Premiere Publications from The Triological Society

Read all three of our prestigious publications, each offering high-quality content to keep you informed with the latest developments in the field.

**The Laryngoscope**

*Founded in 1896*

Editor-in-Chief: Michael G. Stewart, MD, MPH

The leading source for information in head and neck disorders.

[Laryngoscope.com](http://Laryngoscope.com)

**Laryngoscope Investigative Otolaryngology**

*Open Access*

Editor-in-Chief: D. Bradley Welling, MD, PhD, FACS

Rapid dissemination of the science and practice of otolaryngology-head and neck surgery.

[InvestigativeOto.com](http://InvestigativeOto.com)

**ENTtoday**

A publication of the Triological Society

Editor-in-Chief: Alexander Chiu, MD

Must-have timely information that Otolaryngologist-head and neck surgeons can use in daily practice.

[Enttoday.org](http://Enttoday.org)
Topical Protection of Mice Laryngeal Mucosa Using the Natural Product Cashew Gum

Aline A. Figueiredo, MD, PhD; Ana P. M. Santana, PhD; Lucas A. D. Nicolau, PhD; Francisco J. Batista-Lima, PhD; Deysi V. T. Wong, PhD; Larisso T. Lucetti, PhD; Gabriela L. P. Batista, MD; Natália A. Caminha, MD; Jand V. R. Medeiros, PhD; Durcilene A. Silva, PhD; José R. S. A. Leite, PhD; Armenio A. Santos, MD, PhD; Pedro M. G. Soares, PhD; Daniel Sifrim, MD, PhD; Marcellus H. L. P. Souza, MD, PhD

Objectives/Hypothesis: Evaluate the effect of in vitro exposure of mice laryngeal mucosa to solutions that simulated human gastric juice and to assess the topical protective effect of cashew gum on mice laryngeal mucosal integrity in vitro.

Study Design: Animal study.

Methods: Murine (Swiss) laryngeal samples were mounted in Ussing chambers. The luminal side of biopsies was exposed to solutions of different acidity with or without pepsin and/or taurodeoxycholic acid (TDC). Transepithelial electrical resistance (TER) was continuously recorded. The topical protective effect of cashew gum solution was evaluated by precoating the biopsies before the exposure with a solution at pH 5 containing 5 mM TDC. Changes in TER and mucosal permeability to fluorescein were measured.

Results: Exposure of laryngeal mucosa to acidic solutions containing pepsin and TDC provoked a pH-dependent drop in TER with the maximal effect at pH 1, but still present at pH 5 (weakly acidic). The exposure of the laryngeal mucosa to a solution of pH 5 with TDC, but not with pepsin, produced a dose-dependent decrease in TER. Precoating the mucosa with cashew gum prevented the reduction of TER and increased transepithelial permeability by exposure to a solution at pH5 containing TDC.

Conclusions: Weakly acidic solutions containing bile acids can produce impairment of laryngeal epithelial barrier, which may be protected by topical treatment with cashew gum.

Key Words: Larynx, reflux, basic research.

Level of Evidence: NA.

Laryngoscope, 128:1157-1162, 2018

INTRODUCTION

Laryngopharyngeal reflux (LPR) is a condition in which gastric refluxate directly contacts laryngeal epithelium. Studies in human laryngeal biopsies revealed molecular and cellular repercussions of LPR, such as decreased laryngeal mucin, dilated intercellular spaces (DIS), and decreased expression of E-cadherin. These changes can impair the barrier function that prevents noxious components of the refluxate from infiltrating the laryngeal mucosa, thereby culminating in ear, nose, and throat symptoms due to the sensitization of nociceptors. Similarly, in vitro exposure of laryngeal mucosa to acidic solutions can impair the mucosal barrier. This has been demonstrated functionally through the measure of transepithelial electrical resistance (TER). Using healthy porcine larynges in an in vitro model, Erickson and Sivasankar demonstrated that acidic pepsin challenge significantly reduced TER and suggested that this could reflect reflux-related epithelial disruption.

Gastric contents can reach the esophagus and upper airway at a weakly acidic pH (4–7), and this condition is often found in patients taking proton pump inhibitors. Studies show that, at this weakly acidic pH, solutions containing bile acids can damage the esophageal epithelium, explaining, at least theoretically, the persistence of symptoms in patients under proton pump inhibitor therapy.

Recently, it has been demonstrated that the esophageal mucosa can be topically protected in vitro from the damage produced by the components of gastroesophageal refluxate. Cashew gum, a natural polymer obtained from the resin of a Brazilian northeastern tree (Anacardium occidentale L.), is a biomacromolecule with adhesive...
properties and anti-inflammatory and gastro-protective characteristics in animal models.\textsuperscript{13,14} Experiments in our lab showed that precoating human esophageal biopsies with cashew gum solution confers protection against acidic solution containing pepsin and bile acids.\textsuperscript{15}

To the best of our knowledge, there are no studies regarding the effect of any topical protectant solution against the damage caused by reflux components to the laryngeal mucosa. This study aimed to evaluate the effect of in vitro exposure of mice laryngeal mucosa to solutions that simulated human gastric juice and to assess the topical protective effect of cashew gum on mice laryngeal mucosal integrity in vitro.

**MATERIALS AND METHODS**

**Animals**

Male Swiss mice (weight 30–40 g) were obtained from the Federal University of Ceará. The animal use protocol was approved by the Animal Research Ethics Committee of the Federal University of Ceará (protocol: 23/2016).

**Surgical Procedure**

The animals were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and euthanized by exsanguination. The larynx of each animal was removed, dissected, and opened along the dorsal sagittal plane. Proximal and distal fragments of the esophagus were also removed and opened for studying esophageal basal resistance.

**TER**

Laryngeal mucosa fragments were mounted in an adapted Ussing chamber (Mussler Scientific Instruments, Aachen, Germany) containing Krebs solution (pH 7.4), with the anterior commissure placed precisely in the adaptor’s orifice, to record the baseline TER. The esophagus was dissected and the mucosa was mounted in the adapted Ussing chamber containing Krebs solution (pH 7.4). The TER was calculated according to Ohm’s Law from the voltage deflections induced by bipolar current pulses of 50 \(\mu\)A, during 200 ms every 6 s, applied through platinum wires. All experiments were conducted under open-circuit conditions.

**Evaluation of Laryngeal TER Using Different Challenges**

After registering baseline values, the solution in the luminal chamber was replaced with a challenge solution as follows: 1) Krebs solution at pH 1 with 1 mg/mL pepsin and 2 mM taurodeoxycholic acid (TDC); 2) Krebs solution at pH 2 with 1 mg/mL pepsin and 2 mM TDC; 3) Krebs solution at pH 5 with 1 mg/mL pepsin and 2 mM TDC; 4) Krebs solution at pH 5; 5) Krebs solution at pH 5 with 1 mg/mL pepsin; 6) Krebs solution at pH 5 with 0.5, 2, or 5 mM TDC; 7) Krebs solution at pH 5 with 5 mM deoxycholic acid (DC); 8) Krebs solution at pH 5 with 5 mM glycocholic acid (GC); and 9) Krebs solution at pH 7. Over the course of 30 minutes, TER was continuously measured, and the percentage change in TER at 1, 5, 10, 20, and 30 minutes was calculated.

**Transepithelial Permeability**

To assess larynx transepithelial permeability, the biopsies were kept mounted in the same chamber, which functioned as a diffusion chamber for measurements of permeability to fluorescein (332 Da). The solution at the luminal side was replaced by 1 mg/mL fluorescein solution. A sample of 100 \(\mu\)L was removed from the nonluminal side of the chamber at 0, 15, 30, 45, 60, 75, and 90 minutes. The permeability of fluorescein was measured using a fluorometer (Fluorostar Optima; BMG Labtech, Ortenberg, Germany) and expressed as the ratio of fluorescence intensity (i.e., the fluorescence intensity at each time point divided by the fluorescence intensity at the initial time point).

**Extraction and Purification of Cashew Gum**

Crude samples were collected from the trunk of \textit{A. occidentale} trees and purified as a sodium salt using as previously described with adaptations.\textsuperscript{16,17}

**Effect of Cashew Gum on Weakly Acidic Solution Containing TDC-Induced Laryngeal Damage**

After stabilization of the basal resistance, the recording was paused and the chambers were removed and separated for side exposure of the larynx. An aliquot of 200 \(\mu\)L of the protective solution (10% cashew gum) or Krebs solution (control) was applied to the exposed luminal side and left in contact with the mucosa for 5 minutes. The mucosal side chamber was then filled with 3.5 mL Kreb’s solution at pH 5 with 5 mM TDC for the recording of TER for 60 minutes after a new stabilization period. After 60 minutes of challenge, the same method described above was performed for the evaluation of transepithelial permeability.

**Statistical Analysis**

Values are expressed as the mean ± standard error of the mean. The comparisons between groups were analyzed by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison tests, or Kruskal-Wallis and Dunn’s test were used when appropriate. A Mann-Whitney test was used when appropriate. Differences were considered to be significant when \(P < .05\).

**RESULTS**

**Baseline TER in larynx and esophagus.** Baseline TER was significantly lower in the laryngeal mucosa than in the esophageal mucosa (Fig. 1).

**Laryngeal TER varying with pH.** TER decreased significantly after exposure to the test solutions at pH1, pH2, and pH5 when compared to the control solution (Krebs at pH7.4, \(P < .05\)), with the maximal effect at pH 1, but still decreases at pH 5 (weakly acidic). The greatest reduction in TER was observed after 30 minutes (Fig. 2).

**Effect of pepsin and bile acids in a weakly acidic solution.** Compared to the control solution, when a pepsin-only challenge was used, there was no difference in TER (\(P < .05\)). Conversely, TER decreased significantly when the larynx was exposed to a weakly acidic solution with bile acid and pepsin, or bile acid only as compared to TER of the control solution. The reduction in TER with a bile acid only challenge did not differ from a bile acid with pepsin challenge (Fig. 3).

**Effect of TDC at different concentrations, DC at 5 mM, and GC at 5 mM.** Using different concentrations of TDC at pH5, a dose-dependent reduction in TER
was observed, with the greatest drop occurring when a 5 mM TDC was used. The unconjugated DC used at 5 mM reduced the laryngeal TER similar to that of TDC. Conversely, 5 mM GC did not alter laryngeal TER (Fig. 4).

**Effect of pH 5 solution containing TDC (5 mM) on fluorescein permeability in laryngeal mucosa.**

Over the course of 90 minutes, there was a progressive increase in transepithelial permeability when the challenge was performed with the 5 mM TDC solution at pH 5. This permeability was significantly different at 90 minutes (Fig. 5A), when the ratio of fluorescence intensity immediately after exchange of solution was compared to the Krebs solution at pH 7.4 (control) (Fig. 5B).

**Effect of precoating with 10% cashew gum solution on laryngeal mucosa exposed to weakly acidic TDC solution (pH 5).** After 60 minutes of challenge, there was a drop of in TER in the group that received precoating with cashew gum, whereas in the group without cashew gum application there was a verified decrease in TER. The cashew gum also prevented the increase of transepithelial permeability to fluorescein, induced by exposure to a solution of pH 5 with TDC 5 mM (Table I). After the mucosa was placed in the fluorescein solution for 90 minutes, there was a 181-fold increase in transepithelial permeability in the group without cashew gum topical application prior to the challenge. In the group where the topical application of cashew gum before the challenge was performed, there was a 43-fold increase in the permeability to fluorescein, which was similar to the increase in permeability in mucosa exposed to Krebs solution at pH 7.4.

**DISCUSSION**

The present study demonstrated that laryngeal exposure to solutions that mimic gastric refluxate contents, including weakly acidic reflux, can impair the epithelial barrier, and that this phenomenon can be prevented in vitro by the topical application of cashew gum.

First, we verified that crude basal values of laryngeal mucosal TER were around five times lower than the esophageal mucosal TER in mice. Mouse esophageal mucosa is a keratinized stratified squamous epithelium, whereas mouse laryngeal mucosa, at the level of the vocal folds, consists of nonkeratinized stratified squamous epithelium. This may be one of the mechanisms of lower laryngeal epithelial resistance in mice. Therefore, this...
apparent fragility of the functional laryngeal epithelial barrier compared to the esophagus could at least, in part, explain the observation that many cases of LPR occur without classic symptoms of gastroesophageal reflux disease (GERD). It is necessary to consider, however, the difference between murine and human esophageal epithelia, with the former being keratinized stratified squamous epithelium and the latter not being keratinized. Even considering those histological differences, experimental approaches in animals are useful tools that make it possible to elucidate events in humans. Experiments with human laryngeal biopsy are useful, but may be technically limited because of the risk of damaging healthy airways during the procedure.

Our experiments revealed that exposure of laryngeal mucosa to acidic solutions combined with pepsin and TDC provoked a pH-dependent drop in TER, with the maximal effect at pH 1. Our results are in accordance with the literature. Erickson and Sivasankar used a similar tool to evaluate TER, and showed that acidic solutions at pH 3, regardless of the presence of pepsin, significantly impaired porcine laryngeal mucosal integrity. Similarly, Sasaki et al. demonstrated a high inflammation score in rat larynges exposed to hydrochloric acid without pepsin at pH 1.5. Notably, we observed a decrease in TER at pH 5 in a solution containing pepsin and TDC. Farré et al., using rabbit esophageal mucosa in vitro, showed that incubation of the tissue with weakly acidic solutions containing bile acids also decreased TER.

Despite the majority of reflux events in patients who are taking proton pump inhibitors with GERD being acidic in nature, the refluxate pH varies between 4 to 7, which is usually called weakly acidic in clinical practice. This persistent exposure to gastric contents in a weakly acidic milieu is likely responsible for laryngeal and esophageal refractory symptoms in some patients with LPR and GERD who do not respond to traditional antireflux therapy with proton pump inhibitors. We separately studied each component and demonstrated the importance of bile acid as the main harmful component of the refluxate, as measured by changes in laryngeal TER. In weakly acidic solutions, our results showed that only pepsin, as a component of the refluxate mimic, was not able to cause a decrease in laryngeal TER. Conversely, in the presence of bile acid, we observed a decrease in laryngeal TER in a concentration dependent manner, thereby demonstrating that bile acid can be topically injurious to laryngeal mucosa. Two experimental studies using a rabbit esophagus exposed to weakly acidic bile acid solutions, similar to patients undergoing proton pump inhibitor therapy, caused a reduction TER. Using a morphological analysis in a rat model, in which bile acids at different pH were applied to healthy laryngeal mucosa, Sasaki et al. demonstrated a high inflammation score in larynxes exposed to an added acidic solution of 5 mM TDC and an intermediate inflammation score in those exposed to a weakly acidic 5 mM TDC, thereby corroborating that damage is pH dependent. Pepsin is an acidic protease that has maximum activity at pH between 1.9 and 3.6. Erickson and Sivasankar, using porcine larynxes, showed that an acidic environment is necessary for pepsin to compromise the laryngeal epithelial barrier, and there are no tissue changes when pepsin is used at a neutral pH (pH 7). Some studies, however, have demonstrated a pepsin-

<table>
<thead>
<tr>
<th>Table I. Effect of Precoating With 10% Cashew Gum Solution on Laryngeal Mucosal Exposed to Weakly Acidic Solution Containing TDC (pH 5).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TER (%) of Basal Resistance at 30 Minutes</strong></td>
</tr>
<tr>
<td>Kreb’s solution, pH 7.4</td>
</tr>
<tr>
<td>Kreb’s solution, pH 5 + 5 mM TDC</td>
</tr>
<tr>
<td>Cashew gum 10%</td>
</tr>
</tbody>
</table>

*P < .05 versus pH 7.4. †P < .05 versus pH 5 + 5 mM TDC (one-way analysis of variance and Turkey’s multiple comparison test/Kruskall-Wallis and Dunn’s test). TDC = taurodeoxycholic acid; TER = transepithelial electrical resistance.
mediated biological effect of a nonacidic and weakly acidic environment. Even without the effect of pepsin on laryngeal TER in a weakly acidic milieu, it is still possible that pepsin can be used as a reflux marker because it indicates reflux in the respiratory tract despite it not causing laryngeal damage.

Similar results were found using TDC and DC. Both caused a significant decrease in TER following exposure to concentrations of 2 and 5 mM, whereas GC did not promote a change when compared to the control. Studies using esophageal aspirates of patients with GERD show an increase in certain bile acids, including TDC and GC, in those who were taking proton pump inhibitors and an increase in unconjugated bile acids, such as DC, in those who were not using a medication. This increase in unconjugated bile acid in patients undergoing proton pump inhibitor therapy is possibly a result of gastric bacterial overgrowth due to the weakly acidic environment and subsequent deconjugation of bile acids. Our experimental results may provide an interesting hypothesis for refractory laryngitis in a subgroup of patients using proton pump inhibitors. In these cases, it could be possible that weakly acidic conditions lead to an increase in unconjugated bile acid, such as DC, which may continue to damage the laryngeal mucosa.

We studied the effect of a solution containing 5 mM TDC at pH 5 in laryngeal epithelial permeability to fluorescein, which indicates a clear correlation between the decrease of TER and the increase in permeability. Studies using laryngeal biopsies show mucosal changes, such as DIS, and a reduction in E-cadherin expression, in patients with LPR. Together, these changes might indicate an increase in mucosal permeability in patients with LPR due to susceptibility related to a flux of small molecules through the laryngeal mucosa. We did not find any experimental studies evaluating the effect of bile acid on laryngeal mucosal permeability. An in vitro study using rabbit esophageal mucosa showed a great correlation between TER reduction and increased permeability after exposure to a weakly acidic solution containing bile acids. Therefore, permeability to fluorescein could be a valuable marker of a reduction in the laryngeal epithelial barrier. Accordingly, there is great clinical potential of substances that can prevent this loss of the epithelial barrier and the subsequent increase in permeability caused by the contact of bile acid with the laryngeal mucosa.

There is a growing interest in studying substances with bioadhesive properties as a therapeutic option for the treatment of GERD. Woodland et al. demonstrated a protective effect of an alginate solution applied topically to the esophageal mucosa prior to challenge with acidic solution containing TDC, thereby emphasizing that this action derives from its adhesive properties on the mucosa, and could work as a topical treatment for GERD. We used another biomacromolecule with adhesive properties. The topical application of 10% cashew gum prevented a reduction in TER and an increase in transepithelial permeability in the laryngeal mucosa exposed to TDC in a weakly acidic solution. De Lima et al. described the in vitro protective effect of cashew gum applied topically in esophageal mucosa exposed to acidic solution containing pepsin and TDC. These researchers further explained that this effect is likely due to its muco-adhesiveness properties, which are provided by complex polysaccharides. Cashew gum is a nontoxic, stable, easily available, biodegradable, and inexpensive substance. Recent studies have demonstrated that the anti-inflammatory properties and gastrointestinal protective activity of cashew gum is in part associated with the inhibition of oxidative stress. Such pharmacological features may be useful in LPR therapy, because this condition is associated with inflammatory and oxidative stress. Although additional experiments using human larynxes and in vivo models are necessary, and the risk of laryngospasm caused by the application of CG on laryngeal mucosa must be considered, these results may indicate a novel approach to LPR treatment.

CONCLUSION

This in vitro study demonstrated that laryngeal mucosa exposed to solutions that mimic human gastric juice could produce functional changes in the epithelial barrier. The impairment of the laryngeal mucosa barrier produced by weakly acidic solutions containing bile acids could be protected by topical treatment with 10% cashew gum. These findings have provided new perspectives for the treatment of LPR.

BIBLIOGRAPHY


