Effect of Acid and Pepsin on Glottic Wound Healing
A Simulated Reflux Model

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Objective: To evaluate the effects of acid and pepsin on the healing of traumatized vocal folds in a simulated reflux model. Gastroesophageal reflux is related to various laryngeal manifestations. However, there is a lack of established reflux animal models that would ensure longer observation periods.

Design: A prospective randomized animal study.

Interventions: Forty-two rabbits underwent a stripping procedure of the unilateral glottis and catheter insertion under transoral endoscopic guidance. The animals were randomly assigned to a control group (n=21; isotonic sodium chloride was used) or a reflux group (n=21, acid and pepsin were used). They received intrapharyngeal catheter irrigation with 3 mL of isotonic sodium chloride or a solution of acid with a pH of 3 and pepsin, 0.3 mg/mL, twice daily for 4 or 8 weeks after surgery.

Main Outcome Measures: Gross and histologic findings of the preinjured glottides of the 2 groups were compared.

Results: The catheter extrusion rate was significantly low (6%), and any catheter problems were immediately solved by reinsertion or reconnection. The extent of glottic scarring and frequency of granulation formation were higher in the reflux group compared with the control group (P<.05). Histologic inflammation scores and collagen deposition were significantly greater in the reflux group compared with the control group (P<.05).

Conclusions: Our data suggest that glottic wound healing is significantly affected by acid and pepsin. Antireflux treatment can be advocated to minimize further injury caused by gastroesophageal reflux in patients who undergo laryngeal surgery.

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Gastroesophageal reflux (GER) is a common disorder that occurs in 7% to 36% of the population.1 This disorder can result in various esophageal and extraesophageal complications. Reflux of gastric contents can cause esophageal mucosal abnormalities, such as inflammation, ulcers, and strictures, as well as pulmonary and otolaryngologic symptoms.2 It has been reported that GER is related to a variety of laryngeal manifestations: chronic hoarseness or cough, contact ulcer, vocal cord granuloma, intubation granuloma, pachyderma, laryngomalacia, laryngospasm, subglottic stenosis, and carcinoma.3 However, studies of the effects of GER on the airway have depended mainly on clinical observations because of a lack of reliable animal models. Several experimental studies showed that topical application or irrigation of acid or gastric juice on the traumatized subglottic mucosa led to severe inflammation, ulcers, and stenosis.3-6 Koufman3 demonstrated in a canine model that the combination of acid and pepsin created more significant subglottic inflammation than did acid or isotonic sodium chloride alone. Little et al4 also reported that subglottic stenosis developed rapidly in animals with mucosal lesions painted with gastric acid. However, the effects of GER on the healing of traumatized vocal folds have been scarcely investigated.

Recently, Adhami et al7 demonstrated that topical application of acid, pepsin, and conjugated bile acid on the injured glottis resulted in significant inflammation at 4 weeks after injury. In contrast, Cohen et al8 reported that acute wound healing of the canine vocal folds that underwent a stripping procedure did not seem to be influenced by the topical application of acid and pepsin compared with those that had an application of isotonic sodium chloride. However, in these canine models, the

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acid refluxate was administered under the unusual condition of general anesthesia and GER was simulated only with limited numbers of application for 2 to 4 weeks.

Clinically, GER is occurring with greater frequency.9 So, we developed a new animal model simulating reflux, modified from previous reports,10,11 that can ensure longer instillation of the refluxate with greater frequency than prior reports. The catheter was inserted into the lateral wall of the hypopharynx using a minimally invasive technique under transoral endoscopic guidance without external pharyngotomy. In the new simulated model, we evaluated the effects of GER on the healing of injured vocal folds.

METHODS

GLOTTIC WOUNDING AND A SIMULATED REFUX MODEL

Forty-two New Zealand white breeder rabbits were anesthetized with intramuscular injection of xylazine hydrochloride, 5 mg/kg, and ketamine hydrochloride, 35 mg/kg. The rabbits ranged in weight from 2.8 to 3.4 kg, and all were male. Direct laryngoscopy was performed using a Kleinsasser pediatric laryngoscope (Karl Storz GmbH & Co, Tuttlingen, Germany). The rabbit glottis was visualized through a laryngeal telescope (Karl Storz GmbH & Co, Tuttlingen, Germany). The rabbit glottis was evaluated by using a telescope as having the presence of mucosal scarring, formation of granulation tissues, erythema, or ulceration. The glottides of all animals were documented with telescopic photographs taken after careful examination. Each animal was then humanely killed with a lethal dose of sodium pentobarbital administrated by intracardiac injection after anesthesia induction.

After creating the glottic wound, a cut-down tube (polyvinyl chloride: Korea Medical Co, Seoul, South Korea) was inserted into the lateral hypopharynx. The tube was chosen for the catheter insertion because it has a small diameter (inner diameter, 0.6 mm; outer diameter, 1 mm) and a biocompatible nature and is highly resistant to degradation by acid and pepsin. Before surgery, the tube was prepared by attaching an 18-gauge needle to the end of the tube. Then the needle was inserted into the pyriform sinus and drawn out through the mouth with Kelly forceps under endoscopic guidance (Figure 1). A 5 × 5–mm, 1-mm–thick Silastic sheet was pricked with the needle and fixed in the cut-down tube with a cyanoacrylate adhesive (Henkel Korea Co, Seoul). The distal tube and needle were separated from the remaining tube and Silastic sheet. The tube with a sheet was pulled back, and the position of the tube was identified in the larynx and hypopharynx through an endoscopic examination (Figure 2B). The tube was fixed at the skin around the exit and the back of the animals with 3-0 silk sutures. The pharyngotomy catheter was flushed with isotonic sodium chloride to ensure patency. Gastrostomy was not performed in all animals. Intravenous cefazolin sodium (100 mg/kg) was administered to minimize the risk of wound infection. All animals received an intramuscular injection of buprenorphine hydrochloride, 0.05 mg/kg, twice a day for 3 days after surgery.

EXPERIMENTAL GROUPS AND ACID EXPOSURE

Pharyngotomy catheter irrigation was initiated on the second day after surgery. The rabbits were randomly assigned to either a control group (n = 21; isotonic sodium chloride was used) or simulated reflux group (hereinafter, reflux group) (n = 21; acid and pepsin were used). The hydrochloric acid concentration (pH of 3.0) was prepared daily from titrating acid with base by continuous monitoring of the pH until the desired pH was obtained. Porcine pepsin (Sigma-Aldrich Co, St Louis, Mo) was added to the titrated acid solution to produce a final concentration of 0.3 mg/mL. During the study periods of 4 or 8 weeks, the laryngeal mucosa of the animals in the reflux group were slowly irrigated twice daily with 1 mL/kg of a solution of acid and pepsin for 3 minutes whereas those of the control animals were irrigated twice daily with 1 mL/kg of isotonic sodium chloride. Cough, swallowing response, and signs of respiratory distress were carefully observed at irrigation.

When the tube extruded early, it was reinserted into the pyriform sinus under endoscopic guidance after anesthesia induction in as described in the “Glottic Wounding and a Simulated Reflux Model” subsection. The tube was reconnected with the interposition of a catheter with a larger diameter when the outside of the tube was disconnected during the study period. All animals were housed in an approved animal care facility with water and regular rabbit food ad libitum until their date of death. All experiments were performed with the approval of the institutional review board for animal researches.

GROSS AND HISTOLOGIC EXAMINATIONS

Before their death at 4 or 8 weeks after surgery, the animals were reanesthetized under the aforementioned anesthetic method. The glottis was evaluated by using a telescope as having the presence of mucosal scarring, formation of granulation tissues, erythema, or ulceration. The glottides of all animals were documented with telescopic photographs taken after careful examination. Each animal was then humanely killed with a lethal dose of sodium pentobarbital administrated by intracardiac injection after anesthesia induction. All rabbit larynges were harvested and placed in 10% formaldehyde for 24 hours. Sample processing was routine, using alcohol dehydration and paraffin embedment. Representative areas of the vocal folds and the posterior glottis were sectioned with a 5-µm thickness and stained with hematoxylin-eosin. The degree of epithelial and subepithelial inflammation was scored in a semiquantitative fashion from 0 to 17 per glottic side based on the previously established histologic scoring system.3 Masson trichrome staining was used to assess the collagen deposition, fibroblast proliferation, inflammatory leukocytes, and capillary angiogenesis. The collagen content was blindly checked and graded...
from 0 to 3+ by 1 pathologist. The numbers of fibroblasts and infiltrated inflammatory leukocytes were counted in microscopy fields with original magnification ×400. The capillary number was first carefully scanned at low magnification (original magnification ×40) to find the area that showed the most intense vascularization. Individual microvessels in the spot were then counted in a single, original magnification ×200 field, and the highest number of microvessels was identified. Formation of granulation tissues was examined using hematoxylin-eosin.

**STATISTICAL ANALYSIS**

The gross and histologic findings of glottic samples of the control and reflux groups were compared using SPSS statistical software (version 11.0; SPSS Inc, Chicago, Ill). The Fisher exact test was used for categorical data such as the incidence rates of mucosal scarring, granulation tissue formation, erythema, or epithelial erosion on gross examination. The histologic data were expressed as the mean (SD). We used the χ² test for collagen content and the Mann-Whitney test for the inflammation scores and numbers of fibroblasts or capillaries. P<.05 was considered statistically significant.

**RESULTS**

Eight of the 42 animals were excluded from this randomized study for postoperative early death by postoperative day 14 because of poor oral intake (n=4), excessive distress (n=2), and airway obstruction (n=2). Finally, 34 animals were included in this study (Table 1). Eight animals in each group were killed at 4 weeks after surgery. Ten control animals and 8 in the reflux group were humanely killed at 8 weeks. Premature catheter extrusion occurred in 2 animals (6%), and the catheter was immediately reinserted into the lateral wall of the hypopharynx. The outside disconnection of the tube occurred in 4 animals (12%), which was promptly resolved by reconnection of a new catheter with a larger diameter. Pharyngostomy leak and abscess formation did not develop in any animals. Most animals in the reflux group did not present with severe coughing or swallowing responses during catheter irrigation.

On telescopic examination at death, scarring and granulation tissue formation were found in the vocal folds and the posterior glottis that received the stripping procedure. These were more frequent and extensive in the reflux group than the control group (Table 1, Figure 3). The granulation tissues were found mainly on the medial surface of the vocal process or the posterior commissure, with higher incidence rates in the reflux group compared with the control group at 8 weeks after injury (P=.01). The gross scarring of more than one third of
the anteroposterior length in the injured whole vocal folds was more frequently found in the reflux group (P<.05). Erythema and epithelial erosion on the glottic mucosa were found in the noninjured side as well as the injured side in the reflux group but not in the control group (P<.03).

On histologic examination, the focal ulceration and granulation tissue formation were found in the glottides of most of the animals in the reflux group at 4 and 8 weeks after surgery (Table 2; Figure 4A and B). The epithelialization was completed in the injured glottides of both groups within 4 weeks after surgery. When inflammation scores were combined in the right and left vocal folds of each animal,7 mean scores were significantly higher in the reflux group than the control group (P<.001). The degree of inflammation and fibrosis in the injured vocal folds in both groups was compared. The collagen fibers were more densely stained in the lamina propria of the injured vocal folds of the reflux group than in that of the control group (P<.05) (Figure 4C and D). The mean number of fibroblasts was higher in the injured vocal folds of the reflux group than those of the control group (P<.05). The capillary angiogenesis was not statistically different between the reflux and control groups (P>.15).

In this study, the healing of glottic wounds was significantly affected by acid and pepsin. The acid refluxate caused more severe inflammation, granulation tissue formation, and subsequent scarring in the traumatized glottis in the reflux group compared with the control animals that received irrigation with isotonic sodium chloride. In a previous animal model,8 when the vocal folds were stripped and received topical application of acid and pepsin with a pH of 2 or 6 or of normal saline solution every other day for 12 days, no significant differences or trends were identified for inflammation and deposition of fibronectin, or for procollagen I. However, because the study demonstrated acute wound healing only at 12 days after vocal fold injury, it is questionable as to whether the later wound healing is affected by the repeated exposure of the refluxate. Ludemann et al10 reported that epithelial and subepithelial inflammation of the uninjured vocal folds were caused by the application of pepsin and acid (pH of 1.5) in proportion to the number of applications for 14 days in a simulated reflux model. Therefore, the GER seems to affect the intact vocal folds but not the injured vocal folds for the acute phase.

Table 2. Comparison of Histologic Findings*

<table>
<thead>
<tr>
<th>Variable</th>
<th>4 wk After Glottic Injury</th>
<th>8 wk After Glottic Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Group</td>
<td>Reflux Group</td>
</tr>
<tr>
<td>Inflammation score</td>
<td>6.7 (2.6)</td>
<td>14.3 (2.9)</td>
</tr>
<tr>
<td>Collagen deposition</td>
<td>1.1 (0.5)</td>
<td>1.9 (0.8)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>58 (20)</td>
<td>79 (23)</td>
</tr>
<tr>
<td>Capillaries</td>
<td>24 (11)</td>
<td>31 (12)</td>
</tr>
</tbody>
</table>

*All data are given as mean (SD) except where indicated.
†Mann-Whitney test except for collagen content (χ² test), P<.05.
within 2 weeks. Adhami et al. extensively studied the role of gastric and duodenal agents in laryngeal injury in an experimental canine model. They demonstrated that in 15 combinations of pepsin, conjugated and unconjugated bile acid, trypsin, and different pH concentrations (1-2, 4-5, and 6-7), pepsin and conjugated bile acid in a pH of 1 to 2 were the most injurious agents affecting the injured laryngeal tissues during the 4-week observation period. Although our study did not evaluate the effects of duodenal agents in addition to acid and pepsin, our data were similar to those in prior reports that showed that acid and pepsin alone also affected laryngeal wound healing.

In our simulated reflux model, we observed the effects of GER on the healing of glottic wounds for a longer period than those observed in previous reports. The extrusion rate of catheters in our model was much lower (6%) than those in previous reports in which most catheters were extruded within 2 to 3 weeks. The lower rate may be the result of insertion of the catheter through minimally invasive surgery using transoral endoscopic guidance and by use of an internal flange. This avoided invasive external pharyngotomy that was performed in the previous studies and probably led to increased pharyngeal leaks. The internal flange used in our study seemed to prevent the extrusion of the catheter tip from the hypopharynx without further injury or pharyngeal leak. The catheter with a small outer diameter (1 mm) was tolerable and resistant to degradation by acids in most animals without causing significant disturbance of oral intake.

The advantage of this model may be a more accurate simulation of GER as it occurs in humans. In previous animal studies, the multiple induction of general anesthesia for a topical application of refluxate was required, and the number of applications was limited. However, this simulated reflux model is more similar to GER as it occurs in humans. Multiple instillations of refluxates could be very easily performed every day in this reflux model. To our knowledge, our reflux model is the first to apply the refluxates to experimental animals for a longer observation period of a maximum of 8 weeks.

We used a solution of pepsin and an acid with a pH of 3.0 or isotonic sodium chloride in this reflux model because a previous report observed that animals irrigated with a solution with a pH of 4.0 had higher inflammation scores than did those irrigated with a solution with a pH of 1.5 in a simulated reflux. According to the report, it may be that the more acidic solution encouraged an immediate swallow response to clear the pharynx and allowed less time for microaspiration to occur.

Figure 4. Histologic sections showing granulation tissue and fibrosis in the injured glottis. The posterior glottides of the animals in the reflux group presented mucosal ulceration and granulation tissue formation (arrows) at 4 weeks (A) (trichrome, original magnification ×40) and 8 weeks (B) (hematoxylin-eosin, original magnification ×100) compared with the control animals (D) (trichrome, original magnification ×100).
According to other previous reports, pepsin combined with acid (pH of ≤4) caused significantly more laryngeal inflammation than did acid alone or placebo in the same pH ranges. Therefore, because pepsin activity was maximal in the acid state, both pepsin and acid seem to be required in the reflux animal models. Our study confirmed that acid and pepsin were sufficient to affect the wound healing of the injured vocal folds.

The scarred vocal fold reaching a mature phase of wound repair is characterized by an increased, disorganized, and thick bundle of collagen matrix in the lamina propria. The disorganization of collagen and elastin fibers, as well as abundant collagen deposition, may influence the viscoelastic shear tissue properties of the vocal folds. In our study, the gross and histologic findings of the glottides exposed by the reflux materials revealed an ongoing inflammatory process as well as wound remodeling after surgery. The repeated exposure of the refluxate to the injured glottis may cause lasting inflammation and granulation tissue formation and, subsequently, more abundant disorganized collagen deposition and fibrosis compared with the wound healing of the controls who received irrigation with isotonic sodium chloride.

Our animal model of vocal fold stripping and reflux was intended to simulate clinical situations. Laryngeal injuries can be caused by various surgical or nonsurgical etiologies, such as external trauma, burns, or endotracheal intubation. Our study suggests that the wound healing of the injured glottis can be significantly affected by GER. Therefore, antireflux therapy may be helpful for the less scarred wound healing of the injured larynx.

In conclusion, our study reveals that this new animal model of simulated reflux can be used in evaluating the effects of GER on glottic wound healing for a longer observation period than suggested in previous reports. Antireflux treatment is advocated to minimize further injury caused by GER in patients who undergo laryngeal surgery.

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