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THE EFFECTS OF ACIDIFIED PEPSIN ON PORCINE VOCAL FOLD TISSUE: DEVELOPING A PORCINE MODEL OF LARYNGOPHARYNGEAL REFLUX DISEASE

For the degree of   Doctor of Philosophy

Is approved by the final examining committee:

Preeti Sivasankar
Co-chair

Ramesh Vemulapalli
Co-chair

Laurent Couetil

Bonnie Blazer-Yost

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Approved by Major Professor(s): Ramesh Vemulapalli and Preeti Sivasankar

Approved by: Ramesh Vemulapalli   2/2/2016
             Head of the Departmental Graduate Program   Date

THE EFFECTS OF ACIDIFIED PEPSIN ON PORCINE VOCAL FOLD TISSUE:

DEVELOPING A PORCINE MODEL OF LARYNGOPHARYNGEAL REFLUX DISEASE

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Abigail Cox Durkes

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

May 2016

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West Lafayette, Indiana

For Opal & Gretel

May they find sustenance in these pages.

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ABSTRACT

Durkes, Abigail C. Ph.D., Purdue University, May 2016. The Effects of Acidified Pepsin on Porcine Vocal Fold Tissue: Developing a Porcine Model of Laryngopharyngeal Reflux Disease. Major Professor: Ramesh Vemulapalli and Preeti Sivasankar.

Approximately 7.5 million Americans are affected by a voice disorder. In the last year, 7.2% of people missed one or more days of work due to a voice problem. For professional voice users, such as teachers, the rate increases to 20% and an annual cost of $2.5 billion. Voice disorders are complex and multi-faceted, as well as difficult to diagnose and treat. Prospective animal studies are necessary to study the pathophysiology of voice disorders, optimize our understanding of laryngeal pathology, and improve treatment outcomes. The pig provides a unique opportunity to test hypotheses relating to laryngeal disease because porcine vocal folds are most similar to human vocal folds from a structural, biochemical, neuromuscular, and cellular perspective. By utilizing the pig as a model of laryngeal disease, research involving basic, translational, and clinical questions can be investigated through collaborations with experts in the field. This dissertation will present *ex vivo* and *in vivo* data utilizing pig tissue to study a common voice disorder, laryngopharyngeal reflux. *Ex vivo* pig tissue was

challenged with acidified pepsin in different electrolyte environments to test the

innate defense mechanisms of the vocal fold epithelium. From there, an *in vivo*

pig model was designed to mimic the clinical situation of human LPR more

closely by challenging healthy, uninjured laryngeal epithelia with acidified pepsin.

The data suggests that healthy vocal folds are able to defend effectively against

reflux challenges. Future plans are to utilize a similar model to investigate other

common laryngeal diseases that afflict the human population as well as

therapeutic interventions to these disorders.

CHAPTER 1. INTRODUCTION

1.1 Introduction

There are many advantages to using sound as a mechanism of communication. Sound travels a distance, it is effective without vision, it passes through objects, it can give directional cues, and it can express complicated messages if the voice apparatus is designed properly. For 7.5 million people with voice problems in the United States alone, improved understanding of the voice and its anatomical origins are crucial to their quality of life.(1) For these individuals that experience voice problems, innovative translational research techniques can directly help expand the diagnostic acuity, therapeutic performance, and overall understanding of voice disorders and their presentation. Voice disorders often originate in the larynx which houses the vocal folds responsible for phonation. The larynx is positioned behind the root of the tongue and ventral to the esophagus; and is involved in the complicated mechanisms of breath, sound production, and airway protection of amphibians, reptiles and mammals. The anatomical position of the larynx adjacent to the gastrointestinal tract subjects the larynx to possible damage from the contents of the oropharynx, esophagus, or stomach. Laryngopharyngeal reflux (LPR) is a disease often presumed to result from gastric contents refluxing in some capacity

into the larynx causing damage. The research of this dissertation helps to answer some of the questions surrounding LPR in human beings.

1.2   The mammalian larynx

The larynx has a complex role in which it must integrate respiration, sound production, and airway protection due to its anatomic location in the upper respiratory tract. Anatomically, the larynx can be subdivided into three regions: 1) the supraglottis including the epiglottis, false vocal folds, and laryngeal ventricles; 2) the glottis including the true vocal folds and the commissures; and 3) the subglottis which is the region distal to the true vocal folds, extending to the trachea.(2) The subdivided laryngeal regions are important because they are lined by different epithelia in the human being as well as other commonly studied species.(3) In the human being, the true vocal folds and proximal epiglottis are lined by stratified squamous epithelium. The stratified squamous epithelium lining the true vocal fold is conserved in many of the common animal species utilized in comparative laryngology studies.(4) The true vocal folds in the human being also have a microridge pattern on the luminal surface. The increased surface area afforded by the microridges likely plays a role in mucus adherence or traction of the vibrating vocal folds.(5) Excluding the previously mentioned proximal epiglottis and true vocal folds, the epithelium of the human larynx is pseudostratified epithelium. Seromucus glands are positioned within the laryngeal lamina propria, except in the true vocal folds.

The anatomy and histology of the human larynx has been extensively studied; however, there are clear interspecies differences between the human larynx and the animal models that are often studied. Morphological variations among mammalian larynges are largely related to lifestyle and dietary needs, even though the mammalian larynx evolved from the same basic design.(6) The herbivore larynx must reposition toward the nasopharynx during feeding to aid in breathing while the animal consumes large quantities of food over long mastication times. To accomplish this, a circumferential seal is formed by high aryepiglottic folds and bulky arytenoids and protects the airway from a largely liquid diet (regurgitated cellulose).(7) In contrast, carnivore's epiglottis is large and flat and primarily protects the airway from largely unmasticated food and minimal liquid over short periods of time. Cats have no aryepiglottic folds and small arytenoids. The primate larynx lies somewhat between the two previously described with an upright rounded epiglottis and aryepiglottic folds.(7)

1.3   Vocal fold epithelium

Healthy vocal fold epithelium functions as the first line of defense against environmental and systemic challenges to the vocal folds. The vocal fold epithelium lies at the interface between the environment and the underlying functional tissues of the voice production apparatus. Not only does the vocal fold epithelium restrict the movement of solutes from the airway into the delicate tissues by creating a barrier, it also actively maintains homeostasis through ion transport functions and appropriate immunological responses. If perturbations

lead to epithelial dysfunction, then the consequences may extend into the connective tissue and muscle of the vocal fold which may directly impact the overall function of the organ.

The adult human vocal fold is comprised of the thyroarytenoid muscle and the overlying mucosa. From a mechanical perspective, the vocal fold is divided into three layers: cover, transition, and body.(40) Histologically, the mucosa is comprised of the stratified squamous epithelium and the lamina propria which can be subdivided into three layers: superficial, intermediate, and deep. The superficial layer of the lamina propria is also known as Reinke's space, a potential space occupied by ground substance and pliable elastin and collagenous fibers that allow for marked vibration.(41) The intermediate and deep layers are comprised mostly of elastin and collagen, respectively.(19, 20) The vocal fold is devoid of glands and large vessels so as not to interfere with vibration.

The true vocal fold epithelium has a distinct cell layer structure similar to cornea, oral mucosa, and esophagus.(42) The vocal fold epithelium has 3 distinct regions, each with unique molecular markers that likely indicate particular intraepithelial functions. Adult human vocal fold squamous epithelium is characterized by cytokeratin 13 (CK13) positivity and absence of cytokeratin 8 (CK8). The upper 3-6 layers of the vocal fold squamous epithelium is involucrin positive, while the lower 3-4 cell layers are cytokeratin 14 (CK14) positive.(42) Future tissue engineering studies will likely tease out the functional differences in these distinct strata of the human vocal fold epithelium.

In order to perform their barrier function, vocal fold epithelia must tightly adhere to neighboring epithelial cells through a junctional complex. This junctional complex is a circumferential arrangement of proteins present in epithelial surfaces and consist of tight junctions and anchoring or adherens junctions.(43) The human vocal fold is known to have tight junctional proteins, occludin and zonula occludens-1; as well as adherens junctional proteins β-catenin and E-cadherin.(44) The tight junctions are localized immediately below the apical surface of the epithelial cell, while the adherens junctions are subjacent to the tight junctions. The physiology and pathophysiology of junctional complexes is constantly evolving as scientists discover new entities that surround these ubiquitous structures. Not only does the junctional complex of epithelial cells create a physical barrier, it also has a functional significance in adjusting the degree of tightness based on physiologic or homeostatic needs.(45) Injurious agents such as cigarette smoke, gastric reflux, and dehydration all have the potential to negatively impact the functionality of the vocal fold epithelial junctional complex.(46-49)

Besides barrier protection and paracellular fluxes of solute, stratified squamous epithelium of the vocal folds maintains the selective movement of ions and water. The exact mechanisms that regulate vocal fold epithelial ion transport are beginning to be better understood. A vocal fold model of transcellular ion and water fluxes suggests that a basolateral sodium-potassium (Na^+/K^+) ATPase pump is the driving force behind active ion transport across the epithelia.(50) A Na^+/K^+-ATPase on the basolateral membrane of vocal fold epithelial cells

generates an electrochemical gradient for Na$^+$ to enter the cell. The epithelial sodium channel (ENaC) and the cystic fibrosis transmembrane regulator (CFTR) are also important ion transport proteins of Na$^+$ and Cl$^-$, respectively.(51, 52) When Na$^+$ and Cl$^-$ are transported via these membrane proteins, an osmotic gradient is created that propels water across the epithelium.(53, 54). The major contributor of water flux is likely aquaporins, membrane channels found in many epithelial tissues.(55)

Finally, epithelial cells also play an important role in wound healing where they have been shown to synthesize and secrete growth factors important for wound repair.(56) It is clear that vocal fold plays a crucial role in maintaining and defending the physiology of the mechanically active vocal fold.

1.4   Porcine animal model of laryngeal disease

Comparative models of the human larynx most often focus on animal species that have analogous anatomy, physiology, and acoustics of the human larynx. The laryngeal literature cites only a few animal models that fit this criteria. Historically, the dog larynx was used as a reliable animal model due to the relatively similar size to human larynges, as well as the presumed phonatory similarities.(8, 9) However, concerns about the use of domestic animals for research have greatly decreased the availability and increased the cost of using a canine model. Further research has identified differences between human and canine laryngeal structure and function.(10) Rabbit and rat models are commonly used for surgical manipulation and graft studies due to their affable response to

handling, minimal housing requirements and detailed review in the literature.(11-13) Unfortunately, the similarities of rat and rabbit larynges to human larynges are limited and the survival rates for laryngeal manipulation in rats and rabbits is fair to poor.(14, 15) Finally, the size of a rat or rabbit larynx precludes multiple measures on the same larynx.

The porcine larynx offers the greatest structural, cellular, immunologic, and neuroanatomical similarity to human vocal folds, than any other characterized animal model.(10, 16-18) These biological and physiological similarities are likely the same traits that are at play in human laryngeal reflux and thus will hopefully translate to a reliable, reproducible model of human laryngeal reflux.

The pig laryngeal skeleton is similar to human's with comparable gross size, dimensions, and shape.(10) Porcine vocal folds have similar dimensions as human vocal folds, and are of sufficient size to allow for multiple measures (e.g. histopathology, IHC, gene expression, protein synthesis, and biomechanics) to be made from the same larynx. The gross anatomy of the pig larynx share similar intrinsic muscle positioning as in the human larynx and the recurrent laryngeal nerve similarly innervates the posterior cricoarytenoid muscle in both the pig and human being.(18)

Histologically, the human and pig larynx are similar except for the fact that the porcine supraglottis is lined by stratified squamous epithelium, contrasted with pseudostratified columnar epithelium in humans.(17) Theoretically, this might impact the rate at which secretions or particulates are cleared from the larynx in pigs compared with human beings.

Larynx-associated lymphoid tissue (organized epithelial lymphoid tissue in the supra- and sub-glottis) is present in the pig and human larynx and similarly distributed between the species allowing for immunological comparisons as well.(16) Organized collections of leukocytes were found in the mucosal epithelium, around tubuloacinar glands, and less often in the submucosa. Immunofluorescent microscopy was utilized to investigate the immunological architecture of the pig larynx and found similar distribution of MHC class II cells and T-lymphocytes as observed in human larynges.(17)

The research of this dissertation focuses on the epithelium; however, the similarities to human vocal fold tissue in regards to connective tissue proteins, and intrinsic muscles make the porcine larynx appropriate for investigating biological properties, as well as mechanical properties of the connective tissue and muscle in future studies. Hahn et al. determined that the porcine lamina propria collagen and elastin distribution is most similar to that of human compared to the other species investigated.(19, 20)

As noted above, the concern about the use of domestic animals for disease models has led to difficulties in purchasing and housing dogs, cats, or other domestic animals. Use of pigs for a laryngeal reflux model permits large amounts of data to be collected while minimizing the costs of purchasing and housing many animals (*ex vivo* studies can obtain larynges from the slaughterhouse, and *in vivo* studies can purchase domestic swine at a minimal cost compared to dog, cat, etc.).

A potential limitation that is common to all animals is their limited phonatory capability. However, one research group concluded that from a structural perspective the pig is a superior animal model compared to the more commonly used dog.(10) The pig and human larynx had similar cricothyroid muscles size, similar rotational mobility at the cricothyroid joint, similar cartilaginous framework, and similar thickness and stiffness of the vocal fold. Additionally, the fundamental frequency (F0), a correlate of voice pitch, and range of phonation in pigs are closest to those of humans. Perhaps with a little innovation the pig could be a quality model of human phonation if one could tap into their trainability.

Weaknesses of the pig model in application include difficulty accessing the vocal folds via endoscopy, phonotrauma, and risk of subclinical infection. The long oral cavity and oropharynx combined with prominent arytenoids requires a skilled endoscopic hand and anesthesia. In other species, such as the rabbit, mild sedation and an oral speculum are all that are required for laryngeal access. Additionally, pigs vocalize or "squeal" when handled in a way that the pig feels is threatening, i.e. restraint for intramuscular injection. The intensive vocalization causes acute trauma to the vocal folds that is visualized endoscopically as posterior edema and vocal fold erythema (personal observation). Unfortunately, these two gross manifestations of vocal fold trauma are the two most common endoscopic findings in patients with laryngeal reflux, complicating an endoscopic exam. As with any animal study, the risk of infection is present; however, commercial pigs raised for slaughter may possess a higher risk of subclinical

upper respiratory infections that may impact the research findings in the larynx. Prophylactic antibiotics can combat the risk, but the threat remains.

1.5   Introduction to LPR

A variety of laryngeal conditions and symptoms are attributed to laryngopharyngeal reflux (LPR), of which "reflux laryngitis" is perhaps the most common. LPR is a widely recognized disorder; however, there is still debate regarding pathophysiology, diagnosis, and treatment. There are two proposed pathophysiological mechanisms for LPR. The most published mechanism of injury, and often the definition of the disease, is retrograde reflux of gastric contents beyond the esophagus, up to the level of the larynx and pharynx. The direct contact of the gastric contents upon the epithelia of the larynx and pharynx result in the reported symptoms. Compared to the esophageal mucosa that receives daily gastric reflux challenges, laryngeal and pharyngeal epithelium are considered more vulnerable to the caustic effects of the reflux and this possibly explains the development of the disease. Alternatively, LPR symptoms may be a reflexive mechanism brought upon by distal esophageal reflux.(21) In this reflex theory, the LPR symptoms may be initiated by either a chemical stimulation of laryngeal nerve endings (chemoreflex); or possibly by an afferent limb of the vagus stimulated by sensory stimuli of the distal esophagus.(22-24) These reflex mechanisms may contribute to LPR symptoms by stimulating the efferent recurrent laryngeal nerve to spasm, thus leading to general laryngeal signs.

Regardless of the inciting trigger, gastroesophageal reflux has been implicated as a cause of a variety of extraesophageal symptoms including cough, asthma, hoarseness, and globus sensation. These generic laryngeal symptoms are often attributed to a variety of upper respiratory ailments. To frustrate the problem, the most common endoscopic findings in patients with abnormal upper esophageal pH (indicating possible reflux episode) are also nonspecific findings. These endoscopic findings of LPR include edema and erythema of the vocal folds, posterior commissure and arytenoids.(25) Edema and erythema are merely gross manifestations of inflammation in general.(26) Definitive diagnosis of LPR is often difficult because inflammation may be identified in a variety of laryngeal insults including infections, vocal abuse, allergy, smoking, environmental irritants, chronic sinusitis, and laryngeal tumor. Empirical antireflux treatment is often initiated as a trial antidote because the symptoms and endoscopic findings are so vague. The literature on antireflux medication outcomes is highly variable and randomized, control studies reported no significant advantage of antireflux treatment versus placebo in suspected LPR patients.(27) The above discrepancies in pathophysiology, diagnosis and treatment encourage controversy surrounding this disease and even skepticism of its existence.(28)

The damaging events that must occur to elicit LPR in patients is uncertain. Histologically, laryngitis is characterized by hyperplasia of the squamous epithelium with a chronic inflammatory infiltrate in the lamina propria.(29) With progression of the process, the epithelium may become atrophic and ulcerated with deposits of fibrin, granulation tissue, and fibrosis in the lamina propria.

Several animal studies have shown that gastric refluxate applied directly to the vocal folds leads to epithelial damage and inflammation in the lamina propria.(30-32) Delahunty *et al* (1968) was the first to show that gastric reflux applied to dog vocal folds resulted in visible tissue injury.(30) Adhami *et al* (2004) applied components of gastric and duodenal reflux at varying pH to uninjured laryngeal mucosa of dogs for three weeks and found that pepsin at pH 1-2 and pepsin + conjugated bile acids at pH of 1-2 resulted in significant inflammation of the vocal fold tissue.(31) Adhami's group also found significant correlation between laryngeal inflammation and gross erythema and edema.

The studies by Delahunty and Adhami demonstrate acidified gastric contents can lead to vocal fold inflammation (specifically in the lamina propria), but they did not expound upon how. Erickson and Sivasankar (2010) demonstrated a decreased transepithelial resistance in *ex vivo* porcine laryngeal epithelium following acid and acid + pepsin (pH3).(33) Transepithelial resistance represents the ability of the epithelium to restrict solute traffic across the epithelium and is maintained by intercellular junctional proteins. Gill *et al* (2005) reported that laryngeal biopsies of LPR patients show decreased E-cadherin (junctional protein) expression as well as increased paracellular space when compared to normal control patients.(34) Decreased transepithelial resistance, decreased E-cadherin expression, and increased paracellular space suggest a possible breakdown of the epithelial barrier leading to the susceptibility of the underlying lamina propria to injury.

Acidic injury is not the only possible cause of laryngeal injury in LPR. Laryngeal biopsy samples from LPR patients demonstrate the presence of pepsin; not detected in normal control subjects.(35) Pepsin is a proteolytic enzyme secreted by chief cells of the stomach. It is secreted in its inactive form, pepsinogen, which is activated in low pH of the stomach milieu. Johnston *et al* (2009) investigated the expression of 84 cytokines in hypopharyngeal epithelial cells incubated with the pepsin at pH 7.4.(36) A number of inflammatory cytokines and receptors were altered indicating a role of the proteolytic enzyme in vocal fold inflammation. Although the lamina propria was not examined specifically, one can speculate that the up-regulation of inflammatory cytokines in the adjacent epithelial cells could contribute to overall inflammation of the tissue. For example, tumor necrosis factor (TNF) and interleukin-1β (IL-1β), both up-regulated in vocal fold epithelial inflammation, act on endothelial cells of post-capillary venules to induce the coordinated expression of numerous adhesion molecules that are critical to leukocyte homing and extravasation to inflamed tissues.(26, 36) Leukotriene B (LTB) and interleukin-8 (IL-8), also up-regulated in pepsin-induced epithelial inflammation, act as chemoattractants to drive leukocyte recruitment to the site of inflammation.(26, 36) The intimate association of epithelium with lamina propria provides ample opportunity for inflammation in one to impact or even induce inflammation in another. Souza *et al* (2009) has proposed a similar cause of reflux inflammation whereby cytokines are the main mechanism of action.(37) This work focuses on the esophageal epithelium and gastroesophageal reflux; however, the mechanisms may relate to laryngeal reflux

and need to be studied. Souza's group noted that esophagitis induced by reflux in animal models took weeks to develop. Caustic acid injury should develop immediately. To study this discrepancy, rats underwent esophagoduodenostomy and esophageal biopsy at multiple time points. Results showed lymphocytic infiltration into the submucosa 3-days post-operation and epithelial hyperplasia preceded surface erosions by weeks. Lymphocytes were only seen in the epithelium 3 weeks post-operation. These results suggest an alternative hypothesis in which reflux incites a cytokine-mediated immune response that causes the esophageal injury as opposed to a caustic acid injury that would cause direct cell injury and secondary inflammation.

1.6 *In vivo* models of LPR

A few studies have explored the effects of reflux in vivo; however, the focus of research was on traumatized laryngeal mucosa and how reflux relates to healing or propagation of disease. Delahunty et al (1968) was the first to show that gastric reflux applied to dog vocal folds 29 times over 39 days resulted in vocal fold ulcer formation and granulation tissue formation.(30) This group applied cotton swabs moistened with stomach contents from the subjects as the reflux challenge. At the end of the study, histopathology of all the challenged vocal folds were virtually the same and included epithelial necrosis and underlying granulation tissue. One of the strengths of this model is that the dog larynx can be visualized with a simple laryngoscope and blade without the need for expensive endoscopy equipment. The main weakness of the model is that

repeated, direct application of gastric contents to any stratified squamous epithelium will likely results in ulceration. This reflux challenge is not physiologically relevant to LPR. Clinically, LPR episodes rarely reach a pH less than 4 and the constituents of the LPR challenge may not have all the proteolytic activity that resides in gastric juices. A minor additional weakness is that cotton swab application likely traumatizes the superficial layers of epithelium and may affect the outcome of disease.

Koufman et al (1991) injured the subglottic mucosa down to the intact perichondrium and then proceeded to paint the subglottic injury site and the uninjured vocal processes of the arytenoids with acidified pepsin (pH 1.5, 2.5, and 4.0, 0.3 mg/ml) 6 times over 2 weeks.(32) All macroscopic and microscopic findings were confined to the subglottic injury site. Inflammation, necrosis, and delayed healing were observed in the subglottic injury sites of all reflux-challenged animals. Because the lesions were present regardless of pH, injury was determined to be primarily dependent on pepsin. An interesting, however often overlooked outcome of this study is the lack of response in the uninjured laryngeal mucosa. Clearly, this dose, frequency, or application method does not mimic LPR disease in human beings with uninjured mucosa.

The animal model that seems to most closely simulate gastroesophageal reflux was developed by Schopf et al (1997) and divided the oblique fibers at the gastroesophageal junction in pigs (pigs and human beings have identical muscular arrangements at this junction).(38) Gastroespohageal reflux was documented by pH probe and lower esophageal sphincter manometric

attenuation. This animal model is not practical for widespread use in laryngeal reflux experiments given that lower esophageal sphincter malfunction is believed to play a minor, if any, role in human LPR. Additionally, the surgical method is highly invasive with large risk to the health of the animal.

The rabbit is utilized in many laryngeal models including phonation trauma, graft development, and reflux. Ludeman et al (1998) placed pharyngostomy tubes in 16 New Zealand white rabbits and proceeded to challenge the surviving rabbits with acidified pepsin (pH 1.5, 0.3 mg/ml) once, twice, or three times per day for 14 consecutive days.(15) Glottic inflammation was greatest in rabbits that received the challenge three times per day. The major strength of this pharyngostomy model is the delivery of acid to the hypopharynx, thus mimicking the effect of acid on the upper airway. The rabbits were only mildly sedated for the reflux applications, possibly mimicking physiological chemical reflux of cough that may contribute to the overall trauma of LPR. This is the only known model to test reflux on untraumatized epithelium. The major limitations of this paper include an unrealistic physiologic pH (1.5) that does not mimic LPR, and the overall failure rate of the pharyngostomy surgery.

Adhami et al (2004) investigated the specific agents in gastric juice responsible for producing laryngeal signs and symptoms.(31) This group applied pepsin, conjugated bile acid, unconjugated bile acid, and trypsin at varying pH to dogs with both injured and uninjured laryngeal mucosa, for three weeks and found that pepsin at pH 1-2 and pepsin + conjugated bile acids at pH of 1-2 were the most injurious to vocal fold tissue and resulted in significant inflammation.

The strengths of this model are the extensive evaluation of the effects of duodeno-gastric ingredients on intact laryngeal mucosa. Interestingly, this group determined that nonacidic material (such as pepsin proposed by other research groups to be the primary culprit in LPR) has little inflammatory capacity a pH 4. The weaknesses include multiple biopsy sites in a confined anatomical area which undoubtedly contributed to the overall inflammatory score of the larynx, even in the uninjured mucosa. The intimate association of laryngeal mucosa throughout the organ provides ample opportunity for inflammation in one area to impact or even induce inflammation in another.

Finally, a rat model was developed by Shimazu et al (2009) in which chronic esophagitis was induced and the histological changes in the larynx were evaluated.(39) The chronic esophagitis was induced by surgical ligation of the forestomach and glandular portion of the stomach as well as pyloric narrowing to restrict gastric emptying. Three rats were sacrificed every 2 weeks for 20 weeks after operation. Mucosal thickening and inflammation were observed in the pharynx and epiglottis of the rat model 8 weeks after the initial operation. Only after 18 weeks was there any evidence of inflammation in the larynx (specifically the interarytenoids) including mild thickening of the mucosa and proliferation and dilatation of the capillaries. The strengths of this model include maintaining the chronic reflux of endogenous gastric acid in rats over a relatively long time and testing uninjured laryngeal mucosa. Weaknesses involved in this study are the survival rates of 37.5 % and only one area of the larynx was examined (arytenoids).

1.7   Conclusion and Hypotheses

LPR appears to be pervasive in the human population yet the underlying mechanisms of disease, diagnostic acuity, and therapeutic intervention are only vaguely understood. A model is needed to further evaluate the pathological and molecular consequences of repeated challenges mimicking LPR in humans to help clarify the understanding of the human disease. This research was undertaken to address the lack of data related to vocal fold epithelial physiology and defense to varying acidic challenges. An established *ex vivo* pig model was used to examine the buffering effects of bicarbonate on the epithelium's response to acidic challenge. We hypothesized that bicarbonate was a substantial contributor to ion secretions that occurs following an acute acidic challenge and that limiting the amount of bicarbonate during an acidic challenge would negatively impact the buffering capacity of the tissue. Second, a prospective *in vivo* pig model was developed to examine the ability of the vocal fold mucosa to withstand repeated acidified pepsin challenges administered in a liquid or aerosolized form. We hypothesized that repeated administration of acidified pepsin would illicit LPR-like pathologic and molecular changes analogous to the human disease.

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CHAPTER 2. BICARBONATE AVAILABILITY FOR VOCAL FOLD EPITHELIAL DEFENSE TO ACIDIC CHALLENGE

2.1 ABSTRACT

Objectives: Bicarbonate is critical for acid-base tissue homeostasis. This study investigated the role of bicarbonate ion transport in vocal fold epithelial defense to acid challenges. Acidic insults to the larynx are common in gastric reflux, carcinogenesis and metastasis, and acute inflammation.

Methods: Ion transport was measured in viable, porcine vocal fold epithelia. First, vocal folds (n = 18) were exposed to the carbonic anhydrase antagonist, acetazolamide, or vehicle. Second, vocal folds (n = 32) were exposed to control buffer or bicarbonate-free buffer on the luminal, basolateral, or bilateral surfaces. Third, vocal folds were challenged with acid in the presence of bicarbonate-free or control buffers.

Results: Vocal fold transepithelial resistance was > 300 ohms*cm^2 suggesting robust barrier integrity. Ion transport did not change after acetazolamide (P > .05). Exposure to bicarbonate-free buffers did not compromise vocal fold ion transport (P > 0.05). Ion transport increased following acid challenge. This increase approached statistical significance and was the greatest for control and basolateral bicarbonate-free buffers.

Conclusions: Bicarbonate secretion may contribute to vocal fold defense against acid challenge. These data offer a potential, novel role for bicarbonate as a therapeutic agent to reduce pH abnormalities in the larynx and prevent associated pathological changes.

2.2   Introduction

Squamous epithelial cells line the surface of the vocal folds and create a physical barrier protecting the underlying lamina propria and muscle. In addition, epithelial cells actively transport ions and water to regulate cell volume and vocal fold hydration.(1-3) It is known that environmental and systemic insults to the epithelium can disrupt vocal fold epithelial ion transport and barrier function.(4-6) For example, porcine vocal fold epithelial barrier function is impaired by an acidic environment.(4) Similarly, an ephemeral event of raised intensity phonation in rabbits leads to down regulation of epithelial cell junction proteins.(7) Due to their anatomic position and propensity for receiving airborne and systemic challenges, the vocal fold epithelial cells may serve as an important target tissue in the larynx for therapeutic interventions that strengthen both barrier and ion transport functions.

The exact mechanisms that regulate vocal fold epithelial ion transport are beginning to be better understood. Leydon et al (2009) described a vocal fold model of transcellular ion and water fluxes that incorporated a basolateral sodium-potassium (Na^+/K^+) ATPase pump and sodium-potassium-chloride ($Na^+/K^+/2\ Cl^-$) cotransporter. Epithelial sodium channel (ENaC), cystic fibrosis

transmembrane conductance regulator (CFTR) chloride channel, and aquaporins, were immunolocalized to the luminal (air-facing) surface of the vocal fold epithelial cell. The role of bicarbonate in ion transport across vocal fold epithelia was not included in the model. Bicarbonate plays a critical biochemical role in the body's pH buffering system and is produced intracellularly by the interconversion of carbon dioxide and water into bicarbonate ion, carbonic acid, and protons by the enzyme carbonic anhydrase.(8, 9) Bicarbonate is charged and requires transport proteins to facilitate its movement across cell membranes in order to control cellular pH and to regulate fluid movement.(10) Esophageal and duodenal epithelium are both routinely subjected to acidic gastric contents and epithelial bicarbonate secretion is believed to play a critical role in defense. Bicarbonate ion secretion has been identified from esophageal submucosal glands and duodenal columnar epithelium.(11, 12) Additionally, rat esophageal epithelium separated from the underlying submucosal glands also demonstrated bicarbonate secretion following an acid challenge suggesting a role for the stratified epithelium alone to supply the neutralizing bicarbonate.(13) The role of bicarbonate as an acid buffer is especially relevant to vocal fold physiology as low pH environments are routinely encountered during episodes of gastric reflux, carcinogenesis and metastasis, and acute inflammation.(14-18)

Isolating bicarbonate transport is challenging, as there are no specific pharmacological antagonists of bicarbonate channels or transporters. Further, bicarbonate may be transported through anion exchange channels such as CFTR.(19, 20) Therefore, indirect methods of elucidating the role of bicarbonate

in vocal fold epithelial ion transport are necessary. These methods include blocking carbonic anhydrase in order to decrease the production of bicarbonate within the cell or decreasing bicarbonate in the surrounding environment to determine if the presence of bicarbonate impacts epithelial ion transport.

The objective of this study was to investigate the essential role of bicarbonate transport in vocal fold epithelia. First, we exposed excised, viable porcine vocal folds to a carbonic anhydrase inhibitor, acetazolamide, to determine if blocking the production of bicarbonate affected epithelial ion transport. Second, the vocal folds were bathed in bicarbonate-free buffer to determine whether the presence of bicarbonate in the ambient environment affected vocal fold epithelial ion transport. This study also yielded data on whether the location of bicarbonate (luminal vs. basolateral vs. bilateral) impacted the ion transport across the excised vocal fold epithelia. Finally, hydrochloric acid (HCl) was administered to the vocal folds in the bicarbonate-free study to simulate a physiologically-relevant clinical condition. Previous research identified a consistent increase in vocal fold ion transport following a physiologic acidic challenge (pH=4) presumably as a defense mechanism to buffer the acid-base imbalance.(5) We set out to determine if bicarbonate contributes to this observed increase in ion transport following a simulated bout of gastric reflux or other acid-base imbalance. We hypothesized that a lack of bicarbonate in the milieu surrounding the vocal folds would lead to a change in ion transport across the epithelium. In addition, we hypothesized that bicarbonate is a critical component of the increased ion transport observed following a vocal

fold acidic challenge. This study sets the groundwork for quantifying the role of bicarbonate in vocal fold defense and as a potential therapeutic agent to treat clinical conditions of acid-imbalance in the larynx.

2.3 Materials and Methods

2.3.1 Tissue Preparation

Fresh, adult male and female porcine larynges were obtained from commercial abattoirs in accordance with approved protocols from Purdue University's Animal Care and Use Committee. Animals were sacrificed via exsanguination and larynges were removed and immersed in cold saline for transport to the laboratory. A validated laryngeal dissection protocol was utilized to prepare each larynx for experiments upon immediate arrival of the larynx to the laboratory.(5, 21) The mid-sagittal plane of the larynx was bisected creating hemi-larynges in which the vocal fold could be visualized. Vocal fold epithelium, basal lamina, and superficial lamina propria were separated from the underlying connective tissue and muscle. The vocal folds were moistened throughout the dissection with Hanks' balanced salts (HBSS; mM: NaCl, 136.8; dextrose, 5.6; KCl, 5.6; $NaHCO_3$, 4.2; $CaCl_2$, 1.3; $MgSO_4$, 0.8; KH_2PO_4, 0.4; Na_2HPO_4, 0.3; pH 7.0) and prepared for electrophysiology experiments.

2.3.2 Solutions and Chemicals

All solutions and chemicals were obtained from Sigma Aldrich (St. Louis, MO). In experiment 1, dimethly sulfoxide (DMSO) was the solvent used to

dissolve the carbonic anhydrase inhibitor acetazolamide and is hereafter referred to as vehicle. In experiments 2 and 3, the control was HBSS with sodium bicarbonate. Bicarbonate-free HBSS was prepared by not adding the sodium bicarbonate and supplementing with HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) and HEPES sodium salt to maintain osmolarity, isotonicity, and pH (Table 1).

2.3.3 Electrophysiology Protocol

A calibrated Ussing system (model 15362; WPI) and voltage clamp (model DVC-1000) were used to record short-circuit current (Isc) and transepithelial resistance (R_T) across porcine vocal folds epithelia. (4-6) Short-circuit current is a measure of ion transport and transepithelial resistance reflects the integrity of the epithelial barrier. Vocal fold epithelia were mounted on chambers that were subsequently installed in the Ussing apparatus. Both chambers were filled with 5 ml of HBSS buffer (Sigma Aldrich, St. Louis, MO). The circulating HBSS was warmed to 37°C and $95\%O_2 / 5\%CO_2$ was bubbled into the media. Two voltage and two current electrodes ($Ag^+ / AgCl$ electrodes with 3 mol/L KCl/agar salt bridges) were placed on either side of the vocal fold in the Ussing chamber. The electrodes measured open circuit potential differences and Isc across the vocal fold. Once mounted, the vocal folds reached baseline in approximately one hour (stable Isc for approximately 5-10 minutes). R_T was measured using Ohm's Law once the tissues reached baseline. Epithelia that reached a baseline R_T of 300 $\Omega*cm^2$ were deemed acceptable and used for experiments.(22)

2.3.4 *Experiment 1*: Carbonic Anhydrase Antagonism

Vocal folds were prepared as described above for electrophysiology experiments and baseline Isc was established. To determine the dose of acetazolamide in a preliminary experiment using six vocal folds, various concentrations of acetazolamide were tested (range: 0.1mM to 6mM acetazolamide). Based on these data (results not shown), a 4mM acetazolamide dose was selected for further study as this was the largest concentration that preserved vocal fold viability. Previous studies with human, esophageal stratified squamous epithelium successfully inhibited carbonic anhydrase with an acetazolamide concentration of 0.1mM.(23)

Vocal folds (n=18) were mounted on Ussing chambers and baseline Isc was reached prior to administration of either 4mM acetazolamide or the same volume of vehicle alone. Ion transport was then recorded for 30 minutes. R_T greater than 300 Ω*cm^2 was measured throughout the 30 minute experiment to assure tissue viability.

2.3.5 *Experiment 2*: Bicarbonate-free Ion Substitution

Once the tissues reached baseline, the luminal and basolateral chambers of the Ussing system were drained. Each chamber was flushed with the incoming buffer in order to eliminate remaining HBSS and then filled with one of four combinations of buffer: (1) bilateral HBSS (n=8), (2) luminal bicarbonate-free HBSS and basolateral HBSS (n=8), (3) luminal HBSS and basolateral

bicarbonate-free HBSS (n=8), and (4) bilateral bicarbonate-free HBSS (n=8). Ion transport was measured for thirty minutes following the solution exchange. Preliminary experiments (data not shown) in our lab indicated that stable Isc was reached in 30 minutes following a chamber exchange of fluid. R_T greater than 300 Ω*cm^2 was measured throughout the 30-minute experiment to assure tissue viability.

2.3.6 *Experiment 3*: Acid Challenge

Dissected vocal folds mounted on Ussing chambers were bathed in one of four combinations of bicarbonate-free buffer described above for 30 minutes. After the 30 minutes, the pH of the solution in the luminal chamber was measured to ensure a physiologic pH of 7. (24) Thereafter, the luminal surface of each vocal fold was exposed to 2.5 µl HCl causing an acidic environment (pH=3-4). The pH of 3-4 was selected because it is in the range of simulated acidic environments in the larynx.(14) Ion transport was measured immediately following the acid challenge.

2.3.7 Data and Statistical Analysis

DataTrax software (WPI, Sarasota, FL) was used to measure Isc in all electrophysiology experiments. Statistical analysis was completed using SPSS (Version 20, Chicago, IL). The dependent variable in all experiments was normalized Isc (% Isc adjusted to baseline). For experiment 1, a T-test was performed to compare the effects of acetazolamide and vehicle on ion transport.

For experiment 2, the Isc value immediately prior to flushing the systems was taken as the baseline measure. A second Isc value was measured after the vocal fold was bathed in one of the four buffers. A univariate analysis of variance (ANOVA) was used to investigate whether the change in Isc was different between the control and bicarbonate-free groups. For experiment 3, the baseline Isc was the Isc value immediately prior to adding HCl. The post-acid challenge increase in Isc was measured within 60 seconds of adding the HCl. A univariate ANOVA was used to investigate whether the increase in Isc following acid challenge was different between the control and bicarbonate-free groups. An α-level of 0.05 was considered statistically significant for all analyses.

2.4   Results

2.4.1   *Experiment 1*: Carbonic Anhydrase Antagonism

Vocal folds assigned to the acetazolamide group did not significantly differ in baseline Isc from vocal folds in the vehicle alone group ($t = -0.851$, $p = 0.434$). Exposure to acetazolamide decreased Isc by 22% as compared to 19% decrease in Isc for vehicle alone. This effect on Isc was non-significant ($t = -0.533$, $p = 0.617$, Figure 2.1).

2.4.2   *Experiment 2:* Bicarbonate-free Ion Substitution

Baseline Isc did not differ significantly across vocal folds in the four buffer groups ($F = 2.839$, $p = 0.06$). The availability of bicarbonate bilaterally (HBSS condition) reduced Isc by 27% as compared to baseline. Exposure to

bicarbonate-free buffer reduced Isc by 30% (luminal), 15% (basolateral), and 8% (bilateral) as compared to baseline. Overall, the differential availability and location of bicarbonate did not significantly change Isc (F = 1.890, p = 0.154, Figure 2.2).

2.4.3   Experiment 3: Experiment 3: Acid Challenge

Addition of acid increased Isc by 57% in the HBSS condition. The magnitude of Isc increase was reduced in the bicarbonate-free condition. In groups lacking bicarbonate, exposure to acid increased Isc by 18% (luminal), 43% (basolateral), and 9% (bilateral) as compared to pre-acid baseline. The increase in Isc was greatest for the HBSS and low-basolateral bicarbonate groups and approached statistical significance (F = 2.886, p = 0.053, Figure 2.3).

2.5   Discussion

The epithelium of the vocal fold provides the interface between the environment and the underlying connective tissue. As such the epithelium is the foremost defense mechanism to airborne and luminal environmental and systemic challenges.(25) In this study, we sought to investigate an epithelial defense mechanism (bicarbonate transport) to pH insults in the vocal fold environment. Bicarbonate plays a critical biochemical role in the body's pH buffering system. Our data show that exposure to an acidic environment increases ion transport and that the distribution of bicarbonate in the ambient

environment of the vocal folds may influence the magnitude of ion transport response.

The interconversion of carbon dioxide and water into bicarbonate is catalyzed by carbonic anhydrase.(8, 9) In experiment 1 we attempted to block the intracellular synthesis of bicarbonate by antagonizing carbonic anhydrase with acetazolamide. We predicted a drop in ion transport with carbonic anhydrase inhibition, signifying a reduction in bicarbonate ion synthesis and thus possibly a decrease in bicarbonate secretion. In the current study, acetazolamide given at concentrations well above dosages previously reported to block carbonic anhydrase in esophageal stratified squamous epithelium, did not significantly reduce basal ion transport in the vocal fold epithelium as compared to vehicle. Potential reasons for this non-significant effect include acetazolamide concentrations or non-specificity of this inhibitor. It is possible that the concentration of acetazolamide was too low to accumulate sufficiently in the cytoplasm of the epithelial cell where it is needed to antagonize carbonic anhydrase. It is also possible that the carbonic anhydrase isomer most prevalent and active in porcine vocal fold epithelium does not respond as well to acetazolamide. Carbonic anhydrase has 16 isomers with different subcellular locations (cytosolic vs. membranous vs. extracellular) as well as catalytic activity.(26) Minimal knowledge is available for the activity and distribution of carbonic anhydrases in species other than human, mouse, and rat. Future studies should also incorporate positive controls such as esophageal epithelium to more clearly define vocal fold response to acetazolamide.

The presence and/or distribution of bicarbonate in the fluid environment of the vocal folds may influence the extent of the defense response to acidic insults. In esophageal epithelium, a bicarbonate-free fluid environment diminished the ion transport response to low pH.(13) We first demonstrated that exposure to a low bicarbonate environment did not adversely affect vocal fold epithelial viability (experiment 2). Reducing pH in the presence of low bicarbonate increased Isc in vocal fold epithelium. These data approached statistical significance. This increase was greatest when bicarbonate was present on the luminal and basolateral surfaces or the basolateral surface alone. As expected, the bilateral bicarbonate-free buffer had the least amount of ion transport increase following an acidic challenge. Our findings suggest that bicarbonate in the environment may be crucial for buffering as demonstrated by an increase in ion transport following an acidic encounter.(5) The lack of statistical significance may be potentially attributed to small sample size.

We chose to investigate an acidic environment (pH=3-4) because a multitude of disorders involve pH imbalances or insults. Possibly damaging acidic environments are encountered during generic cellular injury such as ischemia, acute inflammation, or necrosis. Any perturbation to cellular respiration can also result in an increase in anaerobic glycolysis followed by increased lactic acid and decreased pH.(27) In fact, anaerobic glycolysis is a common finding in neoplastic cells undergoing metastasis and is believed to be a mechanism of survival for these cells.(16, 17) Most notably in the laryngeal scientific literature, laryngopharyngeal reflux (LPR) involves the retrograde reflux of gastric contents

into the pharynx and larynx.(28) The normal pH of the larynx is 7 while the pH of the gastric contents are between 1.5 and 2.(29) Consequently, the pH of the larynx and vocal fold can drop to a pH less than 5 during an LPR episode. Non-acidic noxious substances in the reflux such as pepsin, bile, and pancreatic enzymes can also increase the damage to the vocal folds during a bout of LPR.(4, 24, 28) While pepsin is optimally active in an acidic environment, stable pepsin may remain in the larynx and be re-activated following another acidic reflux insult.(29) An acidic environment is therefore an important component of the pathophysiology of LPR regardless of the primary causative agent in the refluxate.

In summary, the results of this study indicate a potential role for epithelial bicarbonate ion transport in vocal fold defense to acid challenges. The role of the extracellular matrix and muscle in supporting epithelial defense to acidic insults was not investigated in this study, and future studies will examine the interaction of these tissue layers in vocal fold defense. This investigation focused on the vocal fold epithelium, as this tissue is the primary recipient of airborne and systemic challenges. As such, the vocal fold epithelial cells may serve as an important target for therapeutic interventions that strengthen both barrier and ion transport functions. Future research will examine a role for aerosolized bicarbonate as a prophylactic treatment in reducing the clinical symptoms and pathology associated with acidic changes in laryngeal disease.

2.6   References

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Table 2.1**:** Composition of HBSS and modified bicarbonate-free HBSS

HBSS+NaHCO3				
	g/L	MW	mM	OsM (mOsm/L)
CaCl	0.14	76	1.85	3.70
MgSO4	0.10	120	0.81	1.62
KCl	0.40	75	5.37	10.73
KH2PO4	0.06	136	0.44	0.88
NaCl	8.00	58	136.89	273.79
Na2HPO4	0.05	142	0.34	0.67
C6H12O6	1.00	180	5.55	5.55
NaHCO3	0.35	84	4.17	8.33
HBSS+HEPES				
	g/L	MW	mM	OsM (mOsm/L)
CaCl	0.14	76	1.85	3.70
MgSO4	0.10	120	0.81	1.62
KCl	0.40	75	5.37	10.73
KH2PO4	0.06	136	0.44	0.88
NaCl	8.00	58	136.89	273.79
Na2HPO4	0.05	142	0.34	0.67
C6H12O6	1.00	180	5.55	5.55
HEPES	1.33	238	5.55	5.55
Na-HEPES	1.09	260	4.17	8.33
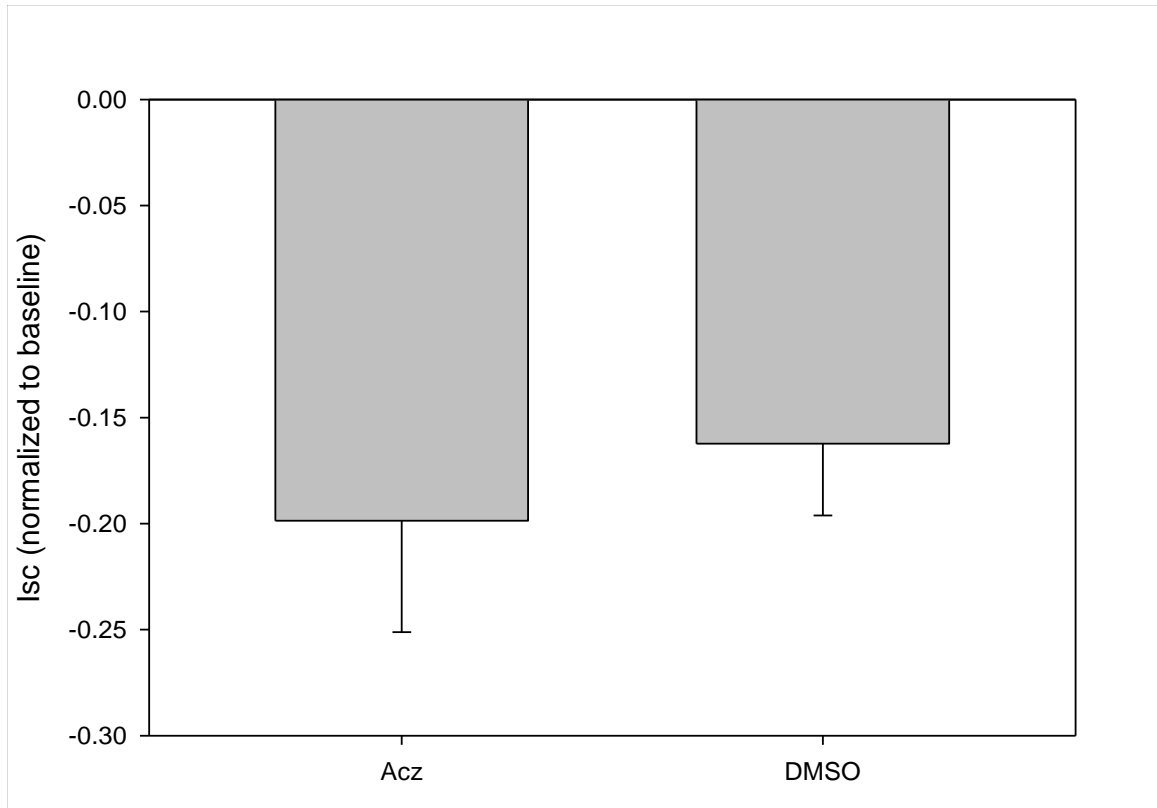
Figure 2.1: Decrease in vocal fold epithelial ion transport when exposed to acetazolamide. Average decrease in normalized percent ion transport in vocal folds exposed to acetazolamide (n = 9) or vehicle alone (DMSO, n = 9). Error bars represent standard deviation of mean.
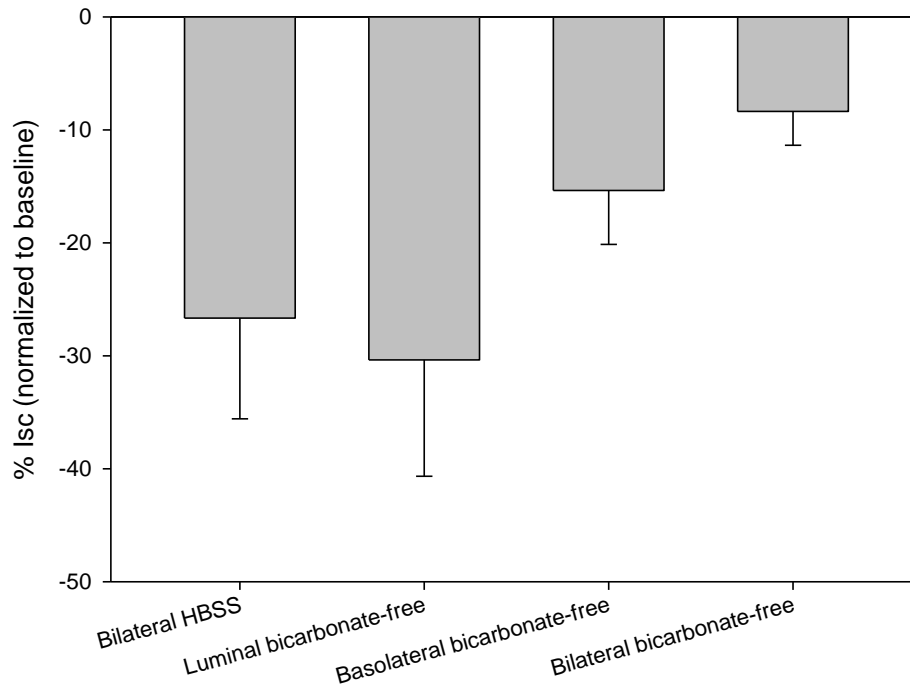
Figure 2.2: Average decrease in normalized percent ion transport in the four buffer groups. Error bars represent standard deviation of mean. (1) bilateral HBSS (n=8), (2) luminal bicarbonate-free HBSS and basolateral HBSS (n=8), (3) luminal HBSS and basolateral bicarbonate-free HBSS (n=8), and (4) bilateral bicarbonate-free HBSS (n=8).
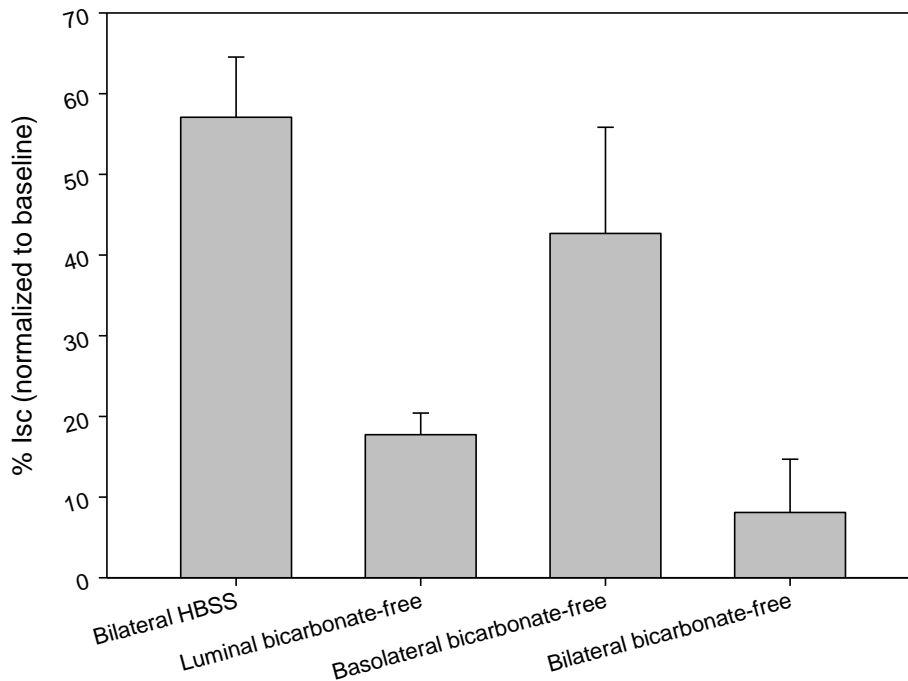
Figure 2.3: Average increase in normalized percent ion transport in the four buffer groups after exposure to acid (pH range: 3-4). Error bars represent standard deviation of mean. (1) bilateral HBSS (n=8), (2) luminal bicarbonate-free HBSS and basolateral HBSS (n=8), (3) luminal HBSS and basolateral bicarbonate-free HBSS (n=8), and (4) bilateral bicarbonate-free HBSS (n=8).

CHAPTER 3. IN *VIVO* INVESTIGATION OF ACIDIFIED PEPSIN EXPOSURE
TO PORCINE VOCAL FOLD EPITHELIA

3.1 Abstract

*Objective***:** The objective of this study was to investigate epithelial changes in response to direct, repeated, acidified-pepsin exposures in an *in* vivo porcine model. We hypothesized that 12 acidified-pepsin applications to simulate reflux would elicit a vocal fold response characterized by inflammation, epithelial proliferation, and increased intercellular space; as well as changes in the gene expression of epithelial junctional proteins, ion transporter proteins, and pro-inflammatory cytokines.

Study Design: Prospective, *in vivo* study

Methods: Eight pigs were randomly assigned to receive acidified pepsin (pH=4) or saline (sham) applied directly to the vocal folds. Larynges were collected following three exposures per week for four weeks. Vocal fold tissue morphology, collagen, and elastin were evaluated histologically by a veterinary pathologist. Gene expression of E-cadherin (Ecad), zona occludin-1 (ZO-1), cystic fibrosis transmembrane conductance regulator (CFTR), epithelial sodium channel (SCNN1α), and inflammatory mediators interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were measured. Ultrastructural

alterations were examined via transmission electron microscopy (TEM); epithelial intercellular space diameter and microridge height were measured.

Results: There were no significant differences in histology, gene transcripts, epithelial ultrastructure, intercellular space, and microridge height after acidified-pepsin exposure.

Conclusions: Twelve simulated reflux challenges were insufficient to elicit epithelial changes which demonstrate the vigor of healthy vocal folds to direct, repeated acidified-pepsin exposures. These data increase our understanding of healthy vocal fold defenses and lay the groundwork for a prospective, uninjured, non-surgical, LPR model where pigs can be exposed directly to acidified-pepsin.

3.2   Introduction

Laryngopharyngeal reflux (LPR) is an extraesophageal manifestation of gastroesophageal reflux disease (GERD) in which gastric refluxate containing acid as well as pepsin directly contact the laryngeal epithelium. LPR is a widely recognized disorder; however, there is still debate regarding pathophysiology, diagnosis, and treatment.(1-4) The current literature relating to LPR is lacking prospective controlled studies in which the outcomes of reflux in healthy subjects is evaluated. Prospective animal studies are necessary to study the pathophysiology of LPR, optimize our understanding of the disease, and improve treatment outcomes. The pig provides a unique opportunity to test hypotheses relating to laryngeal disease because porcine vocal folds are most similar to human vocal folds from a structural, biochemical, neuromuscular, and cellular

perspective.(5-7) Pig vocal folds are of sufficient size to allow for the multiple measures collected in this study. Other animal models that have been used for voice research (namely rabbit and rat) are too small in size for the number of measures proposed in this study.(8, 9)

The minimum acid exposure that results in relevant laryngeal pathology is not well understood presently. There is a discrepancy in the human literature in which some investigators believe that any amount of laryngopharyngeal reflux is abnormal, while others have documented occasional pharyngeal reflux in healthy subjects.(10, 11) Animal studies have documented that a few times per week of gastric contents contacting the injured upper airway can result in significant pathology.(12, 13) *In vitro* research on healthy, porcine vocal folds have revealed that the epithelial barrier function is impaired, and vocal fold ion transport increases, following a single acidic challenge (pH=3).(14, 15) In order to investigate these *in vitro* findings and their repercussions to the larynx in a more realistic manner, we set out to determine if repeated reflux challenges result in a reproducible animal model to study the pathogenesis, diagnostic techniques, and therapeutic intervention relating to laryngeal reflux disease.

In this experiment, liquid refluxate is applied directly on the vocal folds of anesthetized animals via videoendoscopy. This method was chosen instead of previously attempted surgical manipulation because we wanted to simulate a clinical condition of healthy individuals being exposed to reflux during everyday activities, with minimal invasive manipulation. This study focuses on the vocal fold epithelia because the location of the epithelia on the vocal fold establishes it

as the primary recipient of challenges such as reflux. The vocal fold epithelia consists of stratified squamous cells connected by apical junctional complexes that together create a barrier to challenges. In addition, epithelial cells actively transport ions and water to regulate cell volume and vocal fold hydration.(16) During an episode of LPR, gastric reflux makes contact with the vocal fold epithelia. The mechanism by which reflux alters epithelial function and the function of the underlying vocal fold tissue layers is not known. Epithelial changes are an important area to investigate since changes to connective tissue and muscle can cause changes in voice quality and voice disorders, and changes to epithelial tissue may be one route by which these tissue planes are injured.

We predicted that applying acidified pepsin (pH= 3-4) directly to the uninjured vocal fold epithelium three times a week for four weeks would result in quantifiable changes to the vocal fold epithelia. Twelve exposures will double the number of reflux challenges that have been reported in previous animal studies.(12, 13) We hypothesize that 12 reflux exposures will result in histopathologic evidence of inflammation and/or remodeling, as well as up-regulation of inflammatory cytokines. Inflammation and epithelial proliferation are two parameters that are diagnostic for reflux esophagitis.(17, 18) We also hypothesized that gene transcripts of epithelial barrier proteins and ion transporters would be altered following the reflux challenge, as a probable defense mechanism to acidic insult.

3.3   Materials and Methods

3.3.1   Animal Procedure

Eight male and female adult domestic pigs (*Sus scrofa*), weighing between 35kg - 60kg, were involved in this study. Pigs were randomly assigned to a reflux (n = 4) and sham group (n = 4). The Purdue Animal Care and Use Committee approved the animal use protocol and all procedures were performed in the presence of a licensed veterinarian. All animals were sedated with a combination of Telazol and xylazine hydrochloride intramuscularly (IM). This drug combination was prepared by using a 500 mg vial of Telazol powder and reconstituting it with 5 ml of 100 mg/ml xylzaine hydrochloride. Once reconstituted, a dose range of 0.15 to 1.0 ml/100pounds was used on each pig for sedation. Sedation was maintained with inhaled isoflurane (1 to 5%) in 100% oxygen for the duration of the procedure. After sedation, the animals were placed in sternal recumbency and a QIF 160 Olympus endoscope (Olympus USA Corp. Center Valley, PA) was introduced to visualize the larynx and assess gross pathology. A 2.0mm diameter, 190 cm long endoscopic aspiration catheter (MILA International, Inc) was inserted into the endoscope port for delivery of either reflux or sham challenge (Figure 3.1). Animals in the reflux group received 1.5 ml of 1.0 mg/ml solution of acidified pepsin (pH 3-4) sprayed directly on the membranous portions of each true vocal folds. Animals in the sham group received 1.5 ml of saline on each true vocal fold in the same way. After the challenge (reflux or sham) was administered, the endoscope and catheter were subsequently removed and the animal was allowed to recover. Overall

respiratory health including difficulty breathing and cough was assessed every 2

hours following the procedure for the first 12 hours. This procedure was repeated

3 times a week for 4 weeks for a total of 12 challenges (reflux or sham) in each

animal. The animals were humanely sacrificed immediately following the final

challenge with intravenous Beuthanasia-D Special (Schering Plough Animal

Health Corp. Union, NJ). The larynx was immediately removed for sample

processing. All animals received full autopsies to rule out any confounding

diseases.

3.3.2   Histology and Histochemical Staining

A 6 mm punch biopsy of the true vocal fold of each animal was fixed in

10% neutral buffered formalin, processed and embedded in paraffin blocks.

Tissue slices (5μm thick) were stained with hematoxylin and eosin (HE) to

highlight tissue morphology. Additional slides were stained with Masson's

trichrome and Verhoeff-Van Gieson (VVG) stains for collagen and elastin,

respectively. All slides were examined by a board-certified veterinary pathologist

for changes in histopathology. Additionally, in order to try and quantify any subtle

changes due to reflux challenge, evidence of cell proliferation were investigated.

Virtual slides were created using Aperio ScanScope (Aperio Technologies, Vista,

CA). Scanned slides were analyzed using Aperio ImageScope software

(v11.2.0.780) established algorithms. The positive pixel count (PPC) algorithm

was utilized to detect epithelial nuclei (indicator of proliferation) and lamina

proprial nuclei (indicator of cellular infiltrate). The PPC algorithm quantifies the

amount of a specific stain present in a scanned slide by specifying a color, then the algorithm counts the number in a specified area. To compare the epithelial proliferation and lamina proprial cellular infiltrate between sham and reflux vocal folds, the number of nuclei based on pixel counts in the epithelium and lamina propria was quantified and standardized over the analysis area of each vocal fold by using the PPC algorithm. A similar method was used to quantify collagen and elastin in the Masson's trichrome and VVG stained slides, respectively.

3.3.3   Transmission electron microscopy

Samples of vocal fold epithelium from 6 animals (3 sham and 3 reflux) were immediately fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer and later with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Samples were post-fixed in buffered 1% osmium tetroxide containing 0.8% potassium ferricyanide, en bloc stained in aqueous 1% uranyl acetate, dehydrated with a graded series of ethanol, transferred into propylene oxide and embedded in Embed-812 resin. Ultrathin sections were cut on a Reichert-Jung Ultracut E ultramicrotome and stained with 2% uranyl acetate and lead citrate. Each specimen was examined and photographed on a FEI Tecnai G^2 20 electron microscope equipped with a LaB_6 source and operating at 100kV. Ten representative fields from each vocal fold were captured and 10 randomly selected areas of intercellular space distance (ISD) or microridge height (MRH) within each image was analyzed via ImageJ (National Institutes of Health,

Bethesda, MD). The mean value of ISD and MRH was computed for each animal by averaging the 100 spaces in the 10 photographs of each vocal fold.

3.3.4 Real-Time PCR Quantification

Vocal fold epithelium was separated from the underlying lamina propria and immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from frozen homogenized vocal fold epithelial tissue using Nucleospin® RNA isolation kit (Macherey-Nagel, Bethlehem, PA). cDNA was synthesized from total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY), according to the manufacturer's instruction. The reactions were incubated in a thermal cycler for 10 minutes at 25°C, 120 minutes, at 37°C, 5 minutes at 85°C, and then held at 4°C.

TaqMan probes (Applied Biosystems) specific for porcine E-cadherin (Ecad), zona occludens-1 (ZO-1), cystic fibrosis transmembrane conductance regulator (CFTR), epithelial sodium channel (SCNN1α), tumor necrosis factor-1 (TNF-1), interleukin-1β, and interferon-γ (IFN-γ) were added to TaqMan Gene Expression Master Mix and to cDNA samples. Real-time PCR was performed using an Applied Biosystems 7500 Real Time PCR system. Reactions were performed as follows. Step 1: 50°C for 2 minutes; Step 2: 95°C for 10 minutes; Step 3 (40X): 95°C for 15 seconds followed by 60°C for 1 minute. The data obtained by real-time PCR was analyzed using the comparative threshold cycle (CT) method. In this method, the amount of the target gene, normalized to B-actin, and relative to a calibrator (sham vocal fold epithelial tissue), is given by

$2^{\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ (sample)$-\Delta CT$ (calibrator), and ΔCT is the CT of the target gene subtracted from the CT of B-actin. The average of four independent analyses for each gene and sample was calculated and was normalized to the endogenous gene *B-actin.*

3.3.5 Statistical Analysis

Statistical analysis of all dependent variables was evaluated using the Wilcoxon rank sum test or Mann-Whitney U test (Stata® 12.1, Statacorp, College Station, TX). An alpha level of 0.05 was selected for statistical significance.

3.4 Results

3.4.1 Histology

Vocal fold histology was similar across all animals regardless of challenge and representative findings are shown (Figure 3.2). Histological analyses revealed intact stratified squamous cells, 2-7 cell layers thick with few desquamated superficial epithelial cells. The basilar layer of epithelium had 0-2 mitotic figures per high power field. The superficial lamina propria had few lymphocytes and rare plasma cells interspersed within a loose collagen matrix and small caliber vessels. The distribution of lymphocytes was consistent throughout all layers of the vocal fold lamina propria. We confirmed previous research showing that collagen was present throughout all layers of the vocal fold lamina propria, and was denser in the deeper layers of the lamina propria.(19) Elastin fibers were denser in the superficial layer of the lamina

propria, but were present throughout all layers of the vocal fold lamina propria.(20) All histologic findings were similar across sham and reflux vocal folds.

Quantitative analysis of digitally scanned slides of true vocal folds revealed similar epithelial cell proliferation, lamina proprial cellular infiltrate, amount of elastin, and collagen deposition across reflux and sham groups (Figure 3.2 and 3.3). A summary of the quantitative findings are presented in Table 3.1.

3.4.2 Transmission Electron Microscopy

There were no ultrastructural alterations in the vocal fold epithelium. The intercellular space distance (ISD) and microridge height (MRH) of vocal fold epithelium from each animal was similar regardless of the challenge. Representative findings are shown (Figure 3.4.). The mean ISD was 0.25 μm (0.10-0.54) in shams; and 0.13 μm (0.07-0.22) in reflux vocal folds ($P > 0.05$). The mean MRH was 0.30 (0.28-0.34) in shams; and 0.28 (0.24-0.30) in reflux vocal folds ($P > 0.05$).

3.4.3 RT-qPCR

The fold difference of Ecad (1.05 ± 0.36), ZO-1(1.20 ± 0.54), CFTR (2.80 ± 3.90), SCNN1α (1.68 ± 1.56), IL-1β (2.79 ± 3.46), TNF-α (0.79 ± 0.29), and IFN-γ (0.65 ± 0.40) mRNA in the reflux vocal folds was not significantly different than the sham vocal folds ($P > 0.05$; Figure 3.5).

3.5   Discussion

In this study, we sought to investigate whether thrice weekly liquid reflux challenges over four weeks would induce vocal fold epithelial changes in a porcine model similar to human LPR. A major strength of this study is that it attempts to mimic the clinical situation of human LPR more closely by challenging healthy, uninjured laryngeal epithelium in an animal model. Our findings suggest that liquid acidified-pepsin applied directly to pig vocal folds did not significantly compromise epithelial structure or function as compared to a sham challenge. These data demonstrate that healthy vocal folds are robust and not vulnerable to 12 acidified-pepsin challenges. These data are also valuable because they demonstrate the usefulness of a pig model in future LPR studies; and provide a step forward in identifying the minimum threshold needed to elicit an epithelial response to reflux challenge in the healthy larynx.

The paucity of consistent findings regarding the mechanisms of mucosal damage and defense in reflux laryngitis in current literature may be explained by a lack of appropriate animal models. The porcine larynx offers the greatest similarity to the human larynx than any other characterized animal model.(5-7) These biological and physiological similarities are likely the same traits that are at play in human laryngeal reflux and may translate to a reliable, reproducible model of human laryngeal reflux. Although our laryngeal reflux pig model focuses on the epithelia, the similarities to human vocal fold tissue in regards to connective tissue proteins, and intrinsic muscles make the porcine larynx appropriate for investigating biological properties, as well as mechanical

properties of the connective tissue and muscle in future studies. (19) The porcine model will also permit the investigation of the interaction of LPR with vocal fold injury from paralysis or trauma.

The data in this study result from the application of liquid refluxate directly on the vocal folds of anesthetized animals via videoendoscopy. Histological analysis did not reveal differences in vocal fold epithelia, lamina propria, and thyroarytenoid muscle between challenge groups. A mild scattering of lymphoid cells was present in the superficial lamina propria of both sham and reflux pig vocal folds similar to that reported by Barker et al. and were thus considered normal mucosal immunity within the pig larynx.(7) In addition to tissue morphology, elastin and collagen amount, distribution, and morphology were similar between sham and reflux pig vocal folds. It is unlikely that these non-significant findings were related to the sample size used. The sample size selected here is consistent with literature on the porcine animal model.(6, 21)

Dilated intercellular spaces of esophageal epithelium is a hallmark ultrastructural lesion in GERD patients.(22) Intercellular space was significantly increased in laryngeal biopsies of GERD patients as well.(23) The laryngeal biopsies of GERD patients also demonstrated ultrastructural abnormalities including numerous cytoplasmic vacuoles. Previous electron microscopic examination of *ex vivo* pig laryngeal tissue incubated in solution at a pH of 2.0 reported an increase in spaces between vocal fold epithelial cells.(24) However, a pH of 2.0 is not physiologically relevant to LPR pathophysiology. Microridges cover the surface of the superficial squamous epithelium of the vocal fold.(25)

The function of the microridge is unknown, however, they are hypothesized to contribute to the adherence of mucus. Damage to the microridge structure could negatively impact the defense of the epithelia to reflux challenges. The ultrastructural morphology, intercellular space distance, and microridge height of the pig vocal fold epithelium were examined in this study and there were no differences between sham and reflux vocal folds.

The gene transcripts of epithelial barrier proteins (Ecad and ZO1), epithelial ion transporter proteins (CFTR and SCNN1α), and pro-inflammatory cytokines (IL-1β, TNF-α, and IFN-γ were compared in reflux and sham vocal folds. We chose Ecad, ZO-1, CFTR, and SCNN1α because they had been previously identified in the pig vocal fold epithelia and likely play a role in vocal fold epithelial defense to environmental challenges.(26, 27) Pro-inflammatory cytokines IL-1β and TNF-α are mediators of acute inflammation and their downstream effects on inflammatory cytokines and fibroblast proliferation may have important consequences in LPR. Furthermore, pepsin has been shown to induce up-regulation of IL-1 and TNF cytokine gene families in hypopharyngeal epithelial cells.(28) IFN-γ plays an important role in innate immunity as well as adaptive immunity. IFN-γ has been shown to be up-regulated in human reflux esophagitis patients and Barrett's esophagus patients.(29) Adaptive immunity, or immunological memory, is postulated to be involved in reflux esophagitis and thus IFN-γ may be modulated in LPR patients as well.(30) Significant differences between reflux and sham vocal fold epithelia were not identified in any of the gene transcripts examined in this study. Assays to determine changes in protein

synthesis were not completed due to lack of changes in gene expression; however, future studies will include these important investigative endpoints.

Reasons for the lack of significant changes after 12 exposures to acidified-pepsin could include small sample size and the limited frequency of exposure to acidified-pepsin. Individual examination of the data do not suggest that increasing sample size would significantly increase power and the sample size selected here is consistent with literature on the porcine animal model.(6, 21) The frequency and duration of the reflux challenge were based on previous research showing that as few as 3 experimental reflux episodes a week can result in injured laryngeal tissue if there is prior mucosal damage.(12, 13) However clinically, LPR can occur 2-5 times per day in human patients.(31) Increased frequency was precluded in the current study because the multiple sedations that are required to apply liquid challenge directly to the larynx could bring harm to the research animals. We are currently investigating methodologies to expose unanesthetized animals to acidified-pepsin challenges more frequently.

The lack of significant change in epithelial structure and function is particularly striking because single, acute, acidic challenges applied to excised pig vocal fold epithelium, *in vitro* can alter barrier resistance and ion transport.(14, 15) The key difference with the current study is that it was conducted *in vivo*, utilizing intact cardiovascular, immune, and neuromuscular systems that synergistically maintain homeostasis in a perturbed environment. These factors illustrate the importance of animal models to understand the

pathophysiology of LPR and other laryngeal diseases. Previous studies have demonstrated reflux-induced damage to vocal folds that had been biopsied prior to initiation of the study.(12, 32) The role of acidified-pepsin on healthy vocal folds have not been examined. Our data suggest that healthy vocal folds are able to defend effectively against acidified-pepsin challenges.

3.6   Conclusion

Pigs were exposed to thrice weekly challenges with either reflux (acidified pepsin) or saline applied directly to the vocal fold epithelia. Exposure to liquid acidified pepsin did not significantly alter tissue morphology, ultrastructural morphology or epithelial intercellular space distance, gene transcripts of inflammatory cytokines, ion transporters, or epithelial barrier proteins. These data provide the groundwork for further investigations into developing animal models to understand the pathophysiology of LPR.

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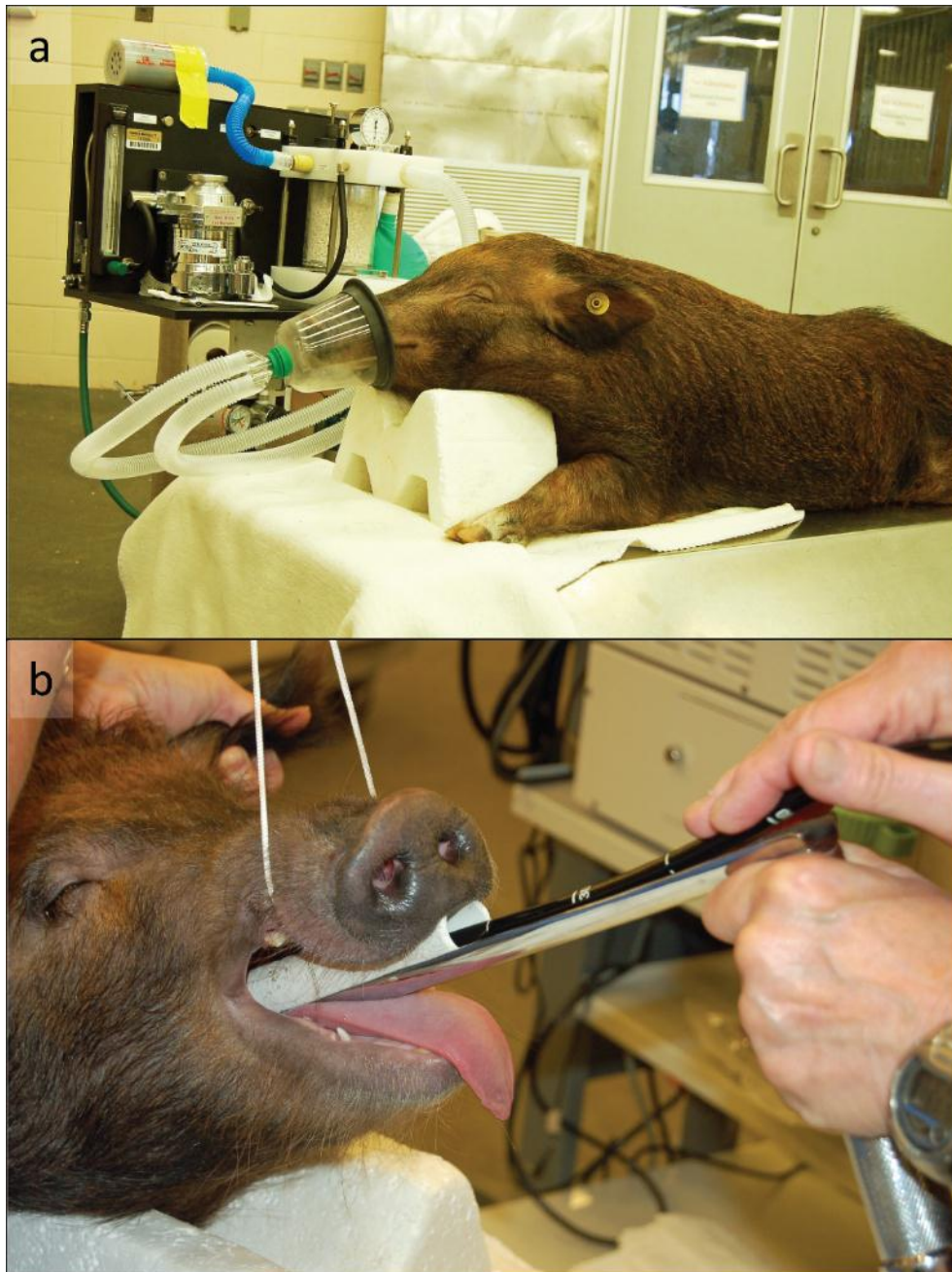
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Figure 3.1: Photo (a) depicting anesthesia requirements prior to endoscopy. Photo (b) depicting endoscopic approach in a pig.
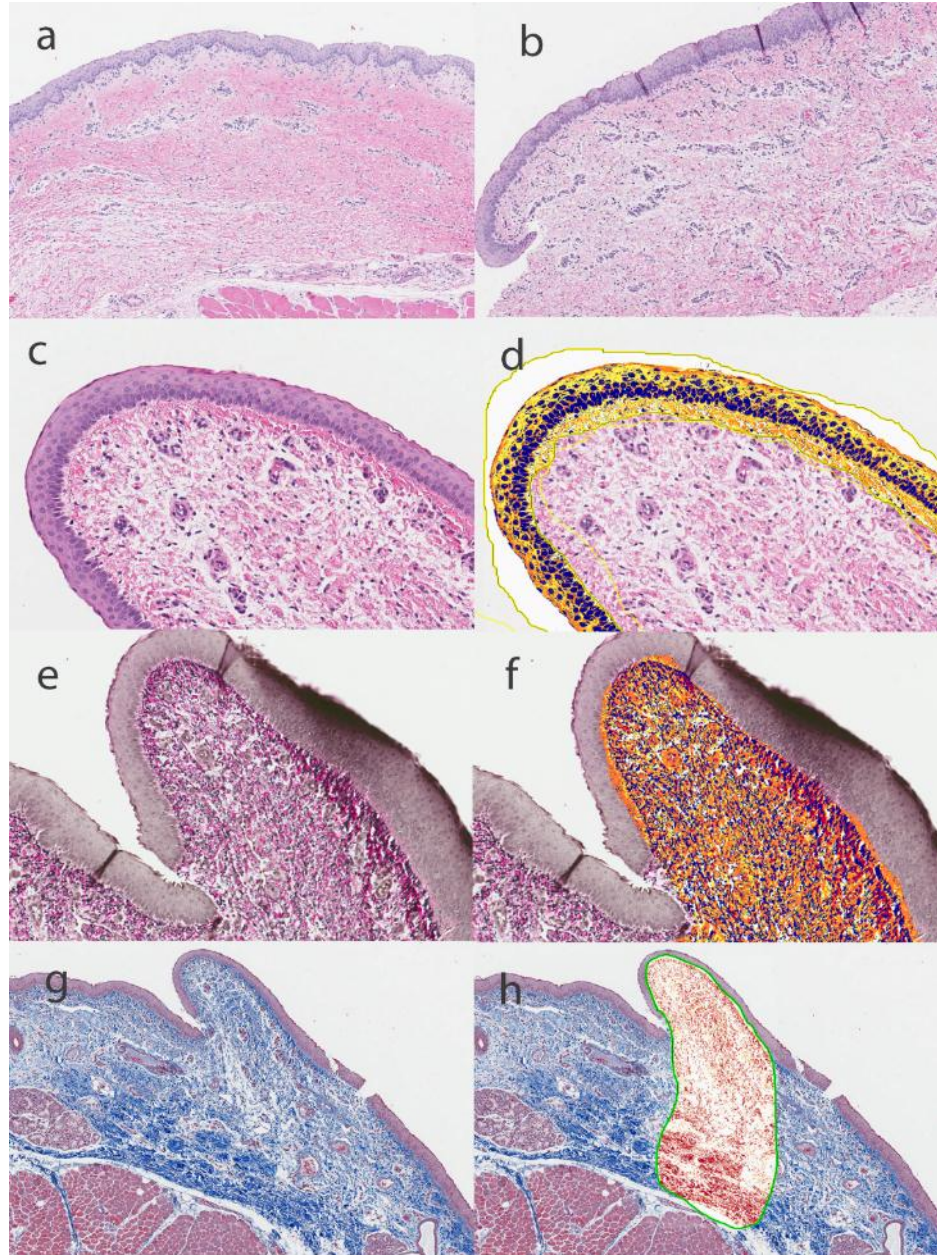
Figure 3.2: Low magnification (a) sham and (b) reflux vocal fold; representative high magnification of reflux vocal fold epithelium and superficial lamina propria [H&E (c) VVG elastin (e) and Masson's trichrome (g)]; overlay of ImageScope software PPC algorithm to count epithelial nuclei (d), VVG elastin (f) and Masson's trichrome (h)
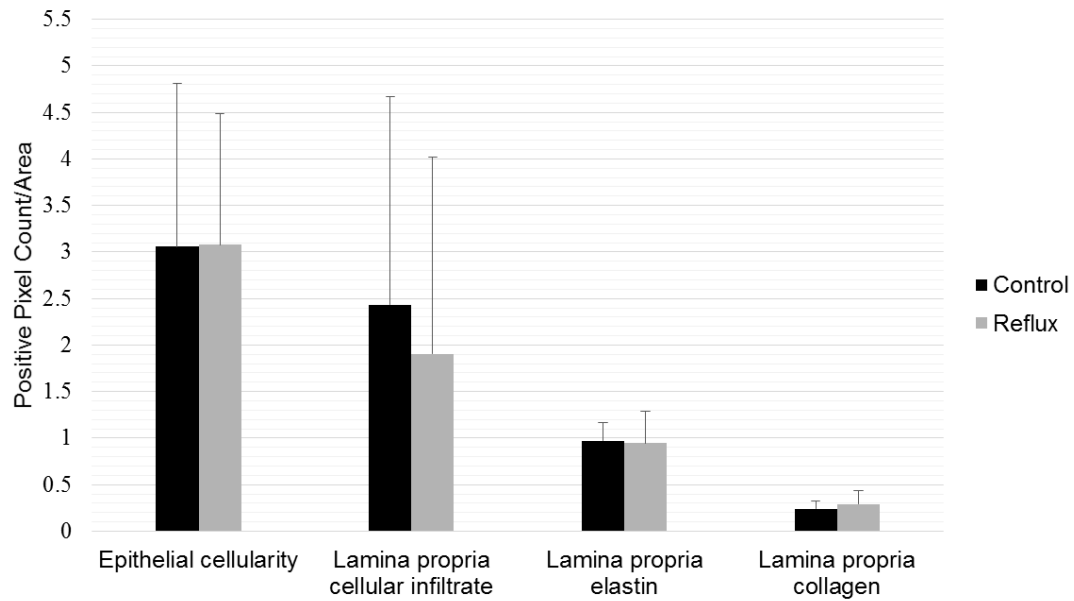
Figure 3.3: Means and standard deviations of histologic findings in sham and reflux vocal folds. Pixels of a given stain color were quantified and standardized over a given area.

Table 3.1: Summary of quantitative histologic findings

	TVF epithelial cells	Lamina propria cellularity	Lamina propria collagen	Lamina propria elastin
Control	Median = 3.326	Median = 1.717	Median = 0.252	Median = 1.05
Reflux	Median = 3.745	Median = 1.175	Median = 0.258	Median = 0.838
Wilcoxon rank sum test	z = -.365, ns	z = -1.461, ns	z = 1.461, ns	z = -0.365, ns

p-value < 0.05, ns = not significant
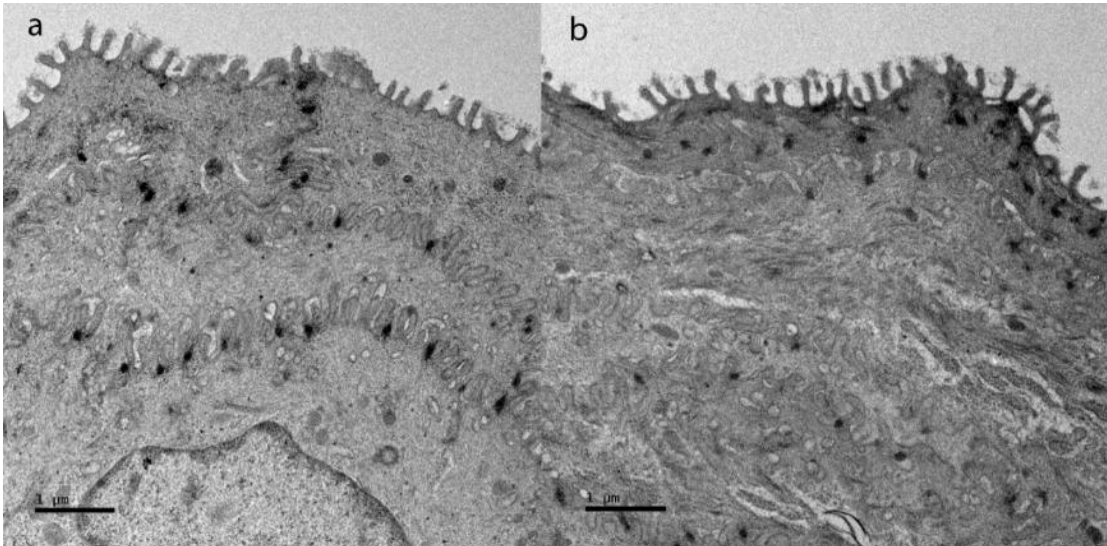
Figure 3.4**:** Transmission electron photomicrograph of (a) sham and (b) reflux vocal fold epithelium. (Original magnification 2550X)
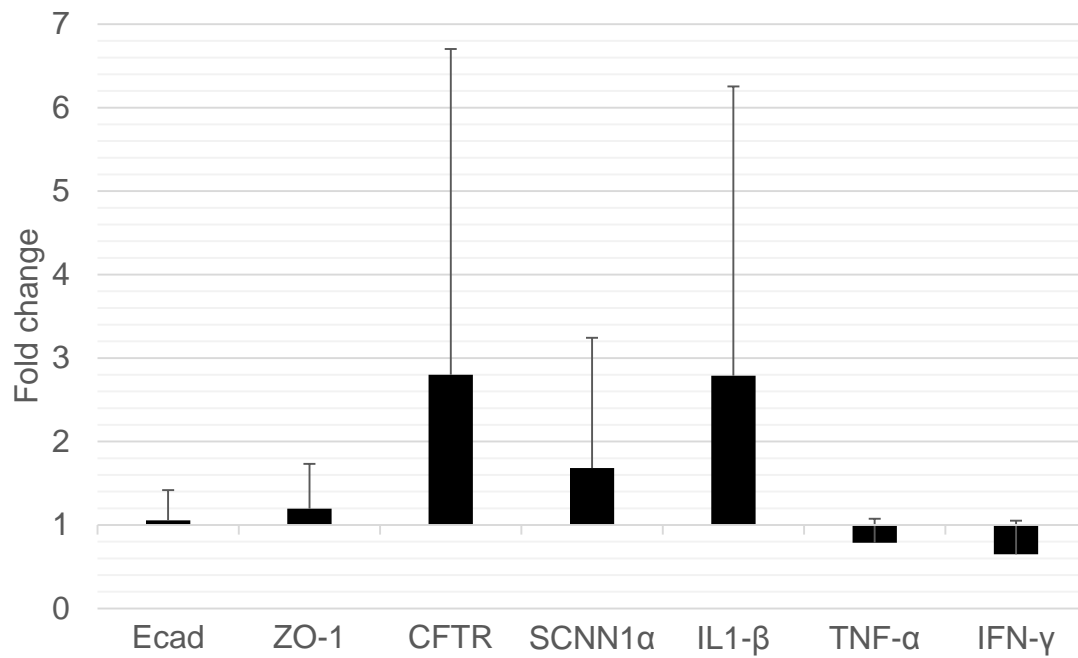
Figure 3.5**:** Mean fold change and standard deviations for gene transcripts in reflux compared to sham vocal folds. Data analyzed using delta CT method. E-cadherin (Ecad), zona-occludens-1 (ZO-1), cystic fibrosis transmembrane conductance regulator (CFTR), epithelial sodium channel (SCNN1α), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ).

CHAPTER 4. NOVEL EXPERIMENTAL PIG MODEL OF AEROSOLIZED ACIDIFIED PEPSIN

4.1   Abstract

Objective: The objective of this study was to investigate epithelial changes in response to repeated, aerosolized acidified-pepsin exposures in an *in vivo* porcine model. We hypothesized that daily inhalation of acidified-pepsin to simulate reflux would elicit a vocal fold response characterized by inflammation, epithelial proliferation, increased intercellular space and decreased microridge height; as well as changes in the gene expression of epithelial junctional and transporter proteins.

Study Design: Prospective, *in vivo* study

Methods: Twelve pigs were randomly assigned to a reflux or sham group in which pigs inhaled acidified pepsin (pH=4) or saline through a nose cone attached to a nebulizer. The pigs were challenged 3 times per day, 5 days per week, for 4 weeks for a total of 60 exposures. Vocal fold, nasal mucosa, trachea and lung tissue morphology were evaluated histologically by a veterinary pathologist. Ultrastructural alterations were examined via transmission electron microscopy (TEM); epithelial intercellular space diameter and microridge height

were measured. Complementary DNA microarray analysis of vocal fold epithelium was conducted and followed up with real-time polymerase chain reaction investigating the gene expression of E-cadherin (Ecad), zona occludin-1 (ZO-1), cystic fibrosis transmembrane conductance regulator (CFTR), and epithelial sodium channel (SCNN1α). Antibodies targeting CFTR, epithelial sodium channel-γ (γENaC), Ecad, and Ki-67 on true vocal fold epithelium were evaluated via immunohistochemistry.

Results: Animals were successfully trained to receive multiple daily inhaled challenges of aerosolized acidified pepsin. There were no significant differences in histology, immunohistochemistry, epithelial ultrastructure, intercellular space, microridge height, or gene transcripts after inhalation of acidified-pepsin.

Conclusions: These data offer a potential novel experimental methodology to test similar inhaled laryngeal challenges on healthy pigs. The success of this methodology could easily transition to chronic inhalation experimentation.

4.2 Introduction

A comparative animal model is needed to better understand laryngopharyngeal reflux (LPR) disease in human patients. A better understanding of LPR's etiology and pathogenesis could provide improved diagnostic criteria and treatment outcomes for the disease.(1) Previous studies investigating largyngopharyngeal reflux (LPR) in an animal model rely heavily on prior injury to the laryngeal epithelium to elicit disease.(2-4) This

injury to laryngeal epithelia prior to challenge may not accurately mimic the disease in human beings. Most patients experiencing LPR have no prior overt injury or symptoms and endoscopic examination reveals intact epithelium.(5) Our lab set out to establish a physiologically relevant animal model of LPR with intact laryngeal epithelium and repeated exposures.

The advent of new technology utilizing a combination of pH and impedance monitoring as a diagnostic tool has allowed for the physical characteristics (liquid, gas, and mixed) of the reflux to be determined.(6) Kawamura et al. determined that gaseous reflux events, regardless of acidity, appear to be more common in suspected LPR patients compared to controls.(7) Published animal studies relating to LPR have all utilized direct contact of simulated reflux containing an acidified pepsin solution or liquefied gastric contents.(2-4, 8, 9) Our study investigates whether repeated challenges with gaseous acidified pepsin can lead to changes in vocal fold epithelium.

As with our previous experiments, this study capitalizes on the comparative similarities between the pig and human vocal fold; as well as the added benefit that overall size of the pig vocal folds are adequate for multiple analyses.(10-12) Aforementioned, studies in our lab utilizing pigs as our animal model relied heavily on repeated sedation to expose the vocal folds to a reflux challenge to simulate LPR (in press). A threshold to elicit disease following direct acid reflux solution exposure may not have been reached. In order to increase the frequency of acid reflux exposure, we set out to design

an animal experiment in which the subject was challenged with simulated reflux multiple times per day for multiple weeks while remaining unanesthetized.

Very little information is available on routine inhalation studies with unanesthetized pigs. Therefore, one of the aims of this study was to develop an inhalation system using pigs as the test animals to obtain an improved, more practical protocol for routine inhalation challenges conducted on unanesthetized animals. Awake and restrained animals are an important combination if investigating repeated challenges in the same animal over a relatively short time period. Sedation and anesthesia has an increased risk of complication if repeated several times a day or even week. Anesthetic agents can also have unintended effects on animal physiology that may confound animal studies.(13, 14) Fasting prior to anesthesia can prove problematic if the animal needs to be anesthetized multiple days in a row. There needs to be time for the animal to recover from anesthesia, eat, digest, and then fast again prior to the next round of anesthesia. For these reasons, we set out to develop a methodology to repeatedly challenge unanesthetized pigs via an inhalation route.

Typical restraint methods utilized on pigs in an agricultural setting include snout tying and hog tying.(15) These methodologies are stressful, potentially painful, and can endanger the animal and handler if the pig decides to be aggressive as a result of the repeated restraint technique. These restraint techniques also block the availability of the snout for normal

breathing necessary for inhalation studies. Fortunately, pigs are easily trained and can be acclimated to a sling apparatus such as the Panepinto Sling (Figure 1).(16) Acclimating and training pigs to the sling is easily accomplished through positive reinforcements such as food and touch. Additionally, pigs are social animals and not only prefer to be amongst their own species but many seek out human contact and visibly enjoy socializing in a laboratory setting. This socialization works well when training and utilizing the restraint sling because the pigs quickly associate being restrained in the sling with positive human attention.

Herein, we introduce a novel experimental methodology to study inhaled challenges to the larynx of pigs. We predicted that normal pigs who inhaled aerosolized acidified pepsin (pH=4) 3 times per day, 5 days per week, for 4 weeks for a total of 60 inhalation challenges would develop observable changes to the vocal fold epithelia. More specifically, we hypothesized that repeated aerosolized reflux challenges would result in histopathologic evidence of inflammation and/or remodeling, as well as changes in immunohistochemical expression levels of specific antigens. We also hypothesized that DNA microarray analysis may highlight specific biomarkers of LPR that have yet to be investigated. To our knowledge, this study is the first to investigate aerosolized simulated reflux in an animal model.

4.3   Materials and Methods

4.3.1   Animal Procedure

Twelve female adult Sinclair minipigs (*Sus scrofa*), weighing between 35kg - 50kg, were randomly assigned to a reflux (n = 6) or sham group (n = 6). The pigs are a strain of pig that was developed specifically for a laboratory setting due to their small size. Six of the 12 animals used in this study were reportedly acclimated to a sling prior to their shipment to Purdue University's animal holding facilities. The Purdue Animal Care and Use Committee approved the animal use protocol and all procedures were performed in the presence of a licensed veterinarian. Two weeks prior to the beginning of the study, all pigs were systematically acclimated through positive reinforcement to voluntarily enter a restraint sling (Panepinto®) and subsequently lifted off of the ground so as to immobilize the pigs without chemical restraint. Once the study began, pigs were individually lifted in the sling and a nose cone was placed over the snout. The animals breathed naturally until 3 ml of reflux or sham solution was aerosolized in the nebulizer (Pari LC® Sprint nebulizer) and delivered to the nose cone (Figure 4.1). The reflux solution was 1.0 mg/ml of acidified pepsin (pH=4) and the sham solution was saline. The pH of the aerosolized reflux solution was verified to be at pH=4 by exposing pH paper to the nebulized reflux solution. After 3ml of solution were nebulized, the animals were lowered to the ground and returned to their pen. This process was repeated 3 times per day, 5 days per week for 4 weeks for a total of 60 aerosolized challenges on each pig. After 4 weeks, the animals

were humanely sacrificed immediately following the final challenge with intravenous Beuthanasia-D Special (Schering Plough Animal Health Corp. Union, NJ). The larynx was immediately removed for sample processing. Full-thickness sections of nasal mucosa taken from the right and left nares, midway up the snout were also sampled. All animals received full autopsies to rule out any confounding diseases.

4.3.2 Histology and Immunohistochemistry

A 6 mm punch biopsy of the true vocal fold, nasal mucosa, and lung of each animal were fixed in 10% neutral buffered formalin, processed and embedded in paraffin blocks. For each punch biopsy of the vocal fold epithelium, 3-μm sections were obtained and stained with Hematoxylin and eosin (HE) as well as select immunohistochemistry (IHC) for CFTR, epithelial sodium channel-γ (γENaC), E-cadherin (Ecad), and Ki-67. Antibodies against CFTR, γENaC, Ecad and Ki-67 were applied to paraffin-embedded tissue sections according to the manufacturer's instructions and performed with a Dako autostainer with incubators at room temperature. Appropriate target-specific positive and negative control tissues were used. After the immunohostochemical procedure, all slides were counterstained with Mayer's hematoxylin and examined by a board-certified veterinary pathologist. Table 4.1 summarizes the protocols for the 4 antibodies used in the study and the immunohistochemical protocols. The expected staining pattern for each antibody was as follows: CFTR (cytoplasm and/or membrane), Ecad

(cytoplasm and/or membrane), γENaC (cytoplasm and/or membrane) and Ki-67 (nucleus).

Virtual slides of immunostained tissues were created using Aperio ScanScope (Aperio Technologies, Vista, CA). Scanned slides were analyzed using Aperio ImageScope software (v11.2.0.780) established algorithms. For all CFTR, γENaC, Ecad, and Ki-67 immunostained slides, a semi-quantitative histochemical score (H score) was calculated by the formula: (3 x percentage of strongly staining) + (2 x percentage of moderately staining) + (percentage of weakly staining), giving a range of 0 to 300. This H score was adapted from the Aperio software.(17)

4.3.3   Transmission electron microscopy

Samples of vocal fold epithelium from 12 animals (6 sham and 6 reflux) were immediately fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer and then transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Samples were then placed in buffered 1% osmium tetroxide containing 0.8% potassium ferricyanide, en bloc stained in aqueous 1% uranyl acetate, dehydrated with a series of ethanol, transferred into propylene oxide and embedded in Embed-812 resin. A heavy metal tracer, lanthanum nitrate (1%) was added at each subsequent stage of electron microscopy processing, including all fixatives and reagents. Lanthanum nitrate can outline the intracellular space for increased accuracy in measurement.(18) Ultrathin sections were prepared on a Reichert-Jung

Ultracut E ultramicrotome. Sections were stained with 2% uranyl acetate and lead citrate. A FEI Tecnai G^2 20 electron microscope equipped with a LaB$_6$ source and operating at 100kV was used to examine and photograph each sample. Ten representative fields from each vocal fold were photographed. Ten randomly selected areas of intercellular space distance (ISD) or microridge height (MRH) within each image was analyzed via ImageJ (National Institutes of Health, Bethesda, MD). The mean value of ISD and MRH was computed for each animal by averaging the distance of 100 intercellular spaces and the height of 100 microridges of each vocal fold.

4.3.4 DNA Microarray and Real-Time qPCR Quantification

Total RNA was extracted from frozen homogenized vocal fold epithelium (n = 12) that was stored at -80°C using Nucleospin® RNA isolation kit (Macherey-Nagel, Bethlehem, PA). A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY) synthesized cDNA from total RNA. The reactions were completed in a thermal cycler programmed for 10 minutes at 25°C, 120 minutes, at 37°C, 5 minutes at 85°C, and then held at 4°C.

For DNA microarray analysis, total RNA (n = 8) was analyzed using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) to assess the quality of the total RNA for each sample. 100 ng of total RNA for each sample was labeled using the standard protocol for the Affymetrix WT Plus kit (Affymetrix, Santa Clara, CA). Individual labeled samples were hybridized to

the Porcine Gene 1.0 ST GeneChips® for 17 hours then washed, stained and scanned with the standard protocol using Affymetrix GeneChip® Command Console Software (AGCC) to generate data (CEL files). Arrays were visually scanned for abnormalities or defects; none were found. All samples were processed in one batch. Eight CEL files were analyzed using Bioconductor - oligo package in order to perform reflux vs. sham comparisons based on expression values. To determine the quality of array files, distribution of raw intensities, and PCA plots; boxplots for 8 CEL files were plotted to get an idea of outliers and distribution of probe intensities. Preprocessing of the array files was done in 3 steps: background correction (to remove non-specific hybridization), normalization (to remove differences in intensity due to artifacts) and summarization (to summarize different probe values per set).

Following DNA microarray analysis, RT-PCR was conducted using TaqMan probes (Applied Biosystems) specific for porcine E-cadherin (Ecad), zona occludens-1 (ZO-1), cystic fibrosis transmembrane conductance regulator (CFTR), and epithelial sodium channel (SCNN1α) were added to TaqMan Gene Expression Master Mix and to cDNA samples. Real-time PCR was performed using an Applied Biosystems 7500 Real Time PCR system. The following steps occurred. Step 1: 50°C for 2 minutes; Step 2: 95°C for 10 minutes; Step 3 (40X): 95°C for 15 seconds followed by 60°C for 1 minute. The data obtained by real-time PCR was analyzed using the comparative threshold cycle (CT) method. In this method, the amount of the target gene, normalized to B-actin, and relative to a calibrator (sham vocal fold epithelial

tissue), is given by $2^{\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ (sample)$-\Delta CT$ (calibrator), and ΔCT is the CT of the target gene subtracted from the CT of B-actin. The average of four independent analyses for each gene and sample was calculated and was normalized to the endogenous gene *B-actin.* Statistical analysis was completed on the average $\Delta\Delta CT$ values of reflux animals compared to sham animals.

4.3.5 Statistical Analysis

Statistical analysis of all immunohistochemical, ultrastructural and RT-PCR dependent variables was evaluated using the Wilcoxon rank sum test or Mann-Whitney U test (Stata® 12.1, Statacorp, College Station, TX). An alpha level of 0.05 was selected for statistical significance.

Statistical analysis of DNA microarrays initiated with RMA normalization at both probe set level (since the array typically consists 4-20 probes per set) and gene set level (since the array consists of transcript clusters per gene). Log2 fold change (log2FC) values after normalization at probe set level for 144,644 probe sets and gene set level for 27,558 gene sets were generated. For reflux versus sham comparison, a linear regression model was fit based on gene set. Differential expression analysis was carried out using limma's empirical Bayes method (Bioconductor package version 3.24.9). Moderated t-test statistic (t value) was calculated along with p-values. These p-values were adjusted for multiple testing using Benjamini and

Hochberg test which resulted in corrected p-values (FDR) for reflux versus

sham comparison.

4.4   Results

4.4.1   Animals

All animals survived without complications following 60 nebulized

acidified- pepsin challenges. Pigs that were reportedly accustomed to a sling

prior to shipment did not show improved acclimation to the sling once the

onsite training began. All pigs responded positively to food rewards and

positive touch from trained animal handlers. All pigs voluntarily loaded in the

restraint sling and tolerated the nose cone long enough to administer 3ml of

nebulized acidified pepsin solution. Two animals, 1 sham and 1 reflux

developed a cough during the 4 week study. Gross autopsy findings were

unremarkable in the larynx of all 12 animals.

4.4.2   Histology and Immunohistochemistry

Vocal fold and nasal mucosa histology were similar in all animals and

comparable to previous studies conducted in our laboratory. Representative

findings are shown in Figure 4.2. Briefly, the stratified squamous epithelium of

the true vocal fold was approximately 5 cell layers thick and morphologically

normal. The lamina propria was made up of loose collagenous matrix with

lymphocytes and fewer plasma cells scattered randomly throughout the

superficial layers. Collagen fibers were comparably denser (fiber thickness as

well as increased numbers of fibers) in the deeper portions of the lamina propria compared with the more superficial layers.

Nasal conchae were lined by a ciliated, pseudostratified columnar epithelium with an underlying lamina propria that contained numerous aggregates of lymphocytes and plasma cells. There were no mitotic figures in the epithelium to suggest hyperplasia. The amount of immune cells scattered throughout the nasal conchae lamina propria was similar in all animals and within normal limits for pig. The submucosal glands were unremarkable.

One pig (#1691) out of 12 had a focal area of consolidation in the right cranial lung lobe. In this pig, approximately 30% of the right cranial lung lobe contained large peribronchiolar lymphoid cuffs that were highly suggestive of *Mycoplasma* spp. infection. *Mycoplasma hyorhinis* was cultured from lung samples. *Mycoplasma hyorhinis* exists in a high percentage of healthy adult swine and can manifest with lesions under stressful conditions, such as a laboratory setting.(19) No other lesions were identified in the animal to suggest further disease; therefore, lung from unaffected lobes was used to evaluate lesions related to the study. In spite of the one pig with suspected focal Mycoplasmosis, no histological differences were noted between animal lungs.

Quantitative analysis of digitally scanned IHC slides labeled with CFTR, γENaC, Ecad, and Ki-67 revealed no significant differences in staining intensity or pattern between sham and reflux vocal fold epithelium. Representative findings are shown in Figure 4.3 and a summary of the semi-

quantitative histochemical scores of each antibody are graphed in Figure 4.4 and listed in Table 4.2.

4.4.3 Transmission Electron Microscopy

Representative ultrastructural micrographs are shown in Figure 4.5. The mean ISD was 0.038 μm (0.030-0.050) in shams; and 0.039 μm (0.030-0.057) in reflux exposed vocal folds (P > 0.05; Figure 4.6). The mean MRH was 0.319 μm (0.232-0.382) in shams; and 0.359 μm (0.258-0.457) in reflux exposed vocal folds (P>0.05; Figure 4.6). Ultrastructural differences were not identified in sham vs. reflux comparison.

4.4.4 DNA Microarray and Real-Time PCR Quantification

No genes in the DNA microarray were considered significantly differentially expressed at FDR 0.05 or 0.1. ΔΔCT values of CFTR (z = 2.051, P = 0.0403) and ZO-1 (z = 2.051, P = 0.0403) were statistically different between reflux and sham vocal folds. ΔΔCT values of Ecad (z = -1.026, P > 0.05) and SCNN1α (z = 1.026, P > 0.05) were not statistically significant between reflux and sham vocal folds. Furthermore, RT-qPCR fold differences of Ecad, CFTR, ZO-1, and SCNN1α in the reflux vocal folds compared with sham vocal folds were less than 2 and thus not considered physiologically relevant (Figure 4.7).[20, 21]

4.5   Discussion

Here we described a novel pig model of aerosolized reflux with an approach focusing on repeated challenges on an alert, comfortable animal void of chemical restraint. In conjunction, we sought to investigate whether thrice daily challenges over 4 weeks with simulated aerosolized reflux would induce vocal fold epithelial changes in a pig model. A major strength of this study is that it attempts to validate whether gaseous reflux events can solely lead to laryngeal changes in an animal model of human LPR. Our findings suggest that aerosolized acidified pepsin inhaled by healthy adult pigs over 60 exposures does not significantly affect vocal fold epithelial function as compared to a sham challenge of saline alone. One of the most common difficulties of *in vivo* experimental design is implementing repeated exposures. Animals, especially large animals, frequently require chemical sedation/anesthesia, also known as chemical restraint, in order to administer a challenge or exposure. Chemical restraint often requires recovery time that can greatly impact the frequency of challenges. Additionally, chemical restraint can impact the biological functions of circulatory and nervous system, as well as cellular homeostasis.(22, 23) In order to model human physiology optimally, iatrogenic manipulation needs to be minimized.

Documenting the pathogenic role of gastric acid refluxate as the causative agent of LPR has been problematic. The clinical symptoms and findings of LPR are nonspecific and often occur in a variety of disease states.(24, 25) Much of the literature investigating the role of pH, gastric reflux

proteases, and physical properties have resulted in conflicting results and thus highlight the need for animal models to investigate suspected disease culprits.(3, 7, 26) The role of gaseous versus liquid reflux states has been more thoroughly investigated in esophageal gastroesophageal reflux disease (GERD) patients, with results that suggest mixed gas/liquid GERD events surpass the number of liquid events and may play an important role in the pathogenesis of disease. Kawamura, *et al.* (2004) examined the physical properties of pharyngeal refluxate in GERD, LPR, and healthy individuals. They reported that gas reflux events are more common among LPR patients compared to GERD patients and control. We set out to test these findings in a comparative pig model.

As previously mentioned, pigs are an ideal animal model of laryngeal disease because of their similar laryngeal anatomy, physiology, and immunology compared to the human larynx.(10-12) One of the limitations of using a large animal model such as the pig is repeated handling without chemical restraint or numerous personnel. This study utilized a restraint system that was introduced decades ago;(16) however, not often cited in the literature. The advantage of the sling as a restraint device is that the pressure applied on the abdominal muscles while the animal is suspended is believed to be calming to the animal, minimizing the stress of restraint.(16) We combined the sling restraint with the pig's natural tendency to train easily in order to administer multiple inhaled challenges in a short period of time, with little to no detriment to the animal. With few personnel and minimal

acclimatization time, pigs were trained to voluntarily enter the sling restraint and wear a nose cone for approximately 15 minutes. This experimental model could easily translate to other inhaled challenges not related to reflux but requiring multiple exposures in an unsedated large animal.

The data in this study result from the repeated exposure of awake animals to aerosolized acidified pepsin (pH =4). Histological analysis of all tissues, but specifically the nasal conchae, laryngeal tissues, trachea, and lungs revealed no differences between sham and reflux pigs. A mild to moderate scattering of lymphocytes in the lamina propria of pig nasal mucosa and larynx is reportedly normal as the nares of the snout and laryngopharyx are the first lines of defense against aerogenous pathogens.(27) In addition to tissue morphology, immunohistochemical markers against CFTR, γENaC, Ecad, and Ki-67 were evaluated on true vocal fold epithelium using a semi-quantitative histochemical score on digitally scanned microscope slides. We chose to evaluate these specific immunohistochemical markers because 1) CFTR and γENaC are epithelial membrane ion transport proteins that have been suggested to be altered in pig vocal fold tissue exposed to an acidic environment;(28, 29) 2) E-cadherin is a tight junctional protein that has been shown to be downregulated in LPR patients compared to healthy human beings;(30) and 3) Ki-67 protein is necessary for cellular proliferation and thus can be quantified via immunohistochemistry to reveal the proliferating growth fraction of a tissue.(31) There were no significant differences between

sham and reflux pigs true vocal fold tissue labeled with the specified immunohistochemical markers.

The ultrastructure, intercellular space and microridge height of true vocal fold epithelium were evaluated in this study as well. As previously reported, transmissions electron microscopy (TEM) on laryngeal biopsies of patients with gastroesophageal reflux disease (GERD) revealed increased intercellular space and increased cytoplasmic vacuolation, presumably due to the increased acidic environment of the larynx.(32) These changes were not identified in the true vocal fold epithelium of sham and reflux pigs in this study. Moreover, microridge height was not significantly different between treatment groups in this study either. Microridges cover the apical surface of the human true vocal fold.(33) Our laboratory has documented microridge morphology of the pig true vocal fold epithelium as well (Figure 4.5). The true function of the vocal fold microridge is not known, but it is posited to play a significant role in mucus adherence to the epithelium, and thus defense against aerogenous challenges to the tissue.(33)

Finally, DNA microarray analysis was completed on the sham and reflux pig true vocal fold epithelium. We had hoped to ascertain differential gene expression profiles comparing sham and reflux tissue that could be further investigated to determine pathogenesis of disease. Unfortunately, DNA microarray analysis in this study did not detect gene differences between treatment groups. Differential gene expression has been reported in humans that smoke cigarette, experience LPR, and develop benign polyps;

and the genetic profiles have been utilized to determine distinctive genetic patterns relating to pathogenesis of these different disease states .(34) Primary human epithelial cell lines exposed to pepsin have also demonstrated distinctive genetic patterns implicated in carcinogenesis.(35) It is our hope that future studies will be able to tease out gene sets that can be implicated and further evaluated for their role in LPR.

In order to further clarify any genetic contribution that aerosolized acidified pepsin may have on mRNA expression of specific membrane ion transporters and junctional proteins of vocal fold epithelium, we selected 4 genes to investigate via RT-qPCR that had previously been characterized in the literature to be likely contributing to LPR disease.(28-30) Significant differences between reflux and sham vocal fold epithelia $\Delta\Delta$Ct were identified in CFTR and ZO-1 ($P < 0.05$), but not in SCNN1α or Ecad. However, physiologically the fold-changes of the mRNA expression of reflux vocal fold epithelia were below 2 when comparing to sham. A fold change of less than 2 is often attributed to normal biological variability and is likely unrelated to a true difference in mRNA expression.(36) Collectively, our mRNA expression data suggest no differences in expression between reflux and sham vocal fold epithelia.

A possible explanation for the lack of significant changes after repeated inhalation exposures of acidified pepsin could be that the inhaled challenge was limited to the nasal turbinates, resulting in little aerosolized challenge reaching the laryngeal epithelium. Perhaps the aerosolized pepsin

condensed and was swallowed decreasing exposure. Or perhaps aerosolized pepsin has little to no impact on the function and physiology of upper respiratory epithelium and that liquid acidified pepsin in combination with gaseous is required to alter epithelial parameters. Previous studies in our laboratory have highlighted the robust defense of the vocal fold epithelia from acidified pepsin challenges.(37) Follow up studies are necessary to elucidate the impact of mixed physical (i.e., liquid, gas, and mixed gas/liquid) properties of the reflux on laryngeal tissue.

4.6   Conclusion

This is the first experimental report of aerosolizing reflux in a pig model of laryngopharygeal reflux. Aerosolized acidified pepsin inhaled three times daily for 4 weeks did not result in significant alterations to morphology, immunohistochemistry, ultrastructual morphology, epithelial intracellular space distance, microridge height, or gene transcripts of select ion transporters nor epithelial barrier proteins. This novel pig model could be helpful to carry out related research involving large animals in an inhalation study or any study necessitating chronic challenges.

4.7   Acknowledgements

4.8   References

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Figure 4.1: Photo depicts the sling-restraint apparatus. Each pig is acclimated to the sling and trained to breathe normally through a standard nose cone.
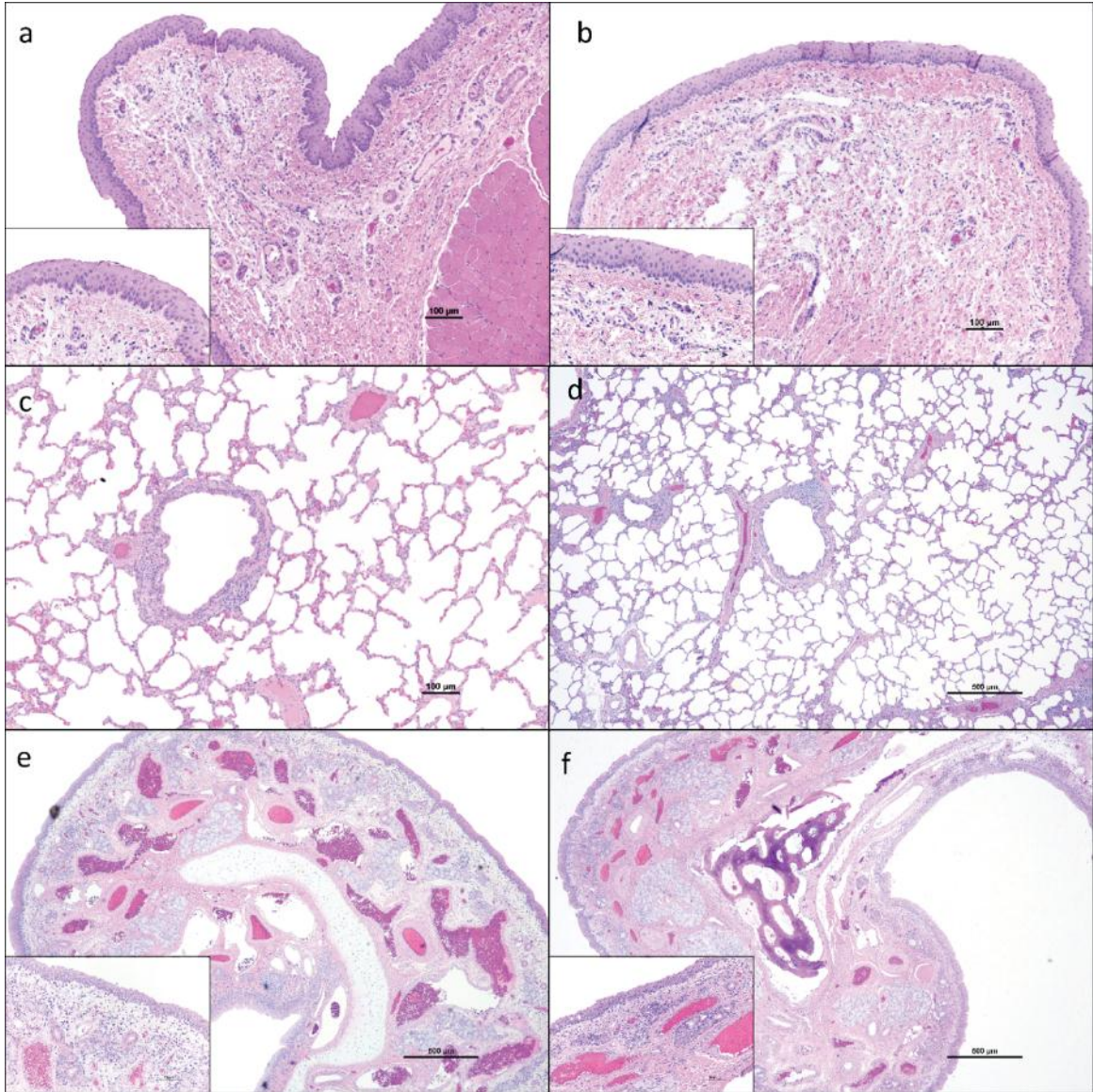
Figure 4.2**:** Photomicrographs of true vocal fold (a,b), lung (c,d) and nasal conchae (e,f). Photomicrographs are representative of sham (a,c,e) and reflux (b,d,f) animals. Insets are higher magnification of the same tissue. Histologic lesions were not identified in sham or reflux true vocal fold, lung, or nasal conchae.

Table 4.1**:** Antibody reagents, antigen retrieval, and detection systems used in immunohistochemistry

Antibody	Dilution	Incubation Time	Pretreatment[d]	Detection system	Source of Antibody
CFTR[a]	1:200	45 min RT	HIER Diva/Biocare Medical	Rabbit-on-Farma-HRP Polymer	Abcam, Cambridge, MA
γENaC[b]	1:100	60 min RT	HIER Borg/Biocare Medical	Rabbit-on-Farma-HRP Polymer	Santa Cruz Biotechnology, Dallas, TX
Ecad[c] (36)	1:50	60 min RT	HIER Diva/Biocare Medical	Mouse-on-Farma-HRP Polymer	BD Transduction, Franklin Lakes, NJ
Ki-67 (7BII)	1:200	60 minutes	HIER citrate, buffer pH 6.0	Mouse-on-Farma-HRP Polymer	Zymed, Carlsbad, CA

[a]CFTR = cystic fibrosis transmembrane conductance regulator
[b] Epithelial sodium channel
[c] E-cadherin
[d]HIER: heat-induced epitope retrieval: Diva/Borg: Antigen retrieval solutions
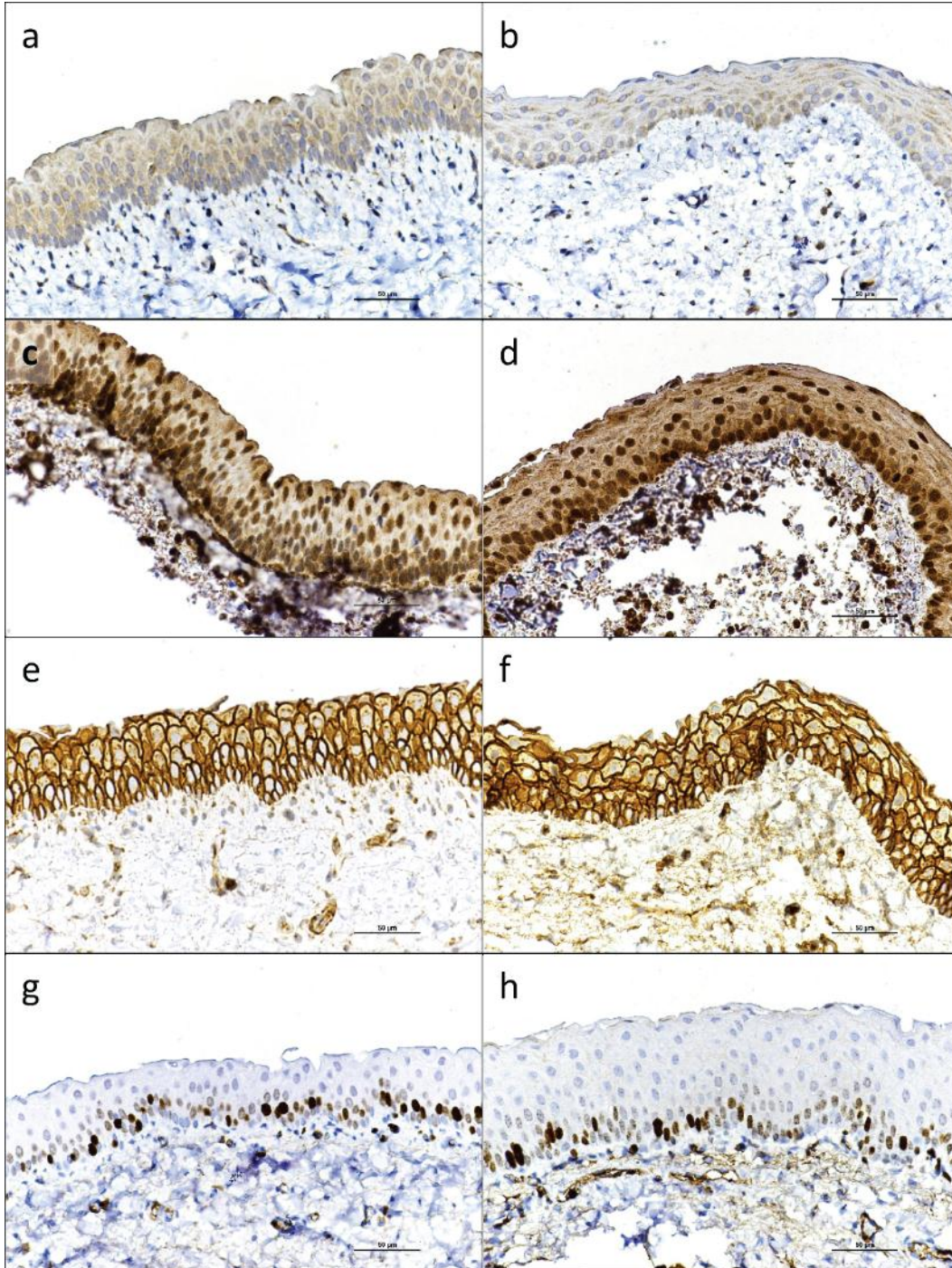
Figure 4.3**:** Photomicrograph of anti-CFTR (a,b), anti- γENaC (c,d), anti-Ecad (e,f), and anti-Ki-67 (g,h) immunohistochemical labeling true vocal fold epithelium. Significant differences between sham and reflux tissues was not identified.
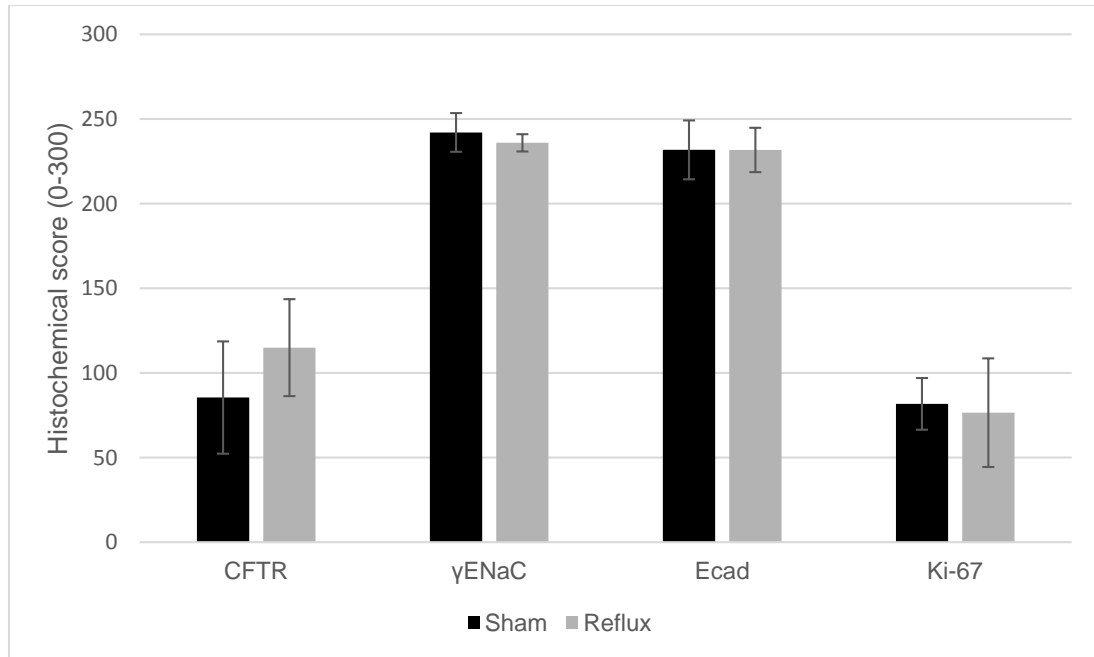
Figure 4.4**:** Means and standard deviations of histochemical scores in sham and reflux vocal folds.

Table 4.2**:** Summary of semi-quantitative immunohistochemistry histochemical scores (H score)

	Anti-CFTR[a]	Anti-γENaC[b]	Anti-Ecad[c]	Anti-Ki-67
Sham	Mean = 85.48	Mean = 242.03	Mean = 231.75	Mean = 81.70
Reflux	Mean = 114.91	Mean = 235.90	Mean = 231.67	Mean = 76.55
Wilcoxon rank sum test	z = -1.441, ns	z = 0.961, ns	z = 0.320, ns	z = 0.961, ns

p-value < 0.05, ns = not significant
[a]CFTR = cystic fibrosis transmembrane conductance regulator
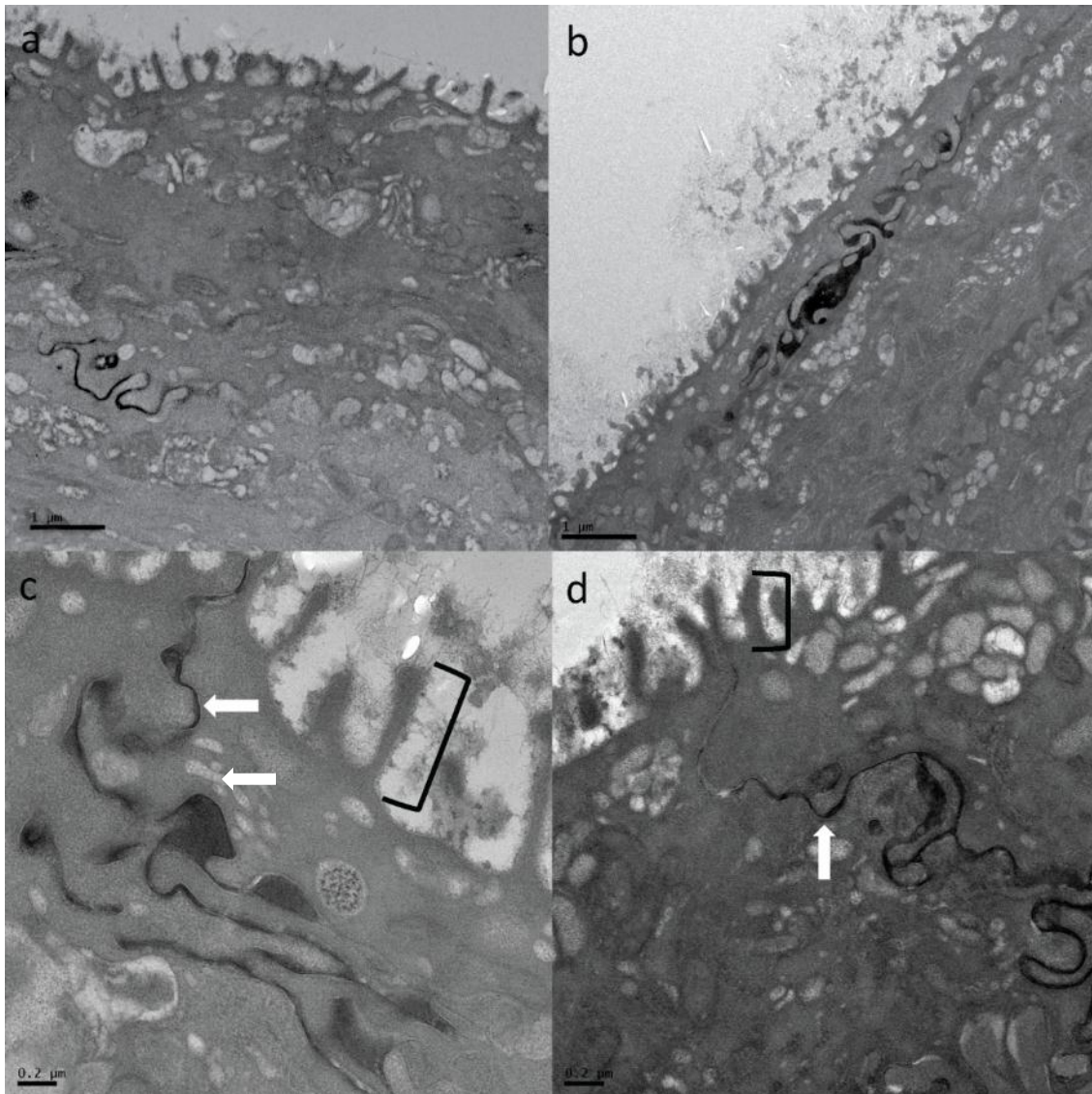[b] Epithelial sodium channel
[c] E-cadherin

Figure 4.5: Transmission electron photomicrograph of (a,c) sham and (b,d) reflux vocal fold epithelium. Photomicrographs (a) and (b) are 2550X magnification. Photomicrographs (c) and (d) are 7000X magnification and highlight the microridges (black brackets) and intercellular space (white arrows). The intercellular space is outlined by lanthanum nitrate, an electron-dense extracellular component of the fixative.
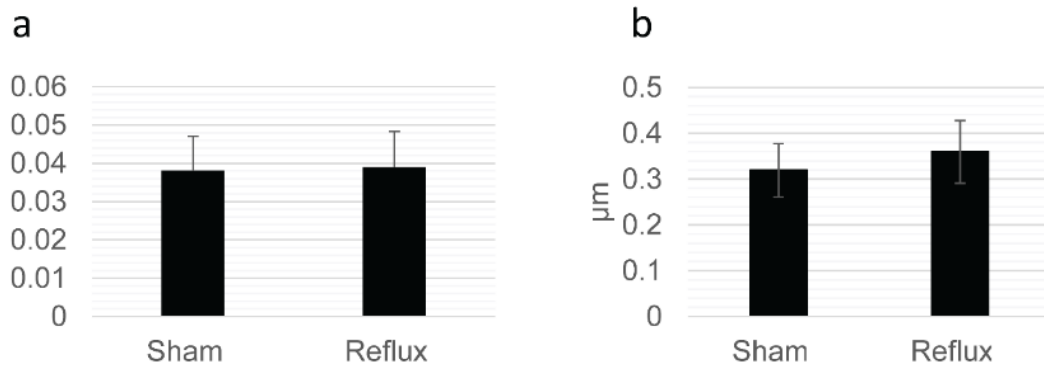
Figure 4.6: (a) Average true vocal fold epithelial intercellular space distance (ISD). (P> 0.05) (b) Average true vocal fold epithelial microridge height (MH). (P>0.05). Error bars represent standard deviation of mean.
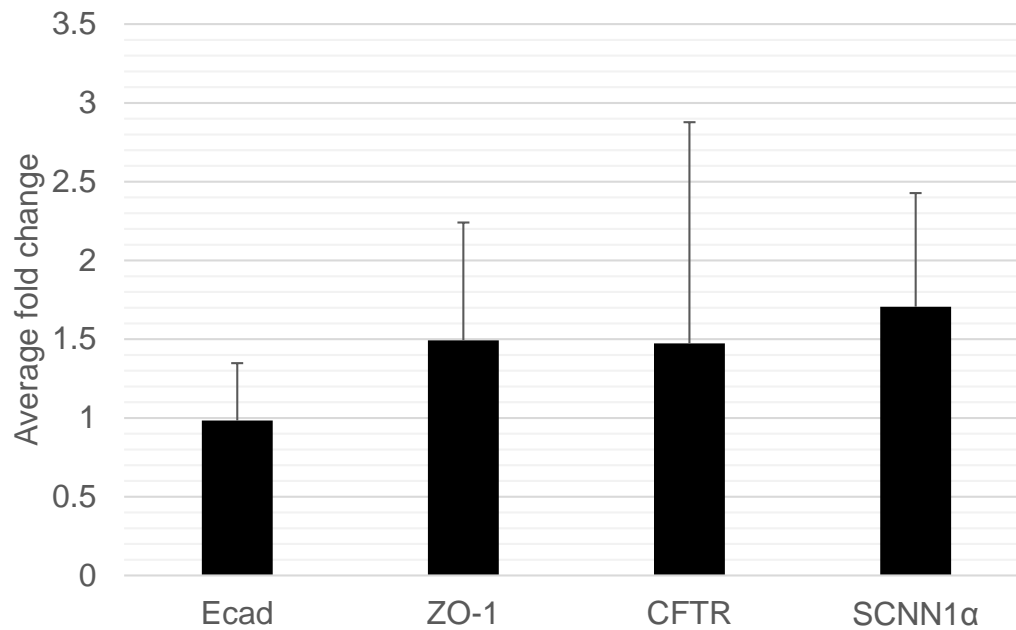
Figure 4.7**:** Mean fold change and standard deviations for gene transcripts in reflux compared to sham vocal folds. Data analyzed using delta CT method. E-cadherin (Ecad), zona-occludens-1 (ZO-1), cystic fibrosis transmembrane conductance regulator (CFTR), and epithelial sodium channel (SCNN1α). (P>0.05)

CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

In conclusion, the studies reported in this dissertation have helped to clarify the role of acidified pepsin on vocal fold tissue. This dissertation contains two major threads of work, one *ex* vivo and one primarily *in* vivo. The *ex vivo* model was utilized to investigate the effects of bicarbonate availability as a possible mechanism of vocal fold epithelial defense against an acidic challenge. We have previously reported that exposing excised porcine vocal fold epithelium to an acidic environment increases ion transport across the epithelium. No specific pharmacological antagonists have been identified against bicarbonate, so we relied on two indirect methods of investigating the role of bicarbonate in vocal fold epithelial ion transport. First, we attempted to block the production of bicarbonate by inhibiting carbonic anhydrase with acetazolamide. Acetazolamide had no effect on the ion transport increase across the epithelium when exposed to low pH. Second, we investigated whether the distribution of bicarbonate in the tissue environment might affect the extent by which the tissue is capable of buffering the acidic insult. We found that low concentrations of bicarbonate in the luminal and basolateral environment of the vocal fold epithelium maximized the increase in ion transport across the epithelium. This finding approached significance and suggests that vocal fold epithelial bicarbonate ion transport is a

possible mechanism of defense against acidic insults in the larynx. Future research will capitalize on this finding and determine if prophylactic treatment with bicarbonate can alleviate some of the clinical symptoms and pathology posed by laryngeal perturbations in pH.

The second major theme of this dissertation was to develop an animal model to simulate human laryngopharyngeal reflux disease. We challenged healthy pig vocal fold tissue with acidified pepsin at a physiologically-relevant pH via two different administration routes, direct liquid application or inhaled aerosolization. Our findings are the first to investigate what is believed to be the leading culprit of LPR in human beings, acidified pepsin, in an animal model that more specifically simulates human anatomy and physiology. Additionally, this model recapitulates realistic scenarios of intact and uninjured vocal fold tissue exposed to moderate acidity (pH = 4). For both exposure routes, clinical data, histology, immunohistochemistry, ultrastructure, and expression of select genes were evaluated. We determined from this data that 12 direct liquid reflux challenges were insufficient to prompt clinical LPR disease, nor microscopic or molecular changes in the healthy vocal fold epithelium. We found that 60 aerosolized reflux challenges were also unable to provoke vocal fold epithelial changes. The aerosolization of acidified pepsin experimental technique is novel and highly translatable to other inhaled toxicants. The limitations of repeated exposures in unanesthetized animals are mostly overcome by acclimating these highly motivated animals to the restraint sling and nose cone. A similar technique could be utilized to test cigarette smoke, environmental pollutants, or even

innovative therapeutic interventions to diseases. The success of the restraint in combination with aerosolized challenge can also be extended to include long-term studies that have previously been inhibited by extensive chemical restraint and potentially unfavorable welfare of the animal.

Our findings suggest that LPR is a chronic, multifactorial disease with yet identified aspects. Future research will explore a combination of agents such as environmental pollutants, upper respiratory infection/inflammation, or diet manipulation that might prime the tissue to respond to the refluxate in a way similar to that of human LPR. Forthcoming inquiries will build upon the foundations set by this dissertation and give rise to further understanding of this multi-faceted disease.

VITA

VITA

<u>Abigail C. Durkes</u>

Current position: Assistant Professor of Veterinary Pathology at Purdue College of Veterinary Medicine

Contact information:
Department of Comparative Pathobiology
Purdue University College of Veterinary Medicine
725 Harrison Street
VPTH 125
West Lafayette, IN 47907
Ph: (765) 494-6063
Email: adcox@purdue.edu

Education:

Degree	Year	Institution	Major Field of Study
M.S.	2011	Purdue University	Comparative Pathobiology
DVM	2008	Purdue University	Veterinary Medicine
M.S.	2004	Purdue University	Animal Sciences
B.S.	2002	Washington University in St. Louis	Biology