Laryngeal Reinnervation Using a Split-Hypoglossal Nerve Graft in a Canine Model

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**IMPORTANCE** Vocal fold immobility following injury to the recurrent laryngeal nerve (RLN) may lead to substantial morbidity. A reinnervation treatment strategy offers several theoretical benefits over static treatment options. This study evaluates the robustness of reinnervation of the larynx using a split-hypoglossal nerve graft in an animal model, with outcomes assessed by independent blinded review.

**OBJECTIVES** To assess whether a full-hypoglossal nerve graft to the RLN after RLN section can provide return of dynamic vocal fold motion in a canine model, and to validate that a split-hypoglossal nerve graft to the RLN may also provide dynamic vocal fold motion to rehabilitate laryngeal function in a canine model.

**DESIGN, SETTING, AND SUBJECTS** A pilot animal study to assess the feasibility and morbidity of laryngeal reinnervation following RLN injury with an end-to-end full-hypoglossal or split-hypoglossal nerve graft was performed at an animal care and research facility in 10 adult female dogs. The study dates were January to July 2013.

**INTERVENTIONS** We performed full-hypoglossal (full XII group [n = 5]) and split-hypoglossal (split XII group [n = 5]) nerve grafts to the RLN in a canine model following RLN section.

**MAIN OUTCOMES AND MEASURES** Morbidity was evaluated through scored feeding observation. Laryngeal function was assessed by video laryngoscopy and evoked laryngeal electromyography was performed at baseline and 6 months after surgery. Video laryngoscopy was graded by independent reviewers blinded to study intervention.

**RESULTS** No clinically significant morbidity was identified after surgery. On review of video laryngoscopy, all 5 animals in the full XII group and all 5 animals in the split XII group demonstrated vocal fold motion by at least 1 independent reviewer. All 3 reviewers agreed on motion in 1 of 5 animals in the full XII group and in 1 of 5 animals in the split XII group. Stimulation of the hypoglossal nerve demonstrated neural connection on evoked laryngeal electromyography in all animals at 6 months.

**CONCLUSIONS AND RELEVANCE** This study confirms that a full-hypoglossal or split-hypoglossal nerve graft may restore vocal fold motion, without significant functional morbidity, following RLN section in a canine model.
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current laryngeal nerve (RLN) dysfunction causing unilateral vocal fold immobility leads to substantial impairment of voice, swallowing, and airway protection in pediatric and adult populations, with profound Effect observed in all dimensions of general health in quality-of-life studies.1-5 Dysfunction of the RLN may arise from several potential etiologies, including infection, congenital malformation, trauma, central nervous system dysfunction, inflammation, malignant neoplasm, surgery (especially notable is thyroid surgery), and idiopathic causes. In children, vocal fold immobility accounts for 10% of all congenital laryngeal lesions and is the second most common cause of neonatal stridor.6

Among adults, the exact prevalence of unilateral vocal fold immobility is unknown, but much of the current data related to morbidity has been obtained from RLN injury during thyroid surgery, with 5% to 10% of patients undergoing thyroid surgery experiencing RLN damage during the initial procedure7-9 and up to 30% during revision thyroid surgery.7,10,11 In patients with known unilateral vocal fold immobility following thyroid surgery, 75% experience difficulties with breathing during daily activities, 56% experience dysphagia, 44% experience aspiration, and 80% have dysphonia,23-24 leading to an estimated $850 million increase in annual health care costs in the United States.15-16

Current treatment options for unilateral vocal fold immobility include injection laryngoplasty, medialization thyroplasty, arytenoid adduction, and reinnervation using the ansa cervicalis. Each strategy has demonstrated reliable improved laryngeal function in pediatric17-19 and adult20-22 populations. However, the ideal surgical treatment has not been established to date. A treatment approach involving reinnervation offers several theoretical advantages over static approaches. These include the following: potential for a one-time procedure, maintenance of viscoelastic properties of the vocal fold, preservation of laryngeal muscle bulk and tone, avoidance of synthetic materials, and theoretically restoration of dynamic vocal fold motion, with improvement in voice and swallow without airway compromise. The use of the ansa cervicalis as a reinnervation approach may successfully restore laryngeal muscle tone but fails to provide restoration of vocal fold motion.23-26 In a series done in 2000, full-hypoglossal nerve reinnervation demonstrated restoration of vocal fold motion in humans,28 although concern regarding donor morbidity has possibly limited its use.

The rationale for using the split-hypoglossal nerve to restore laryngeal function is based on similar temporal activation patterns for the tongue and laryngeal musculature, similar nerve fiber types between the RLN and hypoglossal nerves, and similar target muscle fiber types between the tongue and intrinsic laryngeal musculature.27 In addition, the hypoglossal or split-hypoglossal nerve graft offers substantially more axons than other candidate nerves. Drawbacks of using the hypoglossal nerve relate to potential morbidity of partial loss of function of the tongue. The extent of morbidity has not been fully defined although results of several studies indicated low morbidity in patients who have lost function of one hypoglossal nerve for facial reanimation28-30 or laryngeal reinnervation.25

The first objective of this study was to assess whether a full-hypoglossal nerve graft to the RLN can provide return of dynamic vocal fold motion, as assessed by independent reviewers. A canine model was chosen based on previous literature demonstrating similarities in anatomy and innervation pattern between the human and canine larynx and tongue.25-31-33 The second objective of this study was to validate that a split-hypoglossal nerve graft may also provide dynamic vocal fold motion, as assessed by independent reviewers. The third objective of this study was to identify morbidity of a split-hypoglossal nerve graft through assessment of aspiration and feeding time. We hypothesized that a split-hypoglossal nerve graft to the RLN may provide dynamic vocal fold motion with lower morbidity than a full-hypoglossal nerve graft.

Methods

This study was performed in accord with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act. The animal protocol was approved by the Institutional Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary. The study dates were January to July 2013. Ten randomly selected healthy adult female beagles (Pine Acres Rabbitry Farm) weighing 6 to 10 kg (age range, 2-4 years) were included in the study. The animals were randomly assigned to 2 groups. Five animals underwent transection of the right RLN and the entire hypoglossal nerve, followed by end-to-end anastomosis of the proximal hypoglossal nerve segment to the distal RLN segment (full XII group) (Figure 1A). The remaining 5 animals underwent transection of the RLN in a similar fashion; however, only partial transection of the hypoglossal nerve was performed, leaving approximately 50% of the nerve intact (split XII group) (Figure 1B). When Galen anastomosis was encountered during identification and section of the RLN, it was severed and resected as part of the removed RLN segment.

In all animals, video laryngoscopy and evoked laryngeal electromyography (L-EMG) was performed before injury and after anastomosis at baseline and 6 months after surgery. Evoked L-EMG was obtained using endotracheal tube surface recording electrodes connected to a nerve monitoring system (NIM 3.0; Medtronic). Normative values have been described using the system in canines and in humans.31-34-35 The nerve monitoring system has an adjustable, factory-set EMG filter of 100 Hz to 2 kHz. Evoked L-EMG was performed with a standard Prass probe (Medtronic) applied directly to the nerve through an open neck accessed via a midline incision. Suprathreshold stimuli were delivered at a rate of 4 Hz with a current of 1.0 mA, as determined from prior evoked L-EMG investigations.36 Recordings were obtained with a sweep speed of 50 milliseconds (5 milliseconds per division) and gains of 2000 μV. As evoked L-EMG was performed, quantifiable measures of latency, amplitude, and wave duration were obtained.

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could be identified, and the more distal branch was tran-
sected and rotated inferiorly, representing approximately 50% of the nerve axons. Even with identification of the dominant distal branches, the nerve was split proximally before organization of the distal fascicles in each case to provide sufficient length of nerve to be rotated inferiorly for anastomosis. At the location where the fascicle joined the main nerve in the split XII group, it was necessary to further proximally split the main nerve to obtain sufficient length for the split graft to reach the RLN near its entry point into the larynx, leaving approximately 50% of the original diameter intact. The RLN was then completely transected in both groups 1 to 2 cm before its entry point into the larynx. When Galen anastomosis was encountered during identification and section of the RLN, this nerve was also severed and resected as part of the removed RLN segment. End-to-end neurorrhaphies were performed using 9-0 nylon, with 2 epineural sutures placed in a tension-free manner (Figure 1B).

Assessing Postoperative Morbidity
Before the initial surgical procedure, each animal underwent a baseline feeding evaluation that measured the time it took to finish each meal. This time was calculated as the mean of 3 consecutive days of feeding. In 3 of the animals (2 in the full XII group and 1 in the split XII group), the baseline feeding times were longer than 30 minutes, were found to vary greatly, and were excluded from the feeding study. Baseline weight measurements were also obtained for each animal.

Following the initial surgical procedure, feeding observation was performed at 1 week, 1 month, and 6 months after surgery. At each feeding observation, the time it took to finish the meal was recorded and averaged over several feeds. Witnessed coughing or aspiration events were also recorded. Weight was measured at 1 month and 6 months after surgery.

Obtaining Laryngoscopy and Evoked L-EMG Following Nerve Grafting
Six months following the initial surgical procedure, each animal was anesthetized, underwent video laryngoscopy, and was intubated with the endotracheal tube as described above. The neck was opened and explored. Evoked L-EMG of the right proximal hypoglossal nerve, right hypoglossal nerve before anastomosis, and right RLN after anastomosis was performed. Three consecutive recordings at each point were captured, and quantitative data, such as latency, amplitude, and wave duration, were recorded. The animal was then painlessly euthanized.

Statistical Analysis
Video laryngoscopy was assessed by 3 independent reviewers, each trained in laryngology and blinded to the hypothesis and type of surgery performed. The reviewers were asked to assess the presence of motion as a primary outcome measure by indicating yes or no. The reviewers were also asked to rate vocal fold position (1 if median, 2 if paramedian, and 3 if lateral), right vocal fold edge (0 if normal, 1 if mild bowing, and 2 if pronounced bowing), and overall hemilaryngeal tone (0 if normal, 1 if mildly hypotonic, 2 if moderately hypotonic, and 3 if severely hypotonic). Internal controls

Obtaining Initial Laryngoscopy and L-EMG
The animals were anesthetized with an intravenous dose of toloxolamine hydrochloride and inhalational isoflurane. The isoflurane was then turned off, and direct laryngoscopy was performed to confirm bilateral vocal fold mobility as the animals began to awake from anesthesia. A 4-mm telescope (HOPKINS II; Storz) attached to a video monitor and video recording device was used to capture vocal fold motion. Following video laryngoscopy, the trachea was intubated with a nerve monitoring system 6.0 endotracheal tube containing surface electrodes (Medtronic) and connected to the response monitor. Electrical impedance levels and spontaneous EMG tracings were confirmed, and the use of isoflurane was resumed. A vertical incision was made in the midline, and the right RLN was identified and dissected free. The hypoglossal nerve was then identified and dissected anterograde into the tongue and retrograde to the point of the ansa cervicalis. During the preinjury assessment, evoked L-EMG was performed to capture waveform morphologic structure, and baseline parameters of latency, amplitude, and wave duration were measured.

Performing Full-Hypoglossal and Split-Hypoglossal Nerve Grafts
Following identification of the right RLN and hypoglossal nerve, measurements were obtained of the anticipated length of hypoglossal nerve needed to reach the RLN at its entry point into the larynx. In the full XII group, the hypoglossal nerve was then completely transected distally and rotated inferiorly. In the split XII group, 2 predominant nerve terminal branches could be identified, and the more distal branch was tran-

The white star indicates the nerve anastomosis to the recurrent laryngeal nerve. The asterisk indicates the split.

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The asterisk indicates the split.
Canine Laryngeal Reinnervation Using a Split-Hypoglossal Nerve Graft

Among the 3 reviewers regarding vocal fold position, degree of bowing, and tone; however, the κ statistics remained low at 0.20, 0.50, and 0.42, respectively. The right vocal fold position was described as median in 2 of 5 animals in the full XII group by all 3 reviewers and as median or paramedian in 4 of 5 animals in the full XII group.

At baseline, all 5 animals in the split XII group had normal right vocal fold motion. At 6 months after surgery, all 3 reviewers agreed that 1 of 5 animals in the split XII group demonstrated right vocal fold motion (Table). When asked to qualify this motion, 1 reviewer indicated observing both abduction and adduction, 1 reviewer indicated observing only adduction, while 1 reviewer indicated being unable to determine the type of motion observed. Two of the 3 reviewers reported motion in 2 of 5 animals in the split XII group, with 1 reviewer reporting observation of motion in 5 of 5 animals in the split XII group. The right vocal fold position was described as median in 3 of 5 animals in the split XII group by all 3 reviewers and as median or paramedian in 5 of 5 animals in the split XII group.

Evoked L-EMG and Tongue EMG

All animals demonstrated successful evoked L-EMG at baseline. Six months after surgery, 5 of 5 animals in the full XII group and 5 of 5 animals in the split XII group demonstrated successful evoked L-EMG results through stimulation of the proximal hypoglossal nerve. Evoked L-EMG obtained by stimulation of the right RLN at baseline (Figure 2) and by stimulation of the proximal hypoglossal nerve in a full-hypoglossal nerve graft at 6 months after surgery (Figure 3) demonstrated similar waveform morphologic structure. Figure 4 shows nerve stimulation at various places in an animal in the split XII group at 6 months after surgery, as well as a sample EMG recording from each stimulus. For the split XII group, electrode placement in the right posterior tongue demonstrated evoked tongue EMG waveform and amplitude results comparable to those obtained from the control side, suggesting that neural connectivity was maintained in the uninjured portion of the hypoglossal nerve.

Discussion

We sought to examine the feasibility and morbidity of laryngeal reinnervation following RLN injury with an end-to-end split-hypoglossal nerve graft in an animal model in a preliminary pilot study. In the setting of independent review, reinnervation using a full-hypoglossal nerve graft or a split-hypoglossal nerve graft provided objective vocal fold motion in 1 of 5 animals. In all animals, evoked L-EMG findings sup-

Table. Independent Review of Video Laryngoscopy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Full XII Group</th>
<th>Split XII Group</th>
<th>κ Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motion observed by ≥1 reviewer</td>
<td>5 (100)</td>
<td>5 (100)</td>
<td></td>
</tr>
<tr>
<td>Motion observed by all 3 reviewers</td>
<td>1 (20)</td>
<td>1 (20)</td>
<td>−0.07</td>
</tr>
<tr>
<td>Normal tone or mild hypotonicity</td>
<td>3 (60)</td>
<td>5 (100)</td>
<td>0.42</td>
</tr>
<tr>
<td>Median or paramedian position</td>
<td>4 (80)</td>
<td>5 (100)</td>
<td>0.20</td>
</tr>
<tr>
<td>No or mild vocal fold bowing</td>
<td>4 (80)</td>
<td>5 (100)</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* The number in each column indicates the number of times all 3 reviewers agreed on the category. See the Methods section for a description of the groups.
ported neuromuscular connectivity and integrity on evoked stimulation. Regarding morbidity, neither the full-hypoglossal nerve graft nor the split-hypoglossal nerve graft produced substantial aspiration events, changes in weight, or changes in feeding time.

A canine model similar to the one in this study has been used in laryngeal reinnervation studies to evaluate the use of several potential nerve candidates, including the RLN, hypoglossal nerve, split-hypoglossal nerve, and ansa cervicalis. Vocal fold motion following RLN injury has previously been reported with the use of a full-hypoglossal or split-hypoglossal nerve graft to the RLN. Our study confirms these findings by independent review and supports the notion that vocal fold motion may be restored following reinnervation using a full-hypoglossal nerve graft or split-hypoglossal nerve graft.

With regard to the degree of reinnervation, restoration of vocal fold motion was confirmed by all 3 reviewers in only 1 of 5 full-hypoglossal nerve grafts and 1 of 5 split-hypoglossal nerve grafts. However, at least 2 reviewers identified motion in at least half of the animals being studied. Additional desirable features, such as median vocal fold location, lack of bowing, and normal or near-normal tone, were also identified in most animals, findings that are comparable to those seen with nerve grafts from the ansa cervicalis to the RLN. Evoked L-EMG results supported that neural connectivity was achieved in all animals.

Several important aspects of the study design limit our ability to quantify the success of the split-hypoglossal and full-hypoglossal nerve grafts beyond the feasibility of demonstrating reinnervation. In this study, we used spontaneous vocal fold motion as a proxy for robust reinnervation. However, spontaneous motion may not necessarily translate into volitional motion and a functional voice. In what is to our knowledge the only published human study involving a full-hypoglossal nerve graft, voice outcomes appeared promising. However, more study would be required to determine how a split-hypoglossal nerve graft might translate into speech. The study is also limited by the subjective nature of interpreting a 10-
second video laryngoscopy recording following RLN injury. Although independent blinded expert review is perhaps the most objective strategy for assessing vocal fold motion, agreement among our reviewers regarding motion was lacking in this study. This finding could be related to the small amount of motion observed at 6 months, the limited nature of a 10-second video, or lack of agreement on the definition of motion between laryngologists. Previous studies assessing agreement among observers found only slight to fair agreement between laryngologists assessing vocal fold immobility. Agreement among observers on vocal fold position, degree of bowing, and toxicity was fair in this study, a finding similar to that seen in a previous study that looked at agreement of similar parameters.

An additional limitation of our experimental design was the timing of the measurement of vocal fold motion. If the vocal fold is being driven by the hypoglossal nerve following grafting, it is possible that we would not see vocal fold motion unless the animals were attempting to swallow, an aspect of the design that may have lessened the amount of motion identified by the reviewers. In addition, although previous evidence indicated motion results achieved by 6 months in the canine model, it is also possible that a longer duration would allow for a greater degree of nerve regeneration and perhaps improve the overall motion results. Further study is necessary to better define the ideal experimental parameters and better quantify the overall success of the split-hypoglossal nerve graft in achieving dynamic laryngeal function.

The degree of morbidity remains a central question for potential use of a split-hypoglossal nerve graft. In this model, it is possible that subclinical differences in aspiration or swallow function were present between the study groups and not measurable. In addition, even if differences were seen, canine swallow and airway protection are not a perfect proxy for human swallow and airway protection. However, EMG findings in this study support the notion that the split-hypoglossal nerve graft maintained neural connectivity to the tongue, consistently to the posterior tongue, offering theo-
retinal advantages of maintenance of bulk and function during swallow over the full-hypoglossal nerve graft. Future histopathological studies may corroborate this finding.

The use of a split-hypoglossal nerve graft to the RLN following RLN injury offers potential for a one-time procedure that maintains the viscoelastic properties of the vocal fold, re-establishes laryngeal muscle bulk and tone, restores vocal fold motion, and provides improvement in voice and swallow without airway compromise. This study offers evidence to support the notion that vocal fold motion can be achieved using a split-hypoglossal nerve graft. Questions remain about the degree of success of such a technique, as well as the degree of long-term morbidity involved. In addition, the quality of voice achieved by this split-hypoglossal nerve technique remains unknown. Future studies demonstrating low morbidity of a split-hypoglossal nerve graft may provide support for its use in unilateral vocal fold immobility related to RLN injury.

Conclusions

This study confirms previous reports that a full-hypoglossal or split-hypoglossal nerve graft to the RLN provides neuro-muscular connectivity. It may restore vocal fold motion, with no substantial functional morbidity, following RLN section in a canine model.


