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Laryngeal Mucosa Alterations in Mice Model of Gastroesophageal Reflux: Effects of Topical Protection

Aline A. Figueiredo, MD, PhD; Thiago M. A. L. Sales, MSc; Lucas A. D. Nicolau, PhD; André A. A. Nunes, MD; Humberto B. Costa-Filho, DVM, MSc; Rubens L. R. Moreira, Ms; Renata R. Nascimento, Ms; Maria K. A. Sousa, Ms; Lorena D. Silva, Ms; João P. Carmo-Neto, MSc; Flávio M. N. O. Sidou, MD; Suliana M. Paula, PhD; Jand V. R. Medeiros, PhD; Durcilene A. Silva, PhD; Daniel Sífrim, MD, PhD; Marcellus H. L. P. Souza, MD, PhD ©

Objectives/Hypothesis: The objectives of this study were to evaluate laryngeal inflammation and mucosal integrity in a murine model of reflux disease and to assess the protective effects of topical agents including alginate, hyaluronic acid, and cashew gum.

Study Design: Animal study.

Methods: A surgical murine model of reflux disease was evaluated at 3 or 7 days postsurgery, and laryngeal samples were collected to measure inflammation (wet weight and myeloperoxidase [MPO]) and mucosal integrity (transepithelial resistance [TER] and mucosal permeability to fluorescein). Additional groups of animals were administered one of several topical agents (alginate, hyaluronic acid, or cashew gum) daily, and laryngeal inflammation and mucosal integrity were evaluated at 3 days postsurgery.

Results: At 3 days, and not 7 days postsurgery, we observed increased laryngeal wet weight and MPO, decreased laryngeal TER, and increased laryngeal mucosa permeability. Alginate partially decreased laryngeal inflammation (wet weight and not MPO) and dramatically improved laryngeal mucosal integrity. Conversely, hyaluronic acid eliminated the inflammation; however, it had no effect on laryngeal mucosal integrity impairment. Cashew gum eliminated laryngeal inflammation as well as the impairment in laryngeal mucosal integrity.

Conclusions: This study shows that a surgical model of reflux disease induced laryngeal inflammation and impairment in laryngeal barrier function. These observed alterations were partially attenuated by alginate and hyaluronic acid and completely reversed by cashew gum.

Key Words: Larynx, reflux, basic research.

Level of Evidence: NA

INTRODUCTION

Gastroesophageal reflux is associated with several disorders of the upper aerodigestive tract. When reflux occurs in or beyond the esophageal tube, it is called gastroesophageal reflux disease (GERD), and carries a high economic burden. Clinical manifestations of extraesophageal reflux disease can also include symptoms of laryngopharyngeal reflux (LPR). In patients with LPR, the gastric contents exhibit a retrograde flow into the laryngopharyngeal region, which causes symptoms that include chronic cough and hoarseness. Gastric content is composed of harmful ingredients, such as hydrochloric acid, pepsin, and bile acids, which can lead to an inflammatory response when the refluxate contacts the laryngeal epithelium.

Currently, managing LPR entails a combination of diet and lifestyle changes, followed by pharmacological therapy that includes three classes of drugs: proton pump inhibitors (PPIs), prokinetic agents, and mucosal cytoprotectants. Although PPIs are considered the mainstay of pharmacological therapy, studies show that PPI therapies fail in approximately 50% of patients with LPR. This unpredictability may be due to the failure of established diagnostic criteria, including the inclusion of patients misdiagnosed with LPR, difficulties in dose standardization, the long duration of treatment, and the fact that PPIs are less effective in treating nonacid or mixed refluxes, compared to acid reflux.

Because PPI therapy is known to be ineffective in a large number of patients with LPR, drugs with suggestive topical effects on the esophageal mucosa, such as alginites and hyaluronic acid, are also prescribed by otolaryngologists. Alginites are natural polysaccharides that form a raft on top of the gastric contents to generate a

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mechanical barrier, thereby preventing reflux outside the stomach, and reducing or preventing the contact of gastric contents with the esophageal and laryngeal mucosa.\textsuperscript{16–18} Moreover, an in vitro study has shown that alginate has an inherent ability to effectively inhibit the enzymatic action of pepsin and to protect human esophageal mucosa against acid-induced damage for a prolonged period after application.\textsuperscript{17–19} Another relevant therapeutic agent in the current context is hyaluronic acid, which also has a mucoadhesive effect. However, our literature review found that the effect of hyaluronic acid on the laryngeal mucosa in the context of LPR has not been studied to date.\textsuperscript{20} Additionally, we recently found that a natural Brazilian polymer called cashew gum shows beneficial effects on both the esophageal and the laryngeal mucosa in different experimental settings.\textsuperscript{21,22} An in vitro study challenged the larynxes of healthy mice with an aggressive solution containing pepsin and bile acids in an acidic medium, and found that topically applied cashew gum protected the laryngeal mucosa against this solution.\textsuperscript{21}

Both in vivo and in vitro experimental models of LPR exist, and have used several approaches in various animal species to try to mimic the effect of reflux on the laryngeal mucosa.\textsuperscript{6,23,24} We have also proposed an in vivo experimental model of nonerosive reflux disease (NERD) in mice, which requires a surgical procedure that alters the normal physiologic processes and forces the gastric contents back into the esophageal tube.\textsuperscript{25} However, experimental evidence on the laryngeal effects of this murine model could not be obtained previously. The aim of this study was to investigate the repercussions of experimental NERD on the laryngeal mucosa and to examine the effect of topical agents on NERD using this murine model.

MATERIALS AND METHODS

This study was divided into two experimental arms. The purpose of one arm was to evaluate the effects of GERD-induced surgery on inflammation and integrity in the laryngeal mucosa at different times postsurgery. The purpose of the other arm was to study the efficacy of topical agents in treating laryngeal inflammation and decrease in mucosal integrity induced by GERD surgery.

Animals

Male Swiss mice (30–35 g) were obtained from the Federal University of Ceará. The animals were randomly housed in appropriate cages, at 23 ± 2°C under a 12/12 hours light/dark cycle, with access to food and water. The Animal Use Protocol was approved by the Ethics Committee of Federal University of Ceará (protocol no: 3781301118).

Surgical Procedure

Surgery was performed according to the procedure described by Silva et al.\textsuperscript{25} Mice were deprived of food for 18 hours preoperatively and were subsequently anaesthetized with ketamine (100 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally), followed by an abdominal laparotomy. A siliconized nonotic ring (diameter: 3.25 mm, width: 2.5 mm; Embramed, São Paulo, Brazil) was placed around the duodenum over the pyloric region to promote substenosis and to limit gastric emptying. Then, the transitional region between the fundus and the glandular portion of the stomach was ligated with a 4–0 nylon thread (Point Suture, Ceará, Brazil) to limit gastric compliance. Finally, the abdomen was closed by suturing the abdominal wall and the skin. Animals in the control group (sham) group were also subjected to abdominal laparotomy; however, ligature and substenosis were not performed. After surgery, animals had free access to food pellets and an oral rehydration solution containing 75 mmol/L Na\textsuperscript{+}, 65 mmol/L Cl\textsuperscript{–}, 20 mmol/L K\textsuperscript{+}, 10 mmol/L citrate, and 75 mmol/L glucose, and were euthanized at 3 and 7 days postsurgery.\textsuperscript{25} Laryngeal samples were collected (Fig. 1) to measure inflammation (wet weight and myeloperoxidase [MPO]), mucosal integrity (transepithelial resistance [TER]), and mucosal permeability to fluorescein.

Larynx Wet Weight

The whole larynx was obtained from each murine subject to evaluate the wet weight, which was used as an indicator of edema in this study. The esophagus and larynx were dissected, washed with sterile saline solution, weighed, and measured. The results were expressed in milligrams per centimeter (mg/cm).

MPO Activity

MPO is an enzyme that is present in the neutrophils. The laryngeal samples (set of three) were homogenized in a potassium phosphate buffer solution (pH 6.0) containing 50 mmol/L Na\textsuperscript{+}, 50 mmol/L K\textsuperscript{+}, 10 mmol/L glucose, and 1 mmol/L HEPES. The MPO activity was determined using the chromogenic substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB). The reaction was terminated with 50 mmol/L Na\textsubscript{2}EDTA and 25 mmol/L HCl.

Experimental Design

Inflammatory parameters: Wet Weight and MPO

Entire larynx

Barrier function: TER and mucosal permeability

Anterior commissure of larynx

Esophagus

Trachea

Vocal cord

Epiglottis

Root of tongue

Anterior commissure

Pyriform recess

Vestibular fold

Fig. 1. Scheme of the local of the samples collections of the larynx. MPO = myeloperoxidase; TER = transepithelial resistance.
hexadecyl trimethyl ammonium bromide and were centrifuged at 4,500 rpm for 15 minutes. MPO activity was evaluated by measuring the change in absorbance at 450 nm, using o-dianisidine dihydrochloride and 1% hydrogen peroxide. Results were expressed as units of MPO per milligram of tissue. All the therapeutic agents were administered via the oral route (0.3 mL of each compound) once a day for 3 days, and the animals were euthanized on day 3 postoperation. The larynx was collected to evaluate wet weight, MPO activity, TER baseline, and mucosal permeability. All treatment protocols and surgical procedures followed the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD).

**Experimental Protocol in an Üssing Chamber.**

In this step, laryngeal samples from healthy animals (sham group) and postsurgery animals (3 and 7 days postsurgery) were used. The larynx was dissected, stripped of muscle layers, and opened in a plate containing a Krebs-Henseleit bicarbonate buffer (KHBB; pH 7.4, 118 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM NaH2PO4, 25 mM NaHCO3, and 11 mM glucose). Laryngeal mucosa sections were mounted in a diffusion chamber to measure the permeability of the tissues induced by bipolar current pulses of 50 A (duration 200 ms, every 6 seconds) applied through platinum wires. The electrical system was equilibrated until a stable (30 minutes) TER baseline was obtained. TER was continuously recorded during the exposure time and plotted as Ω/cm².²⁷

**Epithelial permeability.** Laryngeal mucosa sections were mounted in a diffusion chamber to measure the permeability to fluorescein (376 Da; 1 mg/mL, diluted in KHBB pH 7.4). After a 30-minute period of stabilization in KHBB (pH 7.4), the solution in the luminal side was replaced by another solution containing the fluorescent marker. Samples (100 μL) were collected from the serosal side every 30 minutes, for a total duration of 90 minutes. The fluorescent marker was measured using a fluorescence plate reader (Fluostar Omega; BMG Labtech, Ortenberg, Germany) and expressed as the intensity of fluorescence.²¹

**Pharmacological Treatment**

To evaluate the efficacy of mucosal protective agents in the management of the inflammatory response and the protection of mucosal integrity, the following three compounds were examined: alginate (Luftagastropro; Reckitt Benckiser, United Kingdom), hyaluronic acid (Esoxx One; Alfa Wassermann, Bologna, Italy), and cashew gum 10% (Biotec, Federal University of Piauí, Parauaiba, Brazil), which were isolated and purified as previously described. All the therapeutic agents were administered via the oral route (0.3 mL of each compound) once a day for 3 days, and the animals were euthanized on day 3 postoperation.

**RESULTS**

**Laryngeal Inflammation on Experimental NERD in Mice**

Laryngeal inflammation was assessed by wet weight and MPO activity (Fig. 2). The laryngeal wet weight in the NERD operation group with no treatment group (panel A) was elevated on the 3rd postoperative day (31.3 ± 0.9 mg/cm), as compared to that of the sham group (23.9 ± 0.8 mg/cm; P <.001). This parameter had returned to baseline on the 7th postoperative day (26.9 ± 0.7 mg/cm). Moreover, the MPO activity (panel B) showed the same pattern of variation, with increased activity on the 3rd postoperative day (9.0 ± 0.5 U/mg tissue) as compared to the sham group (1.0 ± 0.3 U/mg tissue; P <.001), and decreasing to baseline (0.9 ± 0.2 U/mg tissue) by the 7th postoperative day.

**Laryngeal Mucosal Integrity on Experimental NERD in Mice**

Laryngeal mucosal integrity was assessed by TER and mucosal permeability (Fig. 3). Laryngeal basal TER was significantly lower on the 3rd postoperative day (17.8 ± 1.1 Ω/cm²) when compared to the sham group (29.6 ± 1.9 Ω/cm²). However, the TER values were similar in both groups by the 7th day postoperation.

![Fig. 2. Laryngeal inflammatory response in the murine model of gastroesophageal reflux disease. Laryngeal wet weight (A) and myeloperoxidase (MPO) activity (B) were measured in the sham-operated group at 3 and 7 days postsurgery. The results are expressed as the mean ± standard error of the mean, n = 5 per group. *P < .05 vs. sham and #P < .05 vs. 3 days (one-way analysis of variance and Tukey test).](image-url)
Fig. 3. Laryngeal mucosal integrity in the murine model of gastroesophageal reflux disease (GERD). Laryngeal basal resistance (A) and mucosa permeability after 90 minutes of fluorescein diffusion (B) were measured in sham and GERD animals at 3 and 7 days postsurgery. Results are expressed as the mean ± standard error of the mean, \( n = 5 \) per group. \( *P < .05 \) vs. sham and \( \#P < .05 \) vs. 3 days (one-way analysis of variance and Tukey test). TER = transepithelial resistance.

Fig. 4. Effect(s) of topical agents on the laryngeal inflammation induced by the gastroesophageal reflux disease surgical model. Laryngeal wet weight (A) and myeloperoxidase (MPO) activity (B) after treatment with saline (control), alginate, hyaluronic acid, or cashew gum. Results are expressed as the mean ± standard error of the mean, \( n = 5 \) per group. \( *P < .05 \) vs. sham, and \( \#P < .05 \) vs. control (one-way analysis of variance and Tukey test).

Fig. 5. Effect(s) of topical agents on the decrease in laryngeal mucosal integrity induced by the gastroesophageal reflux disease surgical model. Laryngeal basal resistance (A) and mucosa permeability (B) after treatment with saline (control), alginate, hyaluronic acid, or cashew gum. Results are expressed as the mean ± standard error of the mean, \( n = 5 \) per group. \( *P < .05 \) vs. sham, and \( \#P < .05 \) vs. control (one-way analysis of variance and Tukey test). TER = transepithelial resistance.
(27.7 ± 4.2 Ω/cm²), as displayed in Figure 3A. As shown in Figure 3B, the permeability of the laryngeal mucosa to fluorescein (after 90 minutes) was increased 3 days post-operation in the operated group (389.6 ± 40.1, fluorescein intensity), as compared to the sham group (217.3 ± 11.1, fluorescein intensity). At 7 days postoperation, laryngeal permeability was similar in both groups (157.6 ± 17.2, fluorescein intensity).

**Topical Agents Treatment**

Effect of mucosal protective agents on laryngeal inflammation (Fig. 4) and barrier function (Fig. 5). Alginate administration prevented modification of the inflammatory parameter wet weight (27.03 ± 1.3 mg/cm) and not MPO activity (6.8 ± 1 U/mg tissue), as compared to the non-treated group (31.3 ± 0.9 mg/cm and 9.0 ± 0.5 U/mg tissue, respectively). Alginate also improved TER and mucosal permeability (26.8 ± 1.9 Ω/cm² and 153.5 ± 21.2 fluorescein intensity, respectively), compared to the operated nontreated group (17.8 ± 1.1 Ω/cm² and 389.6 ± 40.1 fluorescein intensity, respectively). Additionally, hyaluronic acid ameliorated both inflammatory parameters of wet weight and MPO activity (27.8 ± 0.9 mg/cm, 2.6 ± 0.3 U/mg tissue, respectively) when compared to the non-treated group. However, hyaluronic acid was not effective in repairing a decrease in TER (18.7 ± 3.1 Ω/cm²) or the increase in laryngeal mucosal permeability (330.3 ± 36.7 fluorescein intensity) associated with the GERD operation. Conversely, cashew gum was able to provide protection against inflammation, as measured by wet weight and MPO activity (26.7 ± 0.3 mg/cm and 2.83 ± 0.6 U/mg tissue, respectively), as well as barrier function, as measured by TER and mucosal permeability (27.5 ± 3.5 Ω/cm² and 119.3 ± 9.7 fluorescein intensity, respectively), when compared to the operated nontreated group.

**DISCUSSION**

LPR is an expensive and highly prevalent disease. Despite its clinical importance, the mechanisms involved in the pathophysiology of LPR are not well-defined. Additionally, the treatment is associated with failure in approximately 50% of patients. Thus, new models to understand the pathophysiology of LPR could be important in the development of new treatments for this condition. In the present study, we used an experimental model to demonstrate that gastroesophageal reflux may lead to laryngeal inflammation and subsequently cause impairments in mucosal barrier function. Furthermore, commercial protecting solutions based on alginate and hyaluronic acid, as well as a noncommercial biopolymer known as cashew gum, were able to fully and partially reverse these pathophysiology mechanisms, respectively. Initially, our results showed that a murine experimental model of NERD induced a laryngeal inflammatory response. Our hypothesis was that the surgical procedure performed in this model forces the gastric contents back into the esophageal tube, and then to the larynx. The gastric contents reflux then induces edema and neutrophil infiltration from the larynx. Our results showed that this inflammatory response peaked at 3 days postsurgery and was resolved by 7 days postsurgery. These results differ from those in the esophageal mucosa, because Silva et al. used the same model to demonstrate that inflammatory processes in the esophageal mucosa were resolved only after 14 days. One possible explanation for this is that a large reflux volume is necessary to reach the larynx, and after 3 days the inflammatory process is decreasing, probably secondary to an adaption of the mice to the surgery. A second possibility is that the laryngeal mucosa of these mice have better defense mechanisms compared to the esophageal mucosa and thus are better adapted for reflux.

This experimental model also demonstrated barrier function impairment in the laryngeal mucosa, which is another issue pertaining to GERD. Our experimental model was based on the model suggested by Silva et al., which demonstrated impairment in the esophageal barrier function as seen by a drop in TER and an increase in mucosal permeability. To the best of our knowledge, ours is the first study to assess TER and mucosal permeability in a murine model of GERD. This subject is clinically relevant because recent treatment protocols recommended for PPI refractory in GERD patients to include topical agents, which have been shown to improve the esophageal barrier function.

The main alginate mechanism to reduce gastroesophageal reflux episodes is to modify the acid pocket. However, recently it was also demonstrated that topical application of the alginate to human esophageal biopsies in Üssing chambers could potentially protect the acid-induced impairment in the epithelial resistance. In addition, Woodland et al. demonstrated that fluorescein labeled with alginate could be seen coating the luminal surface in human esophageal biopsies for at least 1 hour. These experiments confirmed that alginate has also a direct protective effect on esophageal mucosa. According to McGlashan et al., alginate improved the symptom scores and clinical conditions of patients diagnosed with LPR; however, they failed to demonstrate the mechanism through which the alginate achieves this result. Although previous research has established the protective properties of alginate, its anti-inflammatory profile does not seem to be the mechanism responsible for this effect. Our study demonstrated that alginate prevented damage to mucosal barrier function. However, it was not able to prevent all inflammatory events, because there was a decrease in edema and not in neutrophil infiltration in the larynx after the alginate treatment. One possible reason for this is that the alginate used in GERD treatment has only a mild anti-inflammatory effect. By contrast, using a different alginate from Kyosei Pharmaceutical Co. (Eniwa, Japan), Yamamoto et al. demonstrated that alginate had potent anti-inflammatory activity in mice models of colitis. Consequently, we cannot disregard the possibility that alginate composition is important in the potency of anti-inflammatory effects.

Hyaluronic acid, another protective solution used in clinical practice, has been recognized as an adjuvant therapy for inflammatory diseases involving the upper aerodigestive tract. There are both basic and clinical research studies to elucidate the anti-inflammatory mechanisms initiated by hyaluronic acid. Savarino et al.
found a synergistic effect of hyaluronic acid with PPI treatment, suggesting that the mucosal protection associated with gastric acid suppression could improve the symptoms in NERD patients. Based on our data, hyaluronic acid diminished inflammation of the laryngeal mucosa in mice with NERD; however, it was not able to prevent laryngeal barrier function damage. The relationship between a decrease in inflammatory processes and a reversal of impaired esophageal mucosal integrity in experimental NERD has been described previously. However, this is not the case with hyaluronic acid treatment. The inflammatory response is a complex event associated with multiple mediators and cellular mechanisms. In this study, we evaluated only two inflammatory events, edema and neutrophil infiltration. Thus, we cannot rule out the possibility that there are other inflammatory mechanisms involved in decreased laryngeal barrier function, which hyaluronic acid was not able to prevent.

Among the three topical solutions used in the present study, only cashew gum was able to completely prevent both inflammation and impairment in mucosal barrier function. Our group previously demonstrated that acidic solutions in murine ex vivo models can cause impairments in laryngeal epithelial barrier function, which may be protected by topical treatment with cashew gum. Additionally, we have demonstrated that cashew gum decreases inflammation in the esophagus and reverses the decrease in epithelial resistance and increase in mucosal permeability that is associated with esophageal inflammation in experimental NERD models. Consequently, we believe cashew gum is a promising agent for the treatment of LPR, that may work through a regurgitation-associated mechanism similar to alginate. In this mechanism, the topical agent associated with gastric refluxate reaches the upper airway tract and forms a temporary shield, which increases the laryngeal pre-epithelial defense.

A limitation of this study is that LPR is a chronic disease, which requires a long treatment duration. In this experimental model, we observed maximum laryngeal damage in the 3 days postoperation, which is an acute and short time period. Furthermore, the anatomy and physiology of the murine gastrointestinal system is different to the human gastrointestinal system. However, recent comparative structural studies demonstrated that the organization of the mouse and human larynx are similar. Despite these limitations, the results of this study contribute significant understanding of LPR pathophysiology and may assist in pharmacological research for this condition.

CONCLUSION
This study established a surgical model of LPR that induced inflammation and barrier function impairment in the laryngeal mucosa. These observed alterations were partially attenuated by alginate and hyaluronic acid, and completely prevented by cashew gum (Fig. 6).

BIBLIOGRAPHY

Fig. 6. A hypothetical scheme of the effects of topical agents on laryngeal inflammation and mucosal integrity in the murine model of gastro-esophageal reflux disease.

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<tr>
<th>NON-TREATED</th>
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