per measure that were analyzed, and all analyzed slides were treated as individual data points, for a maximum of 12 slides per group. Some expected and unavoidable attrition of individual slide samples occurred during processing due to non-ideal tissue quality, but for all analyses, $n \geq 8$ slide images.

CHAPTER 4

RESULTS

Direct Measures of Dehydration

Hematocrit

A mixed-effects repeated measures regression analysis of hematocrit values between experimental groups over time revealed a significant main effect of condition ($F(1,10)=34.42$, $p=0.0002$) and a significant interaction effect of time and condition ($F(9, 88)=6.325$, $p<0.0001$). Raw hematocrit data is presented in Table 2 and graphical representation in Figure 7.

Table 2. Individual Subject Hematocrit Data Over Study Duration

		Hematocrit (%)									
		0hr	8hr	16hr	24hr	32hr	40hr	48hr	56hr	64hr	72hr
Control	Control-03	42	42.5	42	42	43	40	44	42	40	43
	Control-04	41	40	41	40	38	39	38	40	36	38
	Control-05	38.5	39	37	37	37	36	38	35	34	37
	Control-06	44	41	44	39	42	38	42	43	44	37
	Control-07	41	39	39	40	--	41	39	38	--	38
	Control-08	40	39	41	40	40	38	40	40	40	40
Dehydrated	WD-03	43	45	46	46	47.5	46	47.5	49	49	50
	WD-04	42	44	46	43	45	46	46	49	47	49
	WD-05	43	46	45.5	46	48	46	47	49.5	49	48
	WD-06	41	43	45	47	43	42	44	45	45	43
	WD-07	42	43	44	44	45	42	47	46	46	41
	WD-08	41	44	44	45	44	44	43	44	48	48

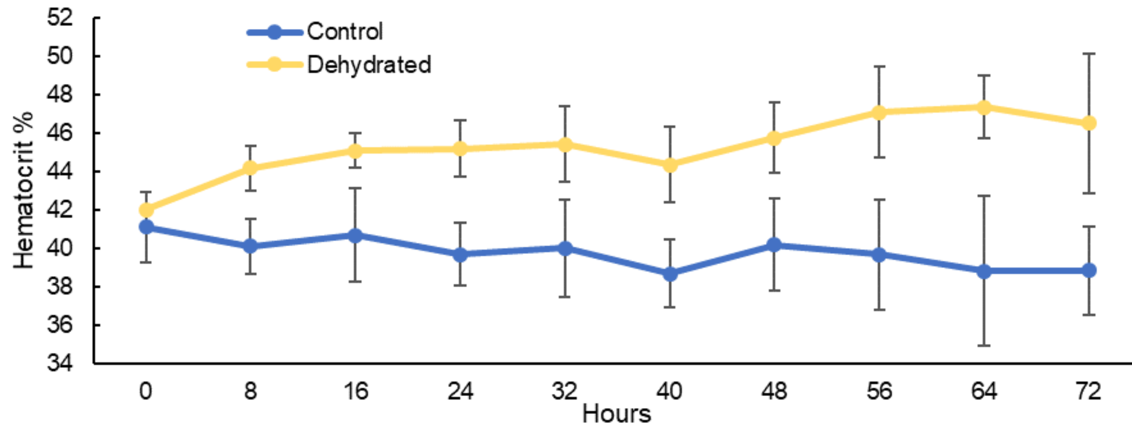

Figure 7. Hematocrit. Graphical representation of hematocrit data, error bars represent standard deviation.

Body weight

A mixed-effects repeated measures regression analysis of animal body weight between experimental groups over time revealed a significant main effect of time ($F(1.104,10.91)=46.38$, $p<0.0001$), a significant main effect of condition ($F(1,10)=13.36$, $p=0.0044$) and a significant interaction effect of time and condition ($F(9,89) = 60.67$, $p<0.0001$). Post-hoc Sidak's Multiple Comparisons testing between groups at individual time points revealed that group differences did not achieve statistical significance until hour 40 of the experiment. Raw body weight data is presented in Table 3 and graphical representation in Figure 8.

Table 3. Individual Subject Body Weight Data Over Study Duration

		Body Weight (kg)									
	Subject	0hr	8hr	16hr	24hr	32hr	40hr	48hr	56hr	64hr	72hr
Control	Control-03	3.87	3.9	3.9	3.8	3.93	3.95	3.82	3.94	3.93	3.91
	Control-04	4.08	4.17	4.16	4.14	4.18	4.19	4.12	4.19	4.17	4.16
	Control-05	3.85	3.94	3.98	3.9	3.96	4	3.93	3.96	4	3.96
	Control-06	3.93	4.03	3.98	3.96	3.98	4.06	3.97	4	4.04	3.93
	Control-07	4.03	4.04	4.02	4	4	4.04	4.02	4.04	--	3.98
	Control-08	3.94	3.83	3.86	3.92	3.85	3.88	3.91	3.88	3.89	3.89
Dehydrated	WD-03	3.79	3.71	3.64	3.62	3.58	3.57	3.52	3.49	3.48	3.44
	WD-04	3.97	3.94	3.84	3.83	3.76	3.75	3.69	3.68	3.67	3.61
	WD-05	3.89	3.88	3.86	3.77	3.76	3.75	3.67	3.65	3.64	3.59
	WD-06	4.08	4.07	4.04	3.94	3.92	3.87	3.82	3.8	3.76	3.74
	WD-07	3.92	3.91	3.88	3.76	3.75	3.75	3.66	3.65	3.64	3.58
	WD-08	4.03	4	3.97	3.84	3.81	3.8	3.75	3.72	3.71	3.65

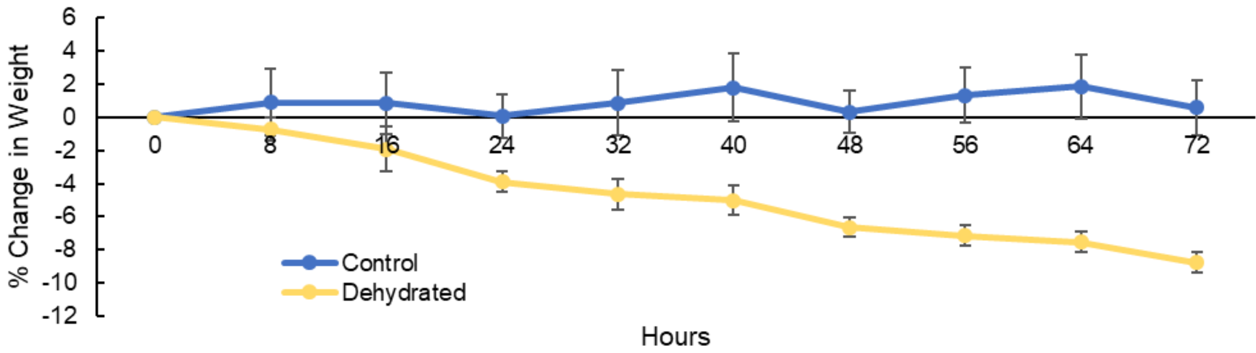

Figure 8. Percent Change in Body Weight. Graphical representation of body weight change over experimental duration, error bars represent standard deviation.

Blood Protein and Ion Concentrations

Summative data of the following sections can be found in Table 4.

Table 4. Summary of Statistical Significance for All Quantitative Analysis

	p-values		
Measure	Main Effect of Condition	Main Effect of Time	Interaction Effect
Hematocrit (%)	**0.0002**	0.14	**<0.0001**
Body Weight (kg)	**0.004**	**<0.0001**	**<0.0001**
Creatinine	0.055	0.61	0.42
Albumin	0.57	0.45	0.57
Calcium	0.77	**0.049**	0.08
Chloride	**0.044**	0.14	**0.0063**
Potassium	**0.014**	**0.042**	0.43
Sodium	**0.002**	0.10	**0.0049**
Basophil Count	0.20	0.11	0.55
Eosinophil Count	0.67	**0.026**	**0.0088**
Monocyte Count	0.15	0.41	0.46
Lymphocyte Count	0.18	**0.017**	**0.0001**
Neutrophil Count	0.11	**0.019**	0.19
Total WBC Count	0.11	**0.0076**	**0.0003**
Laryngeal Gland Size (μm^2)	0.46	-	-
Lamina Propria Depth (µm)	0.67	-	-
Propria Vascularity (%area)	0.62	-	-

Blood Creatinine Concentration

A mixed-effects repeated measures regression analysis of blood creatinine concentration between experimental groups over time revealed no significant main effects of time or condition ($F(2.384,19.87)=0.5668$, $p=0.61$; $F(1,10)=4.705$, $p=0.0553$ respectively). There was also no significant interaction effect between time and condition ($F(3,25)=0.9849$, $p=0.42$). Blood creatinine concentration data is presented in Figure 9.

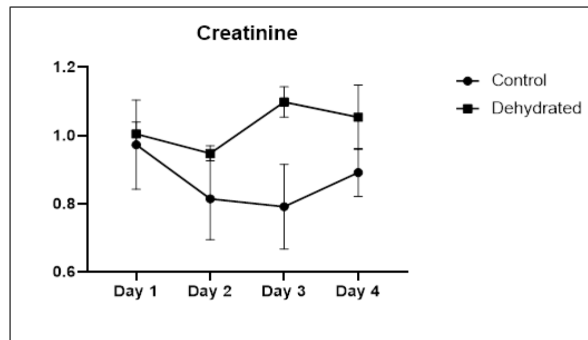

Figure 9. Creatinine Concentration. Measured in mg/dL, error bars represent standard error of the mean.

Blood Albumin Concentration

A mixed-effects repeated measures regression analysis of blood albumin concentration between experimental groups over time revealed no significant main effects of time or condition ($F(1.742,13.94)=0.7990$, $p=0.45$; $F(1,10)=0.3492$, $p=0.57$ respectively). There was also no significant interaction effect between time and condition ($F(3,24)=0.6785$, $p=0.57$). Blood albumin concentration data is presented in Figure 10.

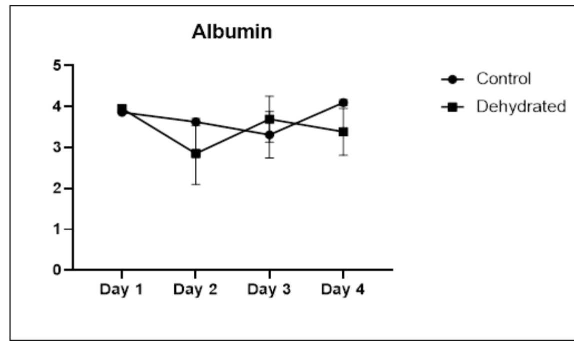

Figure 10. Albumin Concentration. Measured in g/dL, error bars represent standard error of the mean.

Blood Calcium Ion Concentration

A mixed-effects repeated measures regression analysis of blood calcium concentration between experimental groups over time revealed a significant main effect of time (F(1.657,13.26)=4.03, p=0.049). There was no significant main effect of condition or a significant interaction effect (F(1,10)=0.09, p=0.77; F(3,24)=2.49, p=0.084 respectively). Blood calcium concentration data is presented in Figure 11.

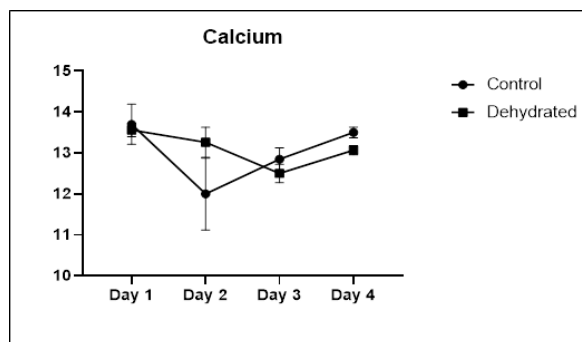

Figure 11. Calcium Concentration. Measured in mg/dL, error bars represent standard error of the mean.

Blood Chloride Ion Concentration

A mixed-effects repeated measures regression analysis of blood chloride concentration between experimental groups over time revealed no significant main effect of time (F(1.699,15.29)=2.265, p=0.14). There was however a significant main effect of condition and a

significant interaction effect ($F(1, 10)=5.285$, p=0.044; $F(3,27)=5.112$, p=0.006 respectively). Blood chloride concentration data is presented in Figure 12.

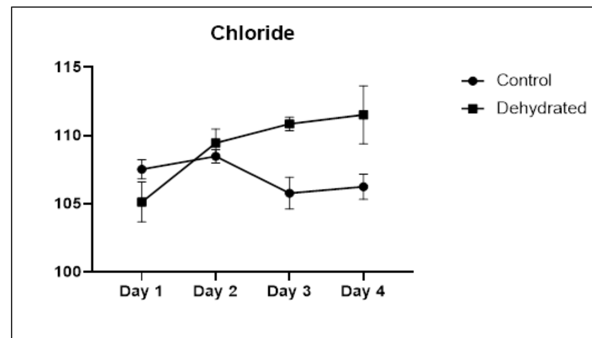

Figure 12. Chloride Concentration. Measured in mmol/L, error bars represent standard error of the mean.

Blood Potassium Ion Concentration

A mixed-effects repeated measures regression analysis of blood potassium concentration between experimental groups over time revealed significant main effects of time and condition ($F(2.605,25.18)=3.300$, p=0.042; $F(1,10)=8.920$, p=0.013 respectively). There was however no significant interaction effect ($F(3,29)=0.9475$, p=0.43). Post-hoc Sidak's Multiple Comparisons testing between groups at individual time points revealed that group differences did not achieve statistical significance until hour Day 4 of the experiment (p=0.026). Raw blood chloride concentration data is presented in Figure 13.

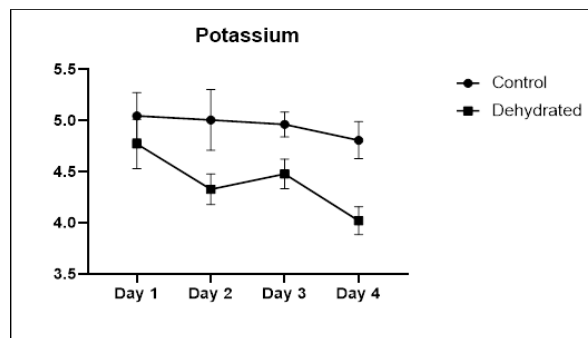

Figure 13. Potassium Concentration. Measured in mmol/L, error bars represent standard error of the mean.

Blood Sodium Ion Concentration

A mixed-effects repeated measures regression analysis of blood sodium concentration between experimental groups over time revealed no significant main effect of time ($F(1.458,14.09)=2.824$, p=0.105). There was however a significant effect of condition and an interaction effect ($F(1,10)=17.29$, p=0.002; $F(3,29)=5.290$, p=0.005 respectively). Blood sodium concentration data is presented in Figure 14.

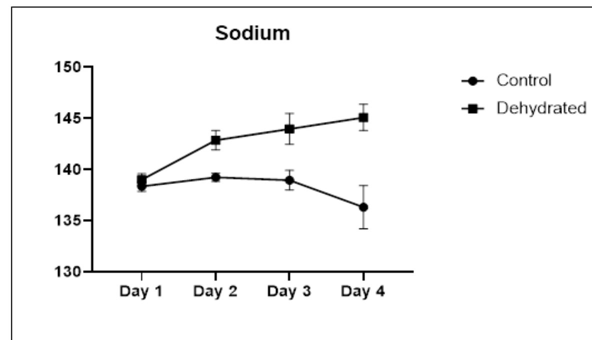

Figure 14. Sodium Concentration. Measured in mmol/L, error bars represent standard error of the mean.

Blood Cell Counts

Basophil Cell Count

A mixed-effects repeated measures regression analysis of basophil cell counts between experimental groups over time revealed no significant main effects of time or condition ($F(1.544,15.44)=2.687$, p=0.11; $F(1,10)=1.919$, p=0.20 respectively). There was also no significant interaction effect between time and condition ($F(3,30)=0.7162$, p=0.55). Basophil cell count data is presented in Figure 15.

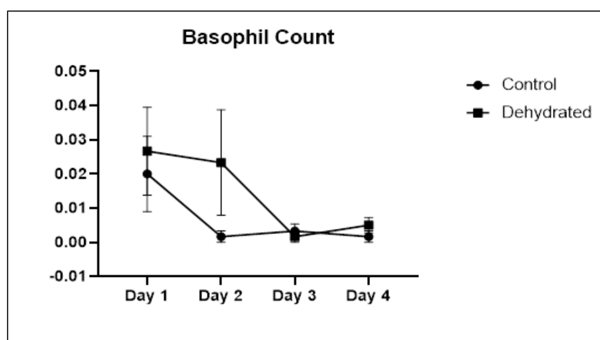

Figure 15. Absolute Basophil Count. Measured in K/μL, error bars represent standard error of the mean.

Eosinophil Cell Count

A mixed-effects repeated measures regression analysis of eosinophil cell count between experimental groups over time revealed a significant main effect of time ($F(1.671,16.71)=4.921$, $p=0.026$), but no significant main effect of condition ($F(1,10)=0.1890$, $p=0.67$). There was, however, a significant interaction effect of time and condition ($F(3,30)=4.642$, $p=0.009$). Post-hoc Sidak's Multiple Comparisons testing between groups at individual time points revealed no significant group differences at individual time points. Eosinophil cell count data is presented in Figure 16.

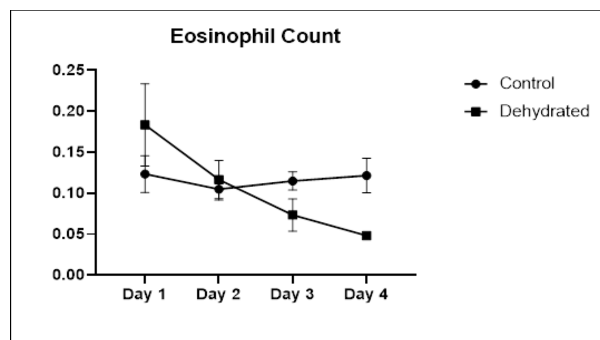

Figure 16. Absolute Eosinophil Count. Measured in K/μL, error bars represent standard error of the mean.

Monocyte Cell Count

A mixed-effects repeated measures regression analysis of monocyte cell counts between experimental groups over time revealed no significant main effects of time or condition (*F*

(1.819,18.19)=0.9231, p=0.41; *F*(1,10)=2.368, p=0.15 respectively). There was also no significant interaction effect between time and condition (*F*(3,30)=0.8943; p=0.46). Monocyte cell count data is presented in Figure 17.

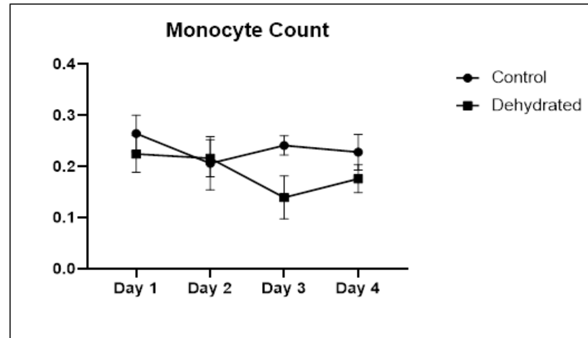

Figure 17. Absolute Monocyte Count. Measured in K/μL, error bars represent standard error of the mean.

Lymphocyte Cell Count

A mixed-effects repeated measures regression analysis of lymphocyte cell count between experimental groups over time revealed a significant main effect of time (*F*(1.857,18.57)=5.324, p=0.017), but no significant main effect of condition (*F*(1,10)=2.122, p=0.18). There was, however, a significant interaction effect of time and condition (*F*(3,30)=9.951, p=0.0001). Post-hoc Sidak's Multiple Comparisons testing between groups at individual time points revealed no significant group differences at individual time points. Lymphocyte cell count data is presented in Figure 18.

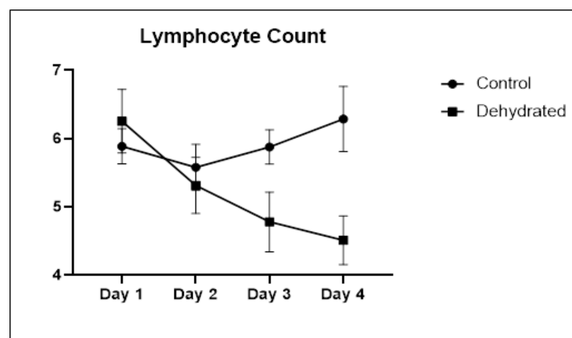

Figure 18. Absolute Lymphocyte Count. Measured in K/μL, error bars represent standard error of the mean.

Neutrophil Cell Count

A mixed-effects repeated measures regression analysis of neutrophil cell count between experimental groups over time revealed a significant main effect of time ($F(1.927,19.27)=4.944$, p=0.019). There was no significant main effect of condition ($F(1,10)=2.989$, p=0.11) or a significant interaction effect of time and condition ($F(3,30)=1.691$, p=0.19). Neutrophil cell count data is presented in Figure 19.

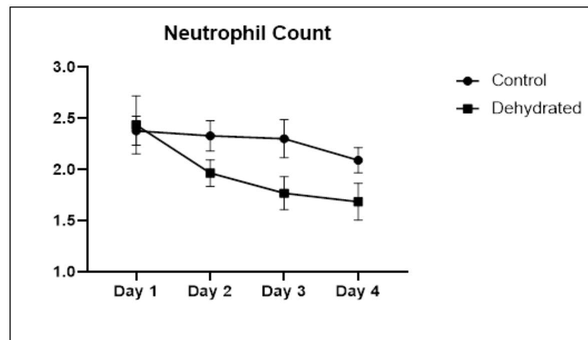

Figure 19. Absolute Neutrophil Count. Measured in K/µL, error bars represent standard error of the mean.

White Blood Cell Count

A mixed-effects repeated measures regression analysis of white blood cell count between experimental groups over time revealed a significant main effect of time ($F(1.427,14.27)=8.146$; p=0.0076), but no significant main effect of condition ($F(1,10)=3.082$, p=0.11). There was, however, a significant interaction effect of time and condition ($F(3,30)=8.355$, p=0.0003). Post-hoc Sidak's Multiple Comparisons testing between groups at individual time points revealed a significant group difference only at Day 4 (p=0.025). White blood cell count data is presented in Figure 20.

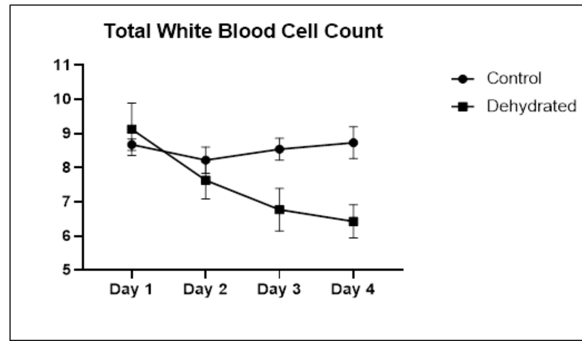

Figure 20. Total White Blood Cell Count. Measured in K/µL, error bars represent standard error of the mean.

Structural Tissue Changes

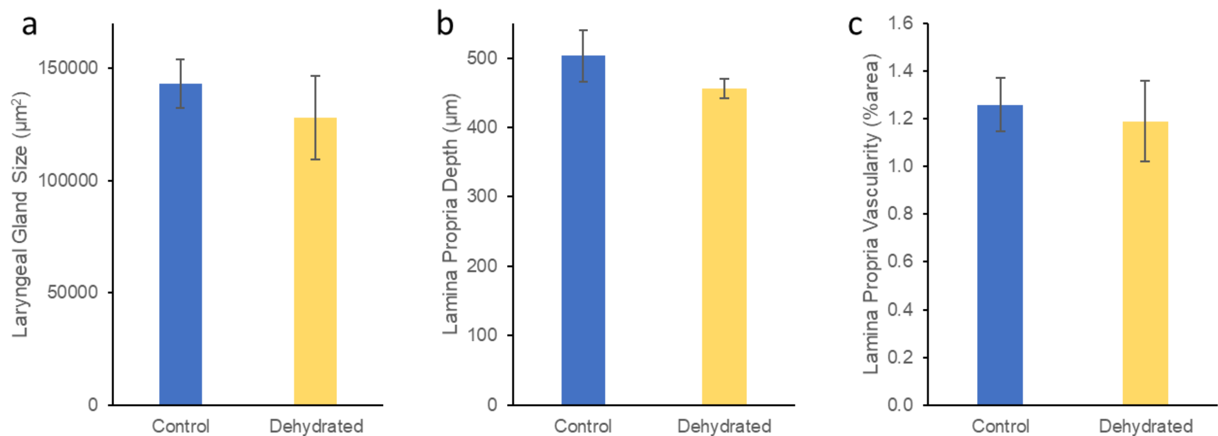

Figure 21. Changes in Laryngeal Structure. a) Laryngeal Gland Size (μm^2), b) Lamina Propria Depth (µm), c) Lamina Propria Vascularity (percent area). Error bars represent standard error of the mean.

Gland Size

Gland size data was analyzed using a non-parametric two-tailed Mann Whitney U. This analysis revealed no significant group differences between the control group and the dehydrated experimental group (U=43, p=0.46). Data presented in Figure 21a, and image example can be found in Figure 5.

Lamina Propria Depth

Lamina propria depth data was analyzed using a non-parametric two-tailed Mann Whitney U. This analysis revealed no significant group differences between the control group and the dehydrated experimental group (U=31, p=0.67). Data presented in Figure 21b, and image example can be found in Figure 6.

Lamina Propria Vascularity

Lamina propria vascularity data was analyzed using a non-parametric two-tailed Mann Whitney U. This analysis revealed no significant group differences between the control group and the dehydrated experimental group (U=42.5, p=0.62). Data presented in Figure 21c, and image example can be found in Figure 4.

Gene Expression Changes

Due to sample loss during processing, data from only two samples per condition are available for gene expression review. Loss of samples occurred during mRNA extraction, where a number of tubes failed and opened during tissue homogenization. Due to small sample sizes, no statistical analysis was completed, and all data are represented in Figure 22.

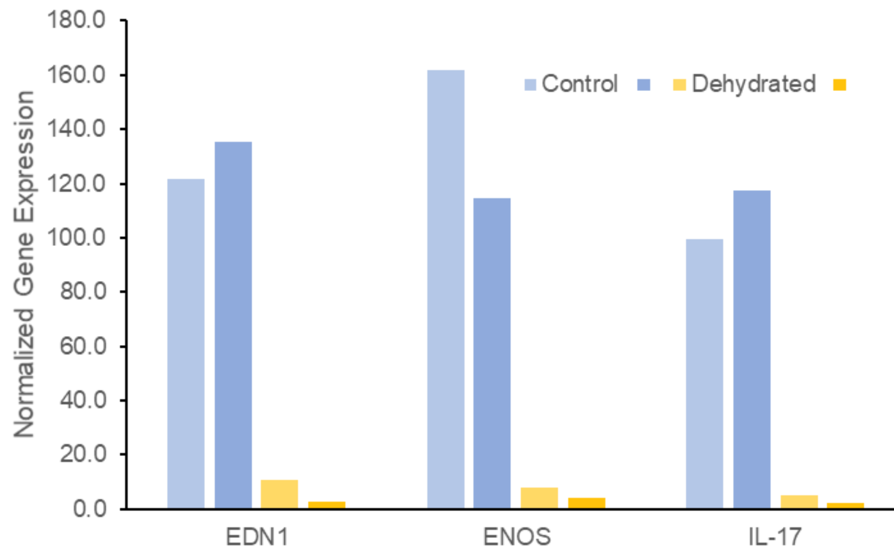

Figure 22. Gene Expression Data. Bars represent individual subject tissue samples.

CHAPTER 5

DISCUSSION

The Rabbit Dehydration Model

In this study, the goal was to identify structural and gene expression vocal fold tissue changes associated with acute systemic dehydration in a rabbit model. This pilot study was essential in the development and validation of a dehydration model that can be used in future studies to investigate dehydration further in the context of voice disorders.

Based on the results of hematocrit and body weight measures, the dehydration protocol utilized in this study was effective. Both hematocrit and body weight measures showed a significant interaction effect of time and condition, showing that the two groups (control and dehydration) were unique, and that the 3 days of water withholding were sufficient to elicit systemic and global changes to the rabbit's homeostatic physiology. These findings are a corroboration of previous literature suggesting that there are significant behavioral and physiologic changes that occur in an acute dehydration model, both specifically in rabbits, and more generally in mammalian systems subjected to acute dehydration conditions.[52,63,65,77]

Subsequent analysis of blood ion concentration was also consistent with other literature, where charged ions became more concentrated in the blood due to a loss of total blood volume. It is expected for dehydration to affect the concentration of blood ions (particularly sodium and chloride), and so findings of the current study confirm the dehydration model used [85–87]. In this study, while other ions (calcium and potassium) had significant differences either over time or between conditions, chloride and sodium concentrations had a significant interaction effect,

suggesting greater change to these concentrations in the dehydrated group over time compared to the control group, and ultimately an effective experimental paradigm to assess acute dehydration. Little was previously known about the New Zealand white rabbit's response to water deprivation, and the findings of this study provide a baseline for future investigation, as well as provide evidence of a similar physiologic and homeostatic response to other mammals when faced with acute dehydration.[86–88]

Interestingly, in addition to an increase in blood sodium and chloride ion concentrations, there was also a significant decrease in the numbers of peripheral blood eosinophils and lymphocytes. This change was also reflected in a decrease in the number of total white blood cells in the peripheral blood. This was a somewhat less anticipated consequence of acute dehydration, but also well supported in the literature. The CBC white blood cell analysis was originally intended to detect increases in white blood cell concentrations, which would indicate possible severe infection of tissue or sepsis within the rabbit system. Of particular concern prior to the study was infection of the colon, with gastrostasis a likely side effect of water deprivation, as the animals tend to also decrease their food intake when water is removed. In the current study, a lack of increase of any white blood cell counts negated this concern. However, eosinopenia (decreased eosinophil count) can also be an indicator of increased blood cortisol, or stress.[89,90] While blood cortisol levels were not assessed during this study, it is reasonable that the burden of acute dehydration would be sufficient to elicit a cortisol response in the animals, thus possibly leading to eosinopenia. Similarly, increased cortisol levels can elicit a decrease in circulating lymphocytes.[91] Lymphocytopenia (decreased lymphocyte counts) can also result from undernutrition, which is equally likely within the experimental design of this study, as the animals in the dehydration condition tended to also discontinue food intake with the removal of water.[92]

Overall, the decrease in presence of two of four measured circulating white blood cells suggests that the dehydrated rabbits reach some level of immunocompromise in response to acute dehydration, such that they may be more susceptible to infection. This may be a response of the system to reallocate resources to the most acute and immediate physiologic pathology, which in the case of this experimental setup is the dehydration itself. The burden of acute dehydration is significant, and it is likely that physiologic resources are being redirected away from ancillary processes and toward more immediate needs, such as in the maintenance of blood pressure and circulation of nutrients to vital organs. This hypothesis is well supported by the findings of this study, as there was no significant change in the blood concentration of creatinine (an indicator of dehydration-related renal failure) and thus no indication of organ failure despite significant changes to systemic hydration levels.[93]

Structural Vocal Fold Changes

Subglottic Laryngeal Gland Size

Despite evidence in the literature that laryngeal mucous production is altered in dehydration[9,34,40,41,94], this change was not reflected in a measurable change to the size of subglottic laryngeal glands in the rabbit model of acute dehydration utilized in this study. It is not imperative that there is structural remodeling of the glands in order for an alteration in mucus production to occur, particularly in response to an acute pathology. An increased viscosity of mucus could lead to a change in overall gland size due to decreased secretion efficiency, but the methodologies employed in this study (small sample sizes and short duration) were not sensitive to such potential changes. Further, size changes to the laryngeal glands themselves would be related to a change in size and/or shape of the existing gland structure and cells. This change is likely due to remodeling

at the macrocellular level, as the turnover rate for most epithelial cells within the airway is between 30 and 50 days due to low mitotic index.[95] Three days of acute dehydration, despite being sufficient to observe systemic changes in organism physiology, may not be sufficient to see *structural* changes within laryngeal glands in response to dehydration. Further, because this was a pilot study, smaller sample sizes may not allow enough statistical power to detect smaller changes. Changes to the gross structure of the glands was not detected, but there may have been undetected changes to the quality and makeup of the produced mucus due to the study design and data collection methods utilized in this study. Future investigation should delve further into other mechanisms by which mucus production can be altered in dehydration through investigation and characterization of the mucus secreted by the glands. Additionally, a more chronic model of dehydration may allow for more long-term changes in gland structure to manifest.

Lamina Propria Depth

Lamina propria depth is another measure that has a fairly large literature base to support its relevance to vocal fold function. However, the vast majority of studies investigating these effects have been *ex vivo* studies, where the dehydration of the tissue is achieved through a hypertonic solution bath that pulls fluid out of the tissue via osmosis.[45,96,97] The level of tissue dehydration achieved via this methodology is certainly outside of physiologic probability, as the extent and severity of local dehydration seen in these *ex vivo* studies is exaggerated, and would be counterbalanced by physiologic homeostatic mechanisms *in vivo*. All subsequent functional vocal fold analyses in these studies is based on tissue viscosity parameters, with the underlying paradigm being that dehydrated tissue has inherently greater viscosity, and thus requires greater pressure and airflow to achieve and maintain vibration. It is yet unclear, however, whether the state of

dehydration achieved via *ex vivo* hypertonic solution baths is representative of an *in vivo* dehydration setting. In the current study, there is not sufficient evidence in this pilot study to support a decrease in vocal fold lamina propria depth in the dehydrated condition. This lack of significant change (although there may be a trend) to tissue volume in the present study suggests that acute systemic dehydration does not have a substantial effect on the viscosity of the vocal fold vibratory structures as has been modeled *ex vivo*. There is one additional *in vivo* study that supports a change in vocal fold water content in response to systemic dehydration in a rat model, but this study also fails to link this change in detected water content to changes in vocal fold volume or viscoelastic properties.[46] It is then appropriate to postulate that in light of the current findings, *ex vivo* methodologies employed to study vocal fold function in the context of dehydration may not be physiologically relevant to the *in vivo* condition and should be evaluated and interpreted cautiously. The current study, where no difference in lamina propria volume was detected, suggests that the conditions created *ex vivo* may not be physiologically relevant. The basis for dehydration in the *ex vivo* model is an assumption that systemic dehydration subsequently causes a reduction in local water content within the vocal fold. In the absence of decreased tissue volume in the present study, it follows that for acute systemic dehydration *in vivo*, the *ex vivo* findings do not generalize.

Lamina Propria Vascularity

Another unanticipated finding of the present study was the lack of significant changes to the vascularity of the vocal fold lamina propria in response to dehydration. Vascularity is known to be widely affected by dehydration, and so vocal fold vascularity (percent positive expression of CD31 in the lamina propria) was expected to be decreased in the experimental condition as a result

of vasoconstriction. Differences in vascularity between conditions was not observed in the current study, and there are a number of possible reasons. The first is that like the other structural tissue assessments conducted in this study, there was not sufficient time allowed for structural remodeling to occur. However, there is other literature to suggest that acute changes to vasculature organization and quantity within the vocal fold lamina propria are possible.[14] Another possibility is that the vocal fold tissue is physiologically protected from the vasodilation predicted and expected elsewhere within the organism in response to acute systemic dehydration. The mechanisms for this possible protection are unknown, but such a process could have significant implications in understanding the unique nature of the vocal fold tissue compared to other connective tissue structures within the body.

Changes in Gene Expression

The results of the gene expression data acquired during this study are somewhat more difficult to interpret due to the inherent complexity of data acquisition and processing using real-time quantitative PCR methodology. Two of the three genes assessed in this vocal fold tissue analysis are classically inversely related to one another. EDN1 is a gene known to be upregulated in conjunction with vasoconstriction of vessels.[82] Conversely, eNOS is associated with an increase in tissue blood flow.[83] In the current study, normalized transcription levels of both of these genes were downregulated in the dehydration condition compared to the control. One possible explanation is that dehydration causes a global downregulation of all non-essential gene transcription within the vocal folds at the cellular level, which may include EDN1, eNOS, as well as the downregulated IL-17 observed in this study. This apparent downregulation may be indicative that this study achieved a level of dehydration such that the subsequent organism and

tissue response was too severe, indicating that a less significant impairment in overall hydration may lend further insight into the subtle homeostatic tissue responses in the absence of a large-magnitude stress response.

There is, however, an alternative explanation for this global downregulation of transcription in the vocal fold tissue of subjects in the dehydrated group. In order to quantitatively and comparatively assess gene transcript levels across samples, a housekeeping gene is utilized as a reference to which all other gene expression values can be normalized. In this study, SDHA was chosen as that housekeeping gene, as there is ample literature to support its stability across a wide range of experimental conditions.[98,99] Despite the stability of SDHA across many conditions, it is no simple task to confirm its consistency within a new experimental comparison in a new model (as is the case in the present study), and due to the paucity of literature both in vocal fold tissue and in rabbit transcriptomics, it is possible that our assumption of consistency of SDHA between hydration conditions in rabbit tissue was false.[100] A relative increase in the expression of SDHA in tissue from subjects in the dehydrated group could also result in the *appearance* of global downregulations of all other genes of interest in the dehydrated group for which SDHA was used to normalize data. In order to confirm or deny this possibility, additional gene expression analyses would have to be completed on the same RNA samples using another or multiple other possible housekeeping genes for comparison.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

This study was a critical first step in a line of basic science investigation of the molecular and cellular mechanisms responsible for vocal fold changes in dehydration. Based on the findings of the current study, there are several study design modifications that are indicated in future studies.

The first target of future studies will be in the development of a chronic dehydration model, such that structural tissue changes have an opportunity to manifest prior to tissue analysis. Factors related to cost and time resources dictated the acute timeline of the current study, and the study effectively provided justification for subsequent studies of a longer duration. Additionally, other more sensitive measures of tissue viscosity, changes in subglottic laryngeal gland mucus production, and vocal fold vascularity will be utilized to better identify subtle differences between groups that may have been missed in the current study design.

The second change in future studies will be in the gene expression methodologies employed to detect transcription alterations. Additional pilot work is necessary to identify the most appropriate housekeeping gene with which to normalize other measures within a dehydration model, such that a clearer distinction can be made between possible global decreased metabolic function of dehydrated tissue versus an upregulation of the normalizing gene.

A larger sample size will also be utilized in future studies in order to achieve greater statistical power to detect differences between experimental conditions. It is clear based on the results of the current study that a physiologically relevant and meaningful severity of dehydration

was achieved. Body weight, hematocrit, blood ion concentrations, and white blood cell counts all support an effective experimental endeavor. However, additional adjustments to study design are necessary in order to tailor the experimental conditions to the dependent variables of greatest interest. It appears that a more chronic model of dehydration will better reflect the *in vivo* dehydration condition in humans, and may facilitate greater structural tissue changes than were appreciated in the present experimental design.

As a pilot, although the data collected and the subsequent interpretation of findings are not directly translatable to human pathology, this study has provided a clear sight into future research needed. Continued work in the investigation of the cellular and molecular vocal fold tissue response to dehydration will elucidate the mechanisms by which systemic dehydration does or does not contribute to the development of chronic vocal fold pathology.

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