ORIGINAL ARTICLE

Simulated Reflux and Laryngotracheal Reconstruction

A Rabbit Model

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Objectives: (1) To test the feasibility of a rabbit model using a pharyngostomy tube to simulate gastroesophageal reflux and (2) to study the effects of gastroesophageal reflux on laryngotracheal reconstruction using a new rabbit model.

Design: Prospective randomized trial.

Subjects: Thirty-three New Zealand white rabbits.

Interventions: Anterior cartilage laryngotracheoplasty and pharyngostomy tube placement into the pyriform sinus were performed in 33 rabbits, 22 of which are included in this analysis. Beginning postoperative day 1, hydrochloric acid at a pH of 1.5 with pepsin (n=7) or at a pH of 4.0 with pepsin (n=8) was irrigated twice daily through the pharyngostomy tube to simulate gastroesophageal reflux, and a control group received twice-daily isotonic sodium chloride solution irrigations (n=7).

Main Outcome Measures: Specimens were scored by a pathologist masked to individual groups using a newly modified inflammation scoring system. In addition, cross-sectional areas of the cartilage grafts and subglottic airway lumina were compared.

Results: Inflammation scores were significantly higher in rabbits receiving hydrochloric acid and pepsin irrigations at a pH of 4.0 ($P=.04$) but not in those in the pH 1.5 group. Cartilage necrosis was prominent in all groups, and airway sizes and cross-sectional areas of the grafts were not significantly different among the 3 groups.

Conclusions: Cartilage necrosis is prominent during the early stages after laryngotracheoplasty. Inflammation can be increased using hydrochloric acid and pepsin irrigations but is difficult to predict based on this study. Although we confirmed the feasibility of this model, further modifications of this study are proposed to improve animal survival and data collection.

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GASTROESOPHAGEAL reflux (GER) in children is a significant health problem that has been implicated in a variety of disease entities, including hoarseness,[1] chronic cough,[2] sinusitis,[3] stridor or recurrent croup,[2,4] central or obstructive apnea,[4] aspiration pneumonia,[4] bronchospasm,[4] and idiopathic subglottic stenosis (SGS).[5] Because there has been difficulty in producing a reliable animal model, research on the effects of GER on the airway has largely been limited to clinical observations.

In adults, treatment of GER has been shown to have a markedly beneficial effect in the treatment of chronic cough, reflux laryngitis, globus pharyngeus, and laryngeal and tracheal stenosis.[6] Furthermore, in adults and children, asthmatic attacks have been linked to the presence of acid in the esophagus.[3,7] In children with GER and respiratory symptoms, the severity of attacks has been directly linked to the acidity of the gastric contents.[7]

Since the advent of mechanical ventilation in neonates, the number of acquired cases of SGS after endotracheal intubation has grown significantly, with reported rates ranging from 1% to 8% of neonates requiring prolonged intubation.[8] During the past 20 years, surgical correction of SGS has focused on increasing the cross-sectional area via laryngotracheal reconstruction (LTR) by placing anterior and/or posterior autogenous cartilage grafts to expand the upper trachea and larynx.

The role of GER in the success of LTR remains controversial. Retrospective studies by Yellon et al[9] and Gray et al[10] have suggested improved results after LTR with GER treatment. However, a retrospective analysis by Zalzal et al[11] showed

MATERIALS AND METHODS

All experiments were approved by the institutional animal care and use committees at Eastern Virginia Medical School, Norfolk, and the Naval Medical Center–Portsmouth, Portsmouth, Va.

Healthy male Pasteurella-free New Zealand white rabbits weighing approximately 2.5 kg (approximate age, 15 weeks) were preanesthetized intramuscularly with acepromazine maleate, 0.75 to 1.0 mg/kg; xylazine hydrochloride, 3 to 5 mg/kg; and ketamine hydrochloride, 35 to 44 mg/kg. Anesthesia was maintained with inhaled isoflurane administered by cone mask. Endotracheal intubation was avoided so that further subglottic injury would not confound results. Perioperative combination trimethoprim and sulfamethoxazole was administered the morning of surgery and for 3 days after surgery.

Surgery was performed as follows: Cartilage from the left costal margin was harvested from an upper midline abdominal incision and placed in isotonic sodium chloride solution (normal saline). Gastrostomy was then performed through the same incision with a 20F Foley catheter brought out through a separate incision. The abdomen was closed in layers with absorbable sutures (**Figure 1**).

Access to the larynx was obtained through a vertical midline neck incision. The strap muscles were separated in the midline, and dissection was carried down to the pretracheal fascia. The pharyngostomy catheter was composed of polyethylene tubing (Intramedic; Clay Adams, Parsippany, NJ) (outside diameter, 0.95 mm), chosen for its tissue nonreactivity. The left pyriform sinus was entered lateral to the thyroid ala, and the pharyngostomy catheter was secured via a 4-0 silk purse-string suture. The catheter was then tunneled subcutaneously and brought out posterolaterally, well away from the midline incision, and anchored to the skin with a 2-0 nylon suture (Figure 1A-B).

An incision was made into the upper 3 tracheal rings and cricoid cartilages; laryngotracheoplasty was performed using a spindle-shaped rib graft, attempting to maintain a uniform size of $2.5 \times 2.5 \times 11.0$ mm. Perichondrium was preserved along the luminal surface of the grafts, which were sewn into place using 6-0 polypropylene sutures. The strap muscles and skin were closed with absorbable sutures. Small Penrose drains were occasionally placed if an air leak was apparent.

After graft placement, endoscopy was performed by inserting a 4.0 ventilating bronchoscope with an appropriately sized telescope into the supraglottic area. The glottis was anesthetized with topical 1% lidocaine, and the position of the pharyngostomy cuff and the patency of the tube were confirmed by flushing saline through the tube. Subglottic luminal diameter in the anteroposterior and transverse dimensions was measured using optical grasping forceps. Animals were then placed in a nonrestrictive jacket to prevent chewing at the catheters.

Postoperative pain relief was provided by subcutaneous administration of buprenorphine hydrochloride, 0.05 mg/kg, every 12 hours for 3 days. Animals were offered rabbit chow and water ad libitum after surgery, with supplemental feedings of "blenderized" rabbit chow and water given 4 times daily if animals were unable to maintain adequate energy intake (5 g of chow per 100 g of body weight per day) by postoperative day 4. Water was also given if inadequate oral intake was noted (<5-10 mL per 100 g of body weight per day).

Pharyngostomy catheter irrigations were initiated on postoperative day 1 and continued for approximately 2 weeks. (For the sake of uniformity, the protocol was shortened to 2 weeks because the catheter extrusion rate was high at 2-3 weeks.) Rabbits were randomly assigned to 3 groups: those receiving acid, pH 4.0, with pepsin, 0.3 mg/mL (group 1, n=8); those receiving acid, pH 1.5, with pepsin, 0.3 mg/mL (group 2, n=7); and those receiving saline (control group, n=7). These hydrochloric acid concentrations are the same as those used by Koufman[6] and were prepared in the same manner and mixed with purified porcine pepsin (Sigma-Aldrich Corp, St Louis, Mo), 3900 U/mL. All animals were irrigated twice daily with solution, 1 mL /kg, and observed for swallowing response, coughing, tachypnea, stridor, or other signs of respiratory distress. Cough response was graded using an objective scoring scale developed by the authors (**Table 1**).

Animals that survived for 2 weeks were humanely killed via a lethal dose of pentobarbital sodium (65 mg/kg intravenously), and their larynges were harvested. Measurements at the narrowest point of the graft were taken to calculate airway cross-sectional area. Larynges were then forwarded to a pathologist (A.L.W.) masked to experimental groups. The degree of inflammation for the graft was scored using a modification of the scale proposed by Koufman[6] (**Table 2**). Mean group inflammation scores were compared using the unpaired *t* test (1-tailed distribution).

The degree of cartilage graft resorption was examined in a manner similar to that used by Cotton.[8] Whole-mount photomicrographs taken at the subglottic level were digitally scanned and analyzed on a desktop computer using NIH Image 1.61 (National Institutes of Health, Bethesda, Md); this program can calculate the area of any object outlined. Cross-sectional areas of cartilage grafts were then computed, and mean cross-sectional areas of cartilage grafts were then compared using 1-way analysis of variance, with the Tukey-Kramer multiple comparison procedure to compare all possible pairs of means.

Cross-sectional areas of the subglottic lumina were calculated from measurements taken endoscopically immediately after surgery and directly at the time the rabbits were killed. Cross-sectional area is calculated using the formula for the area of an ellipse: area $= \pi(A+B)$. Mean reductions in cross-sectional areas were compared using 1-way analysis of variance as well. Uniform error from the 2 different methods of measurement was assumed.

no obvious benefit. Experimental studies[6,12-14] on dogs and pigs showed that gastric juices are deleterious to the healing of injured mucosa in the subglottic airway and that SGS can be induced by applying acid to injured mucosa. The main drawback of these experiments is that they do not mimic the physiologic characteristics of GER.

The focus of this experiment was to study the fate of anterior cartilage grafts placed in rabbits while simulating GER in a recently developed rabbit model.[15] After anterior laryngotracheoplasty and pharyngostomy tube placement with postoperative acid exposure, a newly modified histologic scoring system was used to grade the

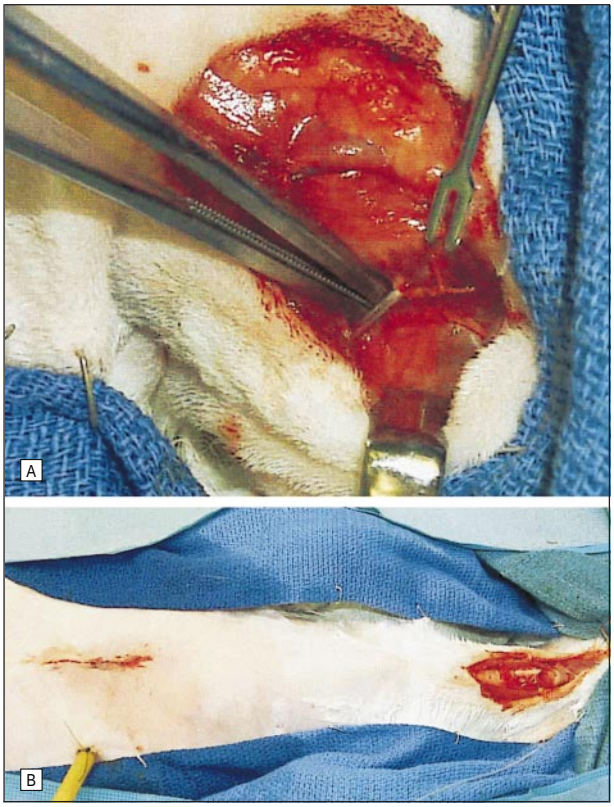

Figure 1. *Intraoperative photographs. A, Forceps under the pharyngostomy tube going into the left pyriform sinus, with the thyroid cartilage retracted by a skin hook. B, Rabbit near the end of surgery, with the abdominal incision closed and gastrostomy in place. The graft has been sewn in place anteriorly, with the pharyngostomy tube tunneled under the skin flap.*

Table 1. Cough Response Scoring System

Grade	Response
I	No coughing, sneezing, or movement
II	Isolated coughing or sneezing, or coughing lasting up to 30 s
III	Persistent coughing lasting 30 s to 2 min
IV	Persistent or paroxysmal coughing lasting >2 min
V (not used)	Severe, persistent coughing mandating euthanasia

degree of inflammation in addition to looking at cartilage resorption.

RESULTS

Pharyngostomy, gastrostomy, and LTR with rib graft were performed in 33 animals. One animal died of a pharyngeal leak on postoperative day 2. Seven animals were killed prematurely: 2 on postoperative days 3 and 5 for severe abdominal wall infection, 2 on postoperative days 7 and 10 for premature catheter extrusion, 1 on postoperative day 3 for excessive pain and distress, 1 for severe enterocolitis, and 1 for a gastrostomy leak and abscess on postoperative day 10. In all, 3 animals were eliminated from data analysis for gastrostomy-related problems, and 1 was eliminated for unilateral vocal cord paralysis and persistent, severe coughing believed to be due to severe aspiration; its larynx was submitted for histologic examination after death. This left 22 animals for analysis: 7 controls, 8 in group 1, and 7 in group 2.

Table 2. Cartilage Graft Histologic Scoring System*

1. Epithelial changes
   - A. Intraepithelial inflammation
     - 0 = ≥Rare PMNs
     - 1 = ≥2 PMN/HPFs in 1 HPF
     - 2 = ≥2 PMN/HPFs in 1-3 HPFs
     - 3 = 2 PMN/HPFs in >3-5 HPFs
   - B. Squamous metaplasia
     - 0 = Absent
     - 1 = Present
   - C. Epithelial covering over cartilage graft
     - 0 = Covered
     - 1 = >75%
     - 2 = <50%
     - 3 = None
2. Subepithelial changes
   - A. Stromal inflammation
     - 0 = Absent
     - 1 = 1-5 PMN/HPFs in 3-5 HPFs
     - 2 = 5-10 PMN/HPFs in 3-5 HPFs
     - 3 = >10 PMN/HPFs in 3-5 HPFs
   - B. Periglandular inflammation
     - 0 = Absent
     - 1 = Rare PMNs per glandular unit
     - 2 = >3 PMNs per glandular unit

**Total possible score is 12. PMN indicates polymorphonuclear neutrophil; HPF, high-power field.*

Complications were mainly those listed in the previous paragraph; 2 animals that were excluded had their pharyngostomy tubes replaced on the first and second postoperative days. Diarrhea occurred in 4 animals, and subcutaneous emphysema occurred in 1. Another animal required operative replacement of the gastrostomy tube on postoperative day 2 after chewing through it.

All animals except 5 (2 in group 1, 2 in group 2, and 1 control) resumed full oral feeds within 1 week of surgery. The control animal that had difficulty was later graduated to full oral feeds. When the graded responses of the animals were compared on postoperative days 7 and 14, there was no significant difference in the reaction among the groups.

Histologic scoring was done by a single pathologist (A.L.W.) masked to the experimental groups. Mean±SD inflammation scores are shown in **Figure 2**. The mean inflammation score for group 1 was significantly higher than that for the control group (8.4 vs 6.3; $P=.04$), whereas the mean score for group 2 was lower, but not to statistical significance ($P=.15$).

Analysis of the cartilage graft showed extensive necrosis in all specimens. The cross-sectional areas of the spindle-shaped grafts were not significantly different among groups ($P=.44$). Units for area measurement of the graft are not given because all images were uniformly magnified with microscopy and photographing. Photomicrographs of the grafts are shown in **Figure 3**A-B. In rabbits with unilateral vocal fold paralysis, no cartilage elements were seen, and the grafts were replaced with fibrous tissue (Figure 3C). Both of

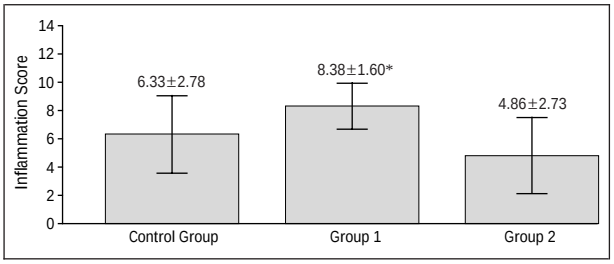

Figure 2. *Mean pathologist inflammation scores by group. Group 1 received hydrochloric acid, pH 4.0, with pepsin; group 2, hydrochloric acid, pH 1.5, with pepsin; and the control group, isotonic sodium chloride solution. Asterisk indicates* P<*.05. Error bars represent SD.*

these animals were irrigated with 4.0-pH hydrochloric acid solution with pepsin.

There was no significant difference among groups with respect to initial airway size, final airway size, and reduction in airway size.

COMMENT

The question of the effects of GER on the success of LTR remains in some dispute. Cotton,[8] Yellon et al,[9] and Gray et al[10] maintain that aggressive treatment of GER improves outcomes, whereas Zalzal et al[11] suggest that there is no objective proof that GER affects the ultimate success of LTR. These groups have published studies based on retrospective reviews and clinical observations of patients treated in a milieu of perioperative treatments, so the effects of antireflux treatment are difficult to isolate. Further evidence for the importance of controlling GER before LTR is presented in recent articles by Halstead[16,17] and Walner et al,[18] which offer convincing supporting evidence that the development of SGS is in many cases linked to GER.

The lack of a reliable animal model remains an obstacle to GER research. To date, the animal model that seems to most closely simulate GER was developed by dividing the oblique fibers at the gastroesophageal junction in pigs; this species was chosen because the muscular arrangement at this junction is identical to that in humans.[14] Although GER was documented by extensive pH probe studies and esophageal manometry, this model has yet to be duplicated and is not practical for widespread use.

The model proposed in this study is based on previous experiments by Ludemann and others at McGill University.[15] The major strength of the pharyngostomy model is the delivery of acid to the hypopharynx, thus mimicking the effect of acid on the upper airway. Other variables, such as reflex-induced bronchospasm, are not shown. Also, the infiltration of acid into the subglottis and trachea cannot automatically be assumed with intact laryngeal reflexes; thus, microaspiration is anticipated and relied on to show a difference among groups.

Another obvious shortcoming of the presented model is that it does not replicate the pathophysiologic characteristics of SGS and thus does not entirely mimic an airway that receives a cartilage graft for SGS. Certainly, concentric scarring and altered structural support in actuality affect the ultimate outcome.

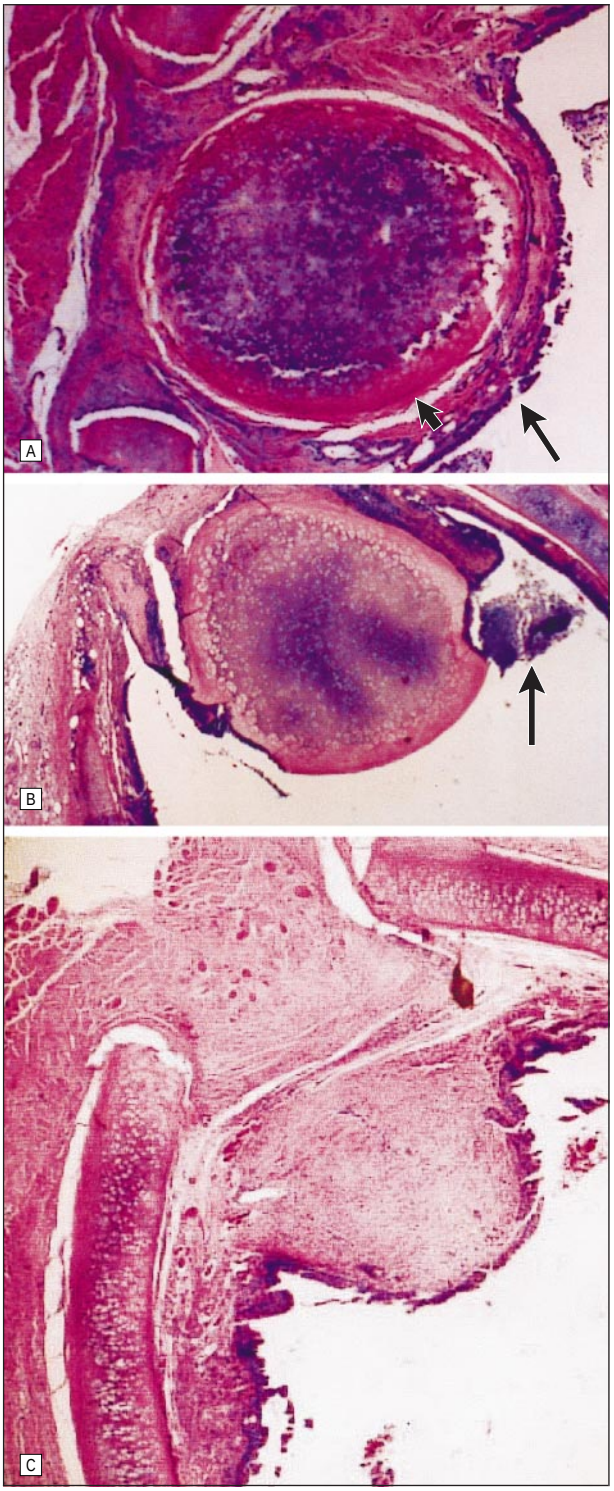

Figure 3. *A, Hematoxylin-eosin–stained section of control group cartilage graft. Note the epithelial overgrowth (long arrow) and the peripheral cartilage necrosis (short arrow) (original magnification ×40). B, Cartilage graft from a group 1 animal (hydrochloric acid, pH 4.0, with pepsin) showing necrosis and no epithelial overgrowth. In addition, granulation tissue is seen at the edge (arrow) (original magnification ×40). (Some prolapse of the graft has occurred with tissue handling.) C, Airway section from a rabbit that had a paretic vocal fold and was irrigated with acid. There are no chondroidal elements seen in the cartilage graft, and its framework has been replaced by fibrous tissue (original magnification ×40).*

The presence of the small pharyngostomy tube did not seem to impair the rabbits' ability to eat and drink; if

tube feeding was withheld, the rabbits would generally resume their diet within 4 days. Three animals were eliminated from data analysis for gastrostomy-related infections, and several chewed through the tubes as well. Considering the fact that only 4 animals could not resume full oral feeds and the extra time and complications associated with operating on the stomach, it seems that gastrostomy is an unnecessary component in this model.

We believe that the proposed scoring system, which was modified from that designed by Koufman,[6] is useful in evaluating airway cartilage grafts in the early postoperative period. While measuring the degree of polymorphonuclear cell infiltration, it also includes epithelial regrowth as a factor for success. Also, glandular hyperplasia might not be relevant during early healing phases.

The relative lack of differences among groups can be attributed to several reasons. As mentioned earlier, we relied on microaspiration of the acid, which is impossible to control, to incite subglottic inflammation. In addition, because of the high catheter extrusion rate after 2 to 3 weeks, the postoperative irrigation period was shorter than we had hoped. A longer period might have better reflected healing, with more mucosal covering, scar tissue formation, stenosis, and additional cartilage resorption.

Animals irrigated with the 4.0-pH solution (group 1) had higher inflammation scores than those irrigated with the 1.5-pH solution (group 2). Because rabbits receiving the stronger acid solution were not noted to cough more than the others, a possible explanation for the lower inflammation scores in the 1.5-pH group is that the more acidic solution encouraged an immediate swallow response to clear the pharynx and allowed less time for microaspiration to occur. If this model is to be used again, one possible way to counteract this response might be to infuse the acid slowly with a pump over several hours. An interesting, surreptitious finding is the extensive resorption of the grafts in the rabbits with paretic vocal folds. Practically, this finding might be relevant to LTR in children because the presence of an endotracheal tube for several days after sugery can cause glottic incompetence and aspiration of secretions onto the surgical site.

The degree of cartilage necrosis might seem surprising, but this has been shown to be an expected phase of the healing process. In a study on LTR in a rabbit model published by Jacobs and others,[19] extensive cartilage necrosis followed by neochondrification occurred during the 10 weeks after surgery. The cartilage remodeling had no apparent effect on the outcome of the graft or the size of the airway lumen. This finding supports those of other studies that have shown early necrosis and neochondrification[8] and preservation of the graft size after long-term healing.[20,21]

CONCLUSIONS

Cartilage necrosis is prominent during the early healing stages after laryngotracheoplasty. Inflammation can be increased by administration of hydrochloric acid and pepsin but is difficult to predict; glottic incompetence most likely exacerbates the inflammatory effects. Further studies are needed to determine whether placing acid into the hypopharyngeal region reliably simulates the effects of GER on airway surgery.

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