Animal Models of Laryngopharyngeal Reflux Disease: A Systematic Review of Mucosal Changes and Voice Disorders☆

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Summary: Background. Over the past decades, various animal models have been developed to elucidate the molecular pathways and tissue alterations associated with laryngopharyngeal reflux disease (LPRD). This systematic review aimed to summarize the current knowledge gained through experimental animal models in understanding LPRD pathophysiology, especially LPRD-related voice disorders.

Methods. According to the PRISMA statements, two investigators systematically search PubMed, Embase, and Web of Science databases for experimental studies investigating mucosa injuries or modifications related to LPRD refluxate, and their potential mechanistic associations with voice quality impairments.

Results. Of 326 retrieved articles, 44 studies documented reflux-induced laryngeal mucosal changes, including 19 animal model studies (canine, porcine, rat, mice, and rabbit models). The exposure of laryngeal tissue to refluxate (pepsin, bile acids, and trypsin) was associated with microscopic tissue alterations, including epithelium disruption and inflammatory infiltrate, metaplasia, erosion, and ulceration. The mucosa alterations vary according to the anatomical sublocation (vocal folds, posterior commissure, and supraglottic and subglottic regions). Several cytokines have been identified as mucosal injury mediators. The microscopic modifications of laryngeal mucosa may cause damage to tissue and changes in cell function. The experimental models were limited to supine-positioned animals exposed to liquid refluxate. No studies addressed the potential effects of elastase, bile salts, trypsin, and lipases in nonacidic (weakly acidic or alkaline) gaseous environment.

Conclusion. Experimental animal models of LPRD supported vocal fold and laryngeal mucosal changes in response to acidic reflux exposure. However, the translational value of these findings is limited by distinct differences in reflux pathophysiology between human subjects and animal models.

Key Words: Laryngeal—Mucosa—Epithelium—Animal—Modification—Injury—Voice—Gastroesophageal—Laryngopharyngeal—Reflux.

INTRODUCTION

Laryngopharyngeal reflux disease (LPRD) is a disease of the upper aerodigestive tract resulting from the direct and/or indirect effects of gastroduodenal content reflux, inducing morphological and/or neurological changes in the upper aerodigestive tract.[1] LPRD is associated with non-specific laryngopharyngeal symptoms, including globus sensation, throat clearing, mucus, or chronic cough.[2,3] The causal relationship between LPRD and dysphonia remains controversial,[4–6] as objective documentation of macroscopic reflux-induced vocal fold lesions is still difficult in clinical practice. It has long been suggested that refluxate content can lead to significant macroscopic and microscopic histopathologic changes in the mucosa of the vibratory margin of the vocal folds, including epithelial cell dehiscence, microtraumas, inflammatory infiltrates, mucosal drying, and epithelial thickening.[7] Due to the difficulties in performing vocal fold biopsies in humans, most histopathological research has been conducted in animal models.[7] In the past few years, the number of studies evaluating subjective and objective voice quality in LPRD patients has substantially grown, with the identification of nonpepsin gastroduodenal enzymes in the upper aerodigestive tract of LPRD patients.[8–10] Over the same period, there has been an increase in studies examining experimental animal LPRD models and laryngeal histological analyses, improving the understanding of pathophysiological mechanisms underlying this disorder.

This systematic review aimed to summarize the current knowledge gained through experimental animal models in understanding LPRD pathophysiology, especially LPRD-related voice disorders.

MATERIALS AND METHODS

This study was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist.[11] The criteria for considering studies were based on population, intervention, comparison, outcome, timing, and setting framework that was used.[12] The Institutional Review Board approval was not required.

Studies

The literature search period was set from January 2000 to January 2025, and included studies published in English-

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language peer-reviewed journals. Eligible studies included prospective and retrospective cohorts, or cross-sectional analyses investigating histological or physiological findings of laryngeal mucosa exposed to LPRD contents in animal models. Clinical studies and nonexperimental studies, such as case reports, letter, and comments, were excluded.

Participants and inclusion criteria

The criteria used for LPRD diagnosis were extracted as well as the method to develop an animal reflux model. There was no restriction regarding the animal model. Mice, porcine, canine, rat, and rabbit models were all considered.

Outcomes

Two independent investigators (G.C. and J.R.L.) extracted data from in vitro animal-derived cell line experiments, and in vivo animal model studies. The association between LPRD and mucosal alterations was systematically evaluated through multiple pathophysiological parameters. Analysis encompassed refluxate-mediated injury mechanisms, including pH variations and gastroduodenal enzymatic activity (specifically pepsin, bile salts, elastase, trypsin, and lipases). The comprehensive assessment of tissue response incorporated cellular morphology and ultrastructural modifications, inflammatory infiltrate characterization, pro-inflammatory cytokine expression patterns, alterations in extra-, intra-, and transmembrane protein expression, epithelial integrity disruption, intercellular junction alterations, and subsequent histoarchitectural reorganization.

Intervention and comparison

No specific intervention criteria were established a priori. For studies investigating treatment-related mucosal modifications in animal models (including acid suppression and enzymatic inhibition protocols), therapeutic interventions were systematically documented. Similarly, all experimental intervention data in cell-based studies were collected with particular attention to methodological parameters.

Time and setting

There were no strict criteria for time and setting in all of those studies.

Search strategy

The two investigators independently conducted the PubMed, Embase, and Web of Science databases for relevant peer-reviewed publications related to laryngeal tissue modification in LPRD. The following keywords were used for the search strategy: Larynx; Laryngeal; Reflux; Laryngopharyngeal; Gastroesophageal; Tissue; Animal; Vocal Cord; and Outcomes. The studies reporting database abstracts, available full texts, or titles with the search terms were considered. The research findings have been reviewed for relevance and the reference lists of some articles (eg, reviews) were examined for additional pertinent studies. The included studies were analyzed for the number of patients, study design, inclusion and exclusion criteria, quality of trial/evidence-based level, demographics, and outcomes.

RESULTS

A total of 1393 articles were retrieved, including 570 from PubMed, 398 from Web of Science, and 425 from Embase (Figure 1). After removing 575 duplicates, 818 articles remained for screening. Initial assessment of titles and abstracts resulted in the exclusion of 429 articles that did not meet the inclusion criteria (case reports, narrative reviews, clinical studies without relevant outcomes, and publications outside the scope of this review). Full-text evaluation of the remaining 389 articles identified 44 eligible studies investigating the association between LPRD and vocal fold tissue impairments. Of them, 19 were experimental animal studies (Table 1).[13–31] The selected studies examined three primary domains: histopathological alterations, cellular functional changes, and molecular expression patterns in canine,[15,16] porcine,[13,17,19–21,23,28] mice,[14,27,29,30] rat,[18,24,31] and rabbit[22,25,26] models (Table 2). The pathological findings were controlled with normal tissue in most studies.[13–16,18,20,22–31] The histopathological or physiological impacts of some treatments have been evaluated in four studies.[14,24,27,30]

Histopathological findings

Histopathological findings have been reported in 17 studies,[13–16,18–20,22–31] using light microscopy, immunohistochemistry, and spectrophotometric analysis (optical density measurements).

The inflammatory cell infiltrate was found in the vocal fold mucosa of most studies and was related to pepsin,[13–16,19,22,24,25] bile acids,[15] and trypsin[15] exposures or unspecified gastric content (Table 2).[29] The reflux content was associated with intercellular space dilatation in four studies, which supported the occurrence of vocal fold mucosa microtraumas.[13,22,26,28] From an anatomical standpoint, Bulmer et al assessed pepsin-induced damage across different laryngeal regions (subglottic, supraglottic, vocal folds, and posterior commissure) at varying pH levels (2.0, 4.0, and 7.4 control). Tissue injury was quantified through spectrophotometric measurements (optical density at 260 and 280 nm) and DNA release analysis.[19] Their findings revealed pH-dependent tissue sensitivity to pepsin, with vocal fold tissue showing vulnerability to pepsin exposure at pH 2.0.[19] Note that there were no significant histological changes between reflux and control animals in only one study.[23]

While most studies focused on pepsin, Lou et al demonstrated in a porcine model that bile salt exposure induced more severe laryngeal mucosal alterations than pepsin alone, characterized by enhanced epithelial desquamation, increased epithelial thickness, and wider intercellular space dilatation at pH 3.0-5.0.[28] However, Ao et al found no significant difference in laryngeal tissue injury between

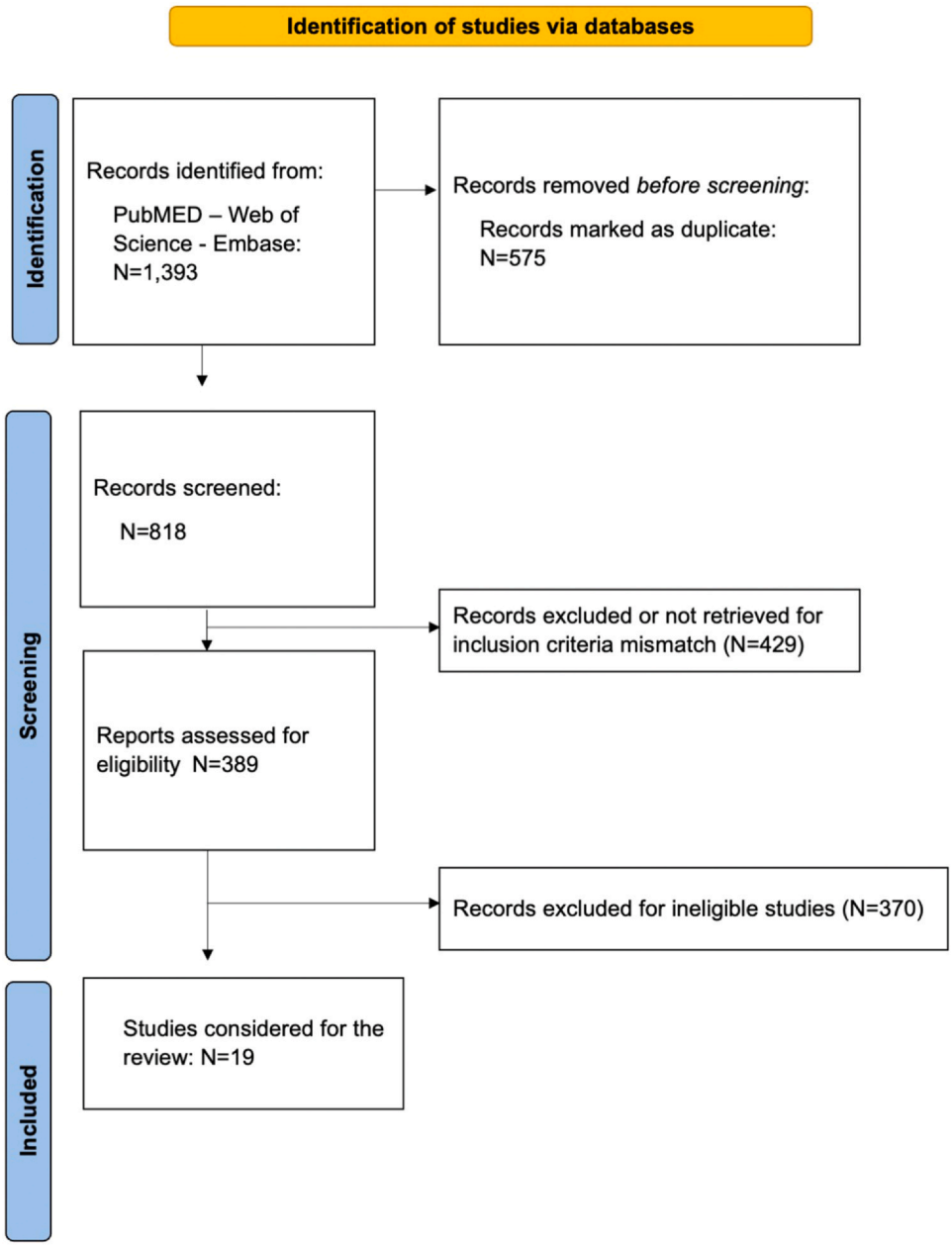

FIGURE 1. *Chart flow.* Two investigators conducted the literature search.

pepsin alone and pepsin combined with bile salts at pH 2.0.[29]

Functional cellular changes

The permeability of vocal fold mucosa was investigated in five studies with transepithelial electrical resistance (TEER) or the Franz diffusing cell system.[14,20,27,28,31] TEER is a quantitative measure of epithelial barrier integrity, which reflects the tissue's ability to restrict ion flow between cells. TEER was significantly decreased when the laryngeal and vocal fold mucosa were exposed to pepsin,[14,20,27,31] or acidity only (reversible phenomenon).[20] Similarly, Lou et al measured vocal fold barrier permeability through the Franz diffusing cell (in vitro system) and reported that gastric contents were associated with significant membrane permeability impairment, with greater impairment observed for combined pepsin and bile salts than pepsin alone.[28] The Ussing chamber technique associated with fluorescein permeability assessment is an in vitro method that quantifies epithelial barrier integrity and transport function by measuring electrical parameters (transepithelial resistance) and molecular permeability (using fluorescein tracer) across mounted tissue specimens under controlled physiological conditions. This approach was used by Figueiredo et al to demonstrate significant impairment in epithelium permeability when tissues were exposed to gastric content.[27]

TABLE 1.
Studies

References	Design	Sample/Animal Characteristics	Analysis	Outcomes	Results
Adhami, 2004 [15]	Prosp.	*Canine laryngeal samples*	Microscopy	Intraepithelial inflammation	Gr1 > Gr2
	Contr.	Gr1: 42 LPRD exposed to bile salts ± pepsin ± trypsin (3t/w) (pH 1-2, 4-5, 6-7)		Squamous metaplasia and erosion	Gr1 > Gr2
				Ulcers, stromal, periglandular	Gr1 > Gr2
		Gr2: 4 CT exposed to saline solution		inflammation, and fibrosis	Gr1 > Gr2
Cohen, 2004 [16]	Prosp.	*Canine laryngeal samples*	Microscopy	Cellular infiltrate	Gr1 = Gr2 > Gr3
	Contr.	Gr1: 3 injured vocal folds exposed to pepsin (pH 2 or 6)		Fibronectin abundance	Gr1 = Gr2 > Gr3
				Procollagen I abundance	Gr1 = Gr2 > Gr3
		Gr2: 3 injured vocal folds exposed to saline sol			
		Gr3: 2 normal vocal folds			
Johnston, 2007 [17]	Prosp.	*Porcine laryngeal cells*	Western	Depletion of CAIII and Sep-70	
	Unc.	Incubated (pH 1.5-6.5+pepsin) during 20 min	Blotting	pH 1.5, 4.5, 5.0, and 6.0	++
				pH 2.0, 2.5	+++
				pH 3.0, 3.5, and 4.0	++
				pH 6.5	-
Shimazu, 2009 [18]	Prosp.	*Rat laryngeal samples (posterior commissure)*	Microscopy	*Laryngeal tissues changes*	
	Contr.	Gr1: 26 GERD rats		Mucosa thickening	16 we > baseline
		Gr2: CT (*N* = NA)		Capillary proliferation and dilatation	16 we > baseline
Bulmer, 2010 [19]	Prosp.	*Porcine laryngeal samples*	Spectro-photometric analysis	*Tissue damages pH 2*	pepsin-/pepsin+
	Unc.	Tissue samples incubated with pepsin at pH 2, 4 *(1-h incubation)*		*Ventricles, vocal folds*	-/+
				Posterior commissure	-/-
				Supraglottic and subglottic	+/+
				Tissue damages pH 4	
				Ventricles, supraglottic	-/+
				Vocal folds, posterior commissure	-/-
				Subglottic	+/+
				Tissue damages pH 7.4	
				All samples	-
Erickson, 2010 [20]	Prosp.	*Porcine laryngeal samples* (*N* = 52)	Voltage clamp	Transepithelial resistance of vocal folds	t0 > t1 > t2 (Gr1)
	Contr.	Exposed to		with acidic pepsin	t0 = t1 = t2 (Gr2)
		Gr1: pH 3 ± porcine pepsin		Transepithelial resistance of vocal folds	t2: Gr1 > Gr2
		Gr2: pH 7 ± porcine pepsin		with acid-only (*reversibility)	Gr1 and 2: t0 > t2*
		t0 = 0, t1 = 15 minutes, t2 = 30 minutes	Microscopy	*Histological changes (t2)*	
				Epithelial shedding, basal,	Gr1 = Gr2
				and intracellular and extracellular edema	Gr1 = Gr2
				Vacuolization	Gr1 = Gr2
Erickson, 2012 [21]	Prosp.	*Porcine laryngeal samples* (*N* = 57)	Electro-physiological	Ions transport (bicarbonate)	t1 > t0
	Unc.	Exposed to pH 3 ± porcine pepsin		*t0 = 0, t1 = 60 minutes*	
Hu, 2013 [22]	Prosp.	*Rabbit vocal fold samples*	Microscopy	Vocal cord intercellular space	Gr1 > Gr2
	Contr.	Gr1: 4 rabbit refluxes		Lymphocytes infiltration	Gr1 > Gr2
		Gr2: 4 CT		*12-week reflux episodes*	
Durkes, 2015 [23]	Prosp.	*Porcine laryngeal samples*	Microscopy	*Vocal folds morphology*	
	Contr.	Incubated (acid + pepsin)		Collagen, elastin structures, epithelial	Gr1 = Gr2

TABLE 1 (*Continued*)

References	Design	Sample/Animal Characteristics	Analysis	Outcomes	Results
		(3 per week (4 w))		cellularity, and lamina propria infiltrate	Gr1 = Gr2
		Gr1 = reflux pigs (*N* = 4)		*Ultrastructural alterations*	
		Gr2 = CT pigs (*N* = 4)		Intercellular space dilatation, microridge height	Gr1 = Gr2
			RT-PCR	*Gene expression*	
				E-cadherin, CFTR, and Zona occludens-1	Gr1 = Gr2
				Epithelial Na+ channel, IL-1β, TNFα, and Ifγ	Gr1 = Gr2
Oyan, 2018 [24]	Prosp.	*Rat laryngeal samples*	Microscopy	Lamina propria edema-vascular dilation	Gr1 = Gr2 > Gr3
	Contr.	Reflux: 30 days before irradiation (20 Gy)		Lamina propria inflammation	Gr1 = Gr2; Gr2 > Gr3
		Laryngeal tissue samples 4 days post radiation		Epithelial inflammation	Gr1 > Gr2 > Gr3
		Gr1 = 8 irradiation		Respiratory epithelium inflammation	Gr2 > Gr1 = Gr3
		Gr2 = 10 reflux + radiation		Loss of cilia	Gr2 = Gr1 > Gr3
		Gr3 = 6 CT		Pseudostratification	Gr2 > Gr3
Lou, 2018 [25]	Prosp.	*Rabbit laryngeal samples*	Microscopy	Laryngeal inflammation (Gr1,2)	4 weeks > baseline
	Contr.	Nasogastric aspiration tube exposure (size #8)		Submucous gland hyperplasia	4 weeks > baseline
		Gr1 = 16 normal nasogastric intubation	qRT-PCR	IL-1β, IL-6,TNF-α, and COX-2	4 weeks > baseline
		Gr2 = 16 modified nasogastric intubation		IL-10	4 weeks > baseline
		Gr3 = 6 CT			
Cao, 2020 [26]	Prosp.	*Rabbit laryngeal samples*	Microscopy	Intercellular space dilatation	Gr1 > Gr2
	Contr.	Vocal cord samples	Histopathology	Congestion, edema, and animal's roughness	Gr1 > Gr2
		Gr1 = 9 LPRD-Gr2 = 9 CT	IHC	Lymphocyte infiltration, IL-8, and VEGF	Gr1 > Gr2
Figueiredo, 2020 [27]	Prosp.	*Mice laryngeal samples*	Wet weight	Mucosal edema, myeloperoxidase	3d: Gr1 > Gr5
	Contr.	Gr1 = GERD	analysis activity (myeloperoxidase)	(3 and 7 days post treatment)	7d: Gr1 = Gr5
		Gr2 = GERD + alginate			Gr1 > Gr2,3,4
		Gr3 = GERD + hyaluronic acid	Ussing chamber technique	Permeability to fluorescein	3d: Gr1 > Gr5
		Gr4 = GERD + cashew gum			7d: Gr1 = Gr5
		Gr5 = CT			Gr1 > Gr2,4
			Voltage deflections	Transepithelial resistance	3d: Gr5 > Gr1
					7d: Gr1 = Gr5
					Gr2 > Gr1; Gr4 > Gr1
Lou, 2021 [28]	Prosp.	*Porcine laryngeal samples*—exposed to	Franz diffusing cell	Fluorescence permeability	Gr1 > Gr2; Gr3 > Gr4 > Gr5
	Contr.	Gr1 = alkaline deoxycholic acid	Microscopy	Epithelial fragmentation, desquamation,	Gr1 > Gr2; Gr3 > Gr4 > Gr5
		Gr2 = acidic pepsin		thickness, and intercellular space dilatation	Gr1 > Gr2; Gr3 > Gr4 > Gr5
		Gr3 = HCL (pH = 3)-Gr4 = HCL (pH = 5)			
		Gr5 = CT			

TABLE 1 (*Continued*)

References	Design	Sample/Animal Characteristics	Analysis	Outcomes	Results
Ao, 2022	Prosp.	*Mice laryngeal samples*	IHC	Epithelial hyperplasia	Gr1 > Gr3; Gr1 > Gr2
[29]	Contr.	Exposed to artificial gastric juice (AGJ)		Glut-1, H+/K+-ATPase α,β	Positive relation
		Gr1: AGJ + pepsin (pH = 2)		2-DG inhibits H+/K+-ATPase α,β	S
		Gr2: AGJ + pepsin + 2 - DG (pH = 2)	RT-PCR and WB	Glut-1, H+/K+-ATPase α,β	Gr1 > Gr3; Gr1 > Gr2
		Gr3: Saline solution (pH = 7)		*15-, 30-, and 45-day evaluations*	
Johnston, 2023	Prosp.	*Mice laryngeal samples*	Microscopy	Reactive epithelium	Gr5: pH 4 > pH 7
[30]	Contr.	Exposition to pepsin and the following drugs:		Intraepithelial inflammatory cells	Gr5 > Gr1
		Gr1 = fosamprenavir-Gr2 = ritonavir		Cell apoptosis	Gr5 > Gr1
		Gr3 = saquinavir-Gr4 = darunavir-Gr5 = CT		Cell apoptosis	Gr4aerosol < Gr5
Kojima, 2024	Prosp.	*In vitro rat laryngeal cells* (*N* = 8)	Electron microscopy	Microprojections	Gr2.1 = Gr2.2 > Gr2.3
[31]	Contr.	Vocal fold epithelium cells			Gr3.1 = Gr3.2 > Gr3.3
		Gr1 = pH = 6.8-Gr2 = pH 4			Gr2 = Gr1 > Gr3
		Gr3 = pH4 + 2 mg/L pepsin	Subgroup	Cells' viability and density	Gr3 = Gr1
		Subgroup.1: before		Cells' viability	Gr3.1 = Gr3.3 = Gr3.2
		Subgroup.2: 0 minute		Cells' density	Gr3.1 = Gr3.3 = Gr3.2
		Subgroup.3: one day after	Transepithelial electrical resistance	Unit area resistance	Gr1 > Gr2 > Gr3 Gr2.3 = Gr2.1- Gr3.3 = Gr3.1
Sales, 2024	Prosp.	*Mice laryngeal samples*	wet weight analysis	Edema and myeloperoxidase activity	Gr1 > Gr4; Gr1 > Gr2,Gr3
[14]	Contr.	Gr1: 6 GERD	Ussing chamber technique	Permeability to fluorescein	Gr1 >;Gr4
		Gr2: 6 GERD + pepstatin			Gr2 = Gr3 = Gr4
		Gr3: 6 GERD + darunavir	Transepithelial electrical resistance	TER	Gr4 > Gr1
		Gr4: 6 CT			Gr2 = Gr3 = Gr4
Shi, 2024	Prosp.	*Porcine laryngeal samples*	Manometry	UES pressure decrease (8 weeks)	Gr1 = S, Gr2 = NS
[13]	Contr.	Gr1 = 4 LPRD	Dx-pH monitoring	Reflux events (pH < 5, 2 weeks)	Gr1 = S, Gr2 = NS
		Gr2 = 4 CT	TEM (8w)	Intercellular gap	Gr1 > Gr2
			Histopathology (8w)	Submucosal gland cell infiltrate	Gr1 > Gr2

Abbreviations: Contr., controlled; CT,controls; DG, 2-deoxy-D -glucose; GERD, gastroesophageal reflux disease; Gr, group(s); IHC, immunohistochemistry; LPRD, laryngopharyngeal reflux disease; *N*, number; NA, not available; Pros., prospective; RT-PCR, reverse transcription polymerase chain reaction; TER, transepithelial resistance; t/w, time per week; Unc, uncontrolled.

TABLE 2.
Histological, Physiological, and Molecular Outcomes

Outcomes	*N*	Primary Findings	References
Histopathological findings			
Epithelial inflammatory cell infiltrate	8	Associated with pepsin exposure	13–16,22,24–26
	2	Associated with bile acids and/or trypsin	15,28
Squamous metaplasia/mucosa thickening	5	Associated with acidic pepsin exposure	15,18,24,27,28
	1	Associated with bile acids, trypsin exposure	15
	1	Associated with unspecified gastric juice content	29
Tissue capillary proliferation and dilatation	2	Associated with pepsin exposure	18,24
Ulcerations	1	Associated with pepsin, bile salts, and trypsin	15
Fibrosis tissue	1	Pepsin exposure increases fibronectin and procollagen I	16
Intercellular space dilatation	4	Associated with refluxate liquid	13,22,26,28
Submucous gland modifications (eg, hyperplasia)	2	Associated with refluxate liquid	15,25
Epithelial desquamation	1	Associated with pepsin exposure	28
Unspecific laryngeal tissue damages	1	Associated with pH 2 and pH 4	19
No histopathological differences between LPRD and CT	1		23
Functional Mucosa Findings			
Mucosa Protective Mechanisms			
Carbonic anhydrase type III depletion	1	Associated with pepsin exposure (pH 1.5-4.0)	17
Sep-70 depletion	1	Associated with pepsin exposure (pH 1.5-4.0)	17
Permeability of epithelium			
Transepithelial electrical resistance	5	Decreases with pepsin exposure	14,20,27,28,31
Transepithelial ion transport	1	Increase with pepsin exposure	21
Transepithelial permeability (chamber technique)	1	Increase with gastric content exposure	27
Molecular Findings			
IL-1β, TNFα tissue expression	2	Increase when long time exposed to gastric content	23,25
IL-6, IL-10, and COX-2 expression	1	Increase when long time exposed to gastric content	25
Glut-1, H+/K+-ATPase α,β	1	Increase when exposed to pepsin	29
IL-8 and VEGF increase	1	Increase with gastric content exposure	26
E-cadherin, CFTR, and Zona occludens-1	1	No difference between GERD and healthy models	23
Epithelial Na+ channel, Ifγ	1	No difference between GERD and healthy models	23

Abbreviations: CR, controls; GERD, gastroesophageal reflux disease; LPRD, laryngopharyngeal reflux disease.

Erickson and Sivasankar[21] indirectly investigated the epithelium permeability through electrophysiological techniques examining ion transport (bicarbonate) in porcine laryngeal samples. In this study, the authors demonstrated a significant reduction in bicarbonate transport 60 minutes after pepsin exposure of vocal folds at pH 3.0.[21] Johnston et al demonstrated that pepsin exposure of porcine laryngeal mucosa significantly depleted two protective proteins: carbonic anhydrase III and SEP-70. This depletion reached maximal levels under strongly acidic conditions (pH 2.0-2.5).[17]

Molecular patterns

Multiple pro-inflammatory cytokines and chemokines have been identified in the laryngeal mucosal inflammatory cascade following exposure to pepsin or gastric contents (Table 2). IL-1β, IL-6, IL-8, IL-10, and TNFα are the primary pro-inflammatory cytokines identified as mediators in the reflux animal models (Table 2).[23,25,26,29] Durkes et al reported no significant differences in gene expression profiles between GERD-affected pigs and control pigs for proteins associated with mucosal barrier defense, epithelial integrity, and structural maintenance (Tables 1 and 2).

Tissue modification in therapeutics

Oyan et al investigated the interaction between reflux disease and radiotherapy in a rat model ($n = 18$), comparing radiation exposure alone versus radiation combined with reflux. Their findings demonstrated synergistic effects of reflux and radiation resulting in enhanced mucosal tissue damage characterized by increased epithelial inflammation and vascular dilatation.[24] Johnston et al explored the benefit of topical fosamprenavir, a pepsin antibody, in laryngeal tissues of mice exposed to pepsin. After the use of fosamprenavir 5 days/week for 4 weeks, the mice demonstrated significantly lower intraepithelial inflammatory cells, and apoptosis compared with controls.[30] Similarly, Sales et al reported a significant improvement in laryngeal epithelial permeability when using pepstatin or darunavir in GERD mice.[14] From a physiological standpoint, the use

TABLE 3.
Animal Model Reflux Features

References	Models	Method for Creating Reflux	Fluid Used	Time/Duration of Incubation	pH Tested
Cohen, 2004[16]	Canine	Surgical vocal fold injury + topical application	Acid + pepsin	12 days (every other day)	pH 2 or pH 6
Adhami, 2004[15]	Canine	Direct application after biopsy injury	Pepsin, bile acids, and trypsin (alone and combined)	9-12 applications over 4 weeks	pH 1-2, 4-5, 6-7
Johnston, 2007[17]	Porcine	Ex vivo organ culture	Pepsin	20 minutes-3 hours	pH 2-7
Shimazu, 2009[18]	Rat	Surgical (pyloric ring placement + fundus-corpus ligation)	Unspecific gastric content	8-20 weeks	Not specified
Bulmer, 2010[19]	Porcine	Ex vivo tissue exposure	Acid alone and acid + pepsin (1 mg/mL)	1 hour	pH 2 and pH 4
Erickson, 2010[20]	Porcine	Ex vivo tissue exposure	Acid alone and acid + pepsin (1 mg/mL)	30 minutes	pH 3 and pH 7
Erickson, 2012[21]	Porcine	Ex vivo tissue exposure	Acid and pepsin	60 seconds	pH 3
Hu, 2013[22]	Rabbit	Total cardiomyotomy	Unspecific gastric content	Not specified	Not specified
Durkes, 2015[23]	Porcine	Direct application to vocal folds	Acid + pepsin (pH 4)	3 exposures per week for 4 weeks	pH 4
Lou, 2018[25]	Rabbit	Nasogastric tube placement	Unspecific gastric content	4 weeks	pH < 4.0
Oyan, 2018[24]	Rat	Direct exposure after surgery	Gastric content	Not specified	Not specified
Cao, 2020[26]	Rabbit	Balloon dilation of lower and upper esophageal sphincters	Unspecific gastric content	8 weeks	Not specified
Figueiredo, 2020[27]	Mouse	Surgical (pyloric ring + fundus ligation)	Unspecific gastric content	3 or 7 days	Not specified
Lou, 2021[28]	Porcine	Ex vivo tissue exposure	Pepsin-bile acids	1 hour	pH 3, 5, 7
Ao, 2022[29]	Mouse	Direct application	Artificial gastric juice + pepsin	15-45 days	pH 2
Kojima, 2023[31]	Rat	Ex vivo tissue culture	Acidic pepsin	5 minutes	pH 2-4
Johnston, 2023[30]	Mouse	Mechanical injury + pepsin application	Pepsin alone (nonacid) and acid + pepsin	3 days/week for 4 weeks	pH 4 and 7
Sales, 2023[14]	Mouse	Surgical model	Gastric content	Not specified	Not specified
Shi, 2024[13]	Porcine	Cricopharyngeal myotomy	Unspecific gastric content	Not specified	pH < 5.0

of alginate, hyaluronic acid, and cashew gum was associated with significant improvement of epithelium permeability in mice models.[27] Similar results were found in mouse GERD models when the laryngeal tissues were exposed to pepstatin and darunavir.[14]

Animal model features and heterogeneity

Types of animal model, reflux process, the fluid used for investigating reflux impact on mucosa, pH of exposure, and the time or the duration of incubation are described in Table 3. Most studies used pepsin or gastric content fluids. Among nonpepsin digestive enzymes, bile acids have been evaluated in three studies,[15,28,29] and trypsin in one study.[15] There was a substantial heterogeneity between studies for the model of reflux (eg, surgical model, ex vivo tissue exposure, and balloon dilatation), fluid used, the pH of fluids, and time/duration of exposure/incubation (Table 3). Most studies used an acidic or weakly acidic pH ranging from 2 to 6,[13,16,19,21,23,25,29,31] but some authors evaluated the pepsin activity and related tissue lesions at pH > 6.0.[15,17,20,28,30] The pH of refluxate fluid was not specified in six studies.[14,18,22,24,26,27] There was a substantial range of incubation time for fluids into the tissue samples, ranging from 60 seconds to 3 hours, administered either once or several times per day or week.

DISCUSSION

The number of studies using animal models to understand LPRD physiology has substantially increased over the past decade. Although there are significant differences across animal model physiologies, reflux processes, exposure fluids, and characteristics, most studies demonstrated that gastric content or pepsin is associated with the development of inflammatory infiltrate in the vocal fold/laryngeal mucosa and intercellular space dilatation (microtraumas) through reduction of some mucosal defense mechanisms against reflux (carbonic anhydrase III, SEP-70). Mechanistic processes have been reported in one study,[26] where the authors developed a rabbit model of reflux disease with animal hoarseness associated with laryngopharyngeal reflux events, inflammatory infiltrate of the vocal folds, and cytokine expression.

The transposition of animal findings to humans is, however, limited for many reasons. First, in all animal models without ex vivo tissue exposure, the reflux disease consisted of liquid and acidic backflow of gastric content into the laryngopharyngeal regions of four-legged animals. In humans, most gastro-esophago-pharyngeal reflux events occur in upright position and during daytime, and are gaseous and weakly acidic or alkaline.[32,33] It has been observed in hypopharyngeal-esophageal multichannel intraluminal impedance-pH monitoring (HEMII-pH) studies that even a few gaseous events per day can be sufficient to develop significant symptoms and findings.[34,35] The concentration of digestive enzymes in liquid fluid versus gaseous droplets could substantially impact the development of lesions in exposed mucosa.

Second, recent studies have suggested that nonpepsin enzymes, such as elastase, bile salts, and trypsin, can play a substantial role in the development of mucosal lesions in the context of more alkaline salivary pH in LPRD patients compared with asymptomatic individuals.[8,9] The alkaline salivary pH of LPRD patients has been observed in three studies and strengthens the evidence for potential activity of alkaline-activated enzymes (eg, elastase, nonconjugated bile salts, and trypsin).[36] Animal models using acidic pepsin on laryngeal tissue may, therefore, yield different results from those observed in humans.

Third, recent data suggest that mucosa injuries induced by gastroduodenal content could be associated with modifications of the laryngopharyngeal microbiome and the related reduction in the protective/recovery role of certain commensal bacteria.[37,38] While this area is still under investigation, it represents a major limitation in the use of animal models with different reflux patterns and microbiome compositions. In other words, currently, there is no animal model that closely resembles human physiopathology of LPRD (eg, type of reflux events, proportion of gaseous/liquid events, pH of most events, and duration of tissue exposure). Similar limitations are commonly observed in human tissue sample studies,[7] with most authors focusing on acidic pepsin effects on laryngeal mucosa/cells.

Finally, it is important to note that the vast majority of available studies in the literature have investigated reflux impact on laryngopharyngeal mucosa. However, in clinical practice, LPRD can be associated with oral, nasal, and ophthalmic (dry eye) symptoms and findings,[39–42] which remain uninvestigated in most animal models.

The primary limitation of this review is the substantial heterogeneity across studies in terms of animal models, reflux simulation methods, exposure fluids, pH levels, and incubation times, limiting the generalizability of findings. The artificial nature of many experimental models (ex vivo cells/tissues) may not accurately reflect the complex in vivo conditions of human LPRD, where factors such as gaseous reflux events, microbiome interactions, autonomic nerve dysfunction, and tissue recovery mechanisms play crucial roles.

CONCLUSION

Experimental animal models of LPRD supported vocal fold and laryngeal mucosal changes in response to acidic reflux exposure. However, the translational value of these findings is limited by distinct differences in reflux pathophysiology between human subjects and animal models.

Author Contributions

Guangjin Chen: Patients were recruited from the Reflux Consultation of the author of the paper. Contributions: Design, acquisition of data, data analysis and interpretation,

drafting, final approval, and accountability for the work; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. **Jerome R. Lechien**: Patients were recruited from the Reflux Consultation of the author of the paper. Contributions: Design, acquisition of data, data analysis and interpretation, drafting, final approval, and accountability for the work; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of Competing Interest

About the paper entitled: "**Animal Models of Laryngopharyngeal Reflux Disease: A Systematic Review of Mucosal Changes and Voice Disorders.**" (by Chen and Lechien). The authors have no financial interest in the subject under discussion. All authors have read and approved the paper. Would you be so kind to consider the present paper and send us the reviewer's comments.

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