Establishing TEM Markers of Laryngeal Nerve Injury in a Translational Mouse Model

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Dysphagia (swallowing dysfunction), dysphonia (voice dysfunction), and dyspnea (respiratory dysfunction) are common complications after surgical procedures targeting the anterior neck, such as cervical spinal surgery and thyroidectomy. During these procedures, the recurrent laryngeal nerve (RLN), a branch of the vagus (10th cranial nerve), may become inadvertently damaged. RLN injury causes ipsilateral vocal fold (VF) paralysis that contributes to dysphagia, dysphonia, and dyspnea. These conditions can be devastating for patients and are associated with poor quality of life, major depression, decreased general health, and increased financial burden [1]. Current treatments for these adverse surgical outcomes are palliative rather than curative. As a result, physicians who perform anterior neck surgical procedures are at high risk for medical litigation.

RLN injury is impossible to systematically investigate in human patients; therefore, we have created a translational mouse model to specifically address this major clinical problem. Our preliminary work with C57BL/6J (i.e., B6) mice shows that unilateral RLN compression injury causes ipsilateral VF paralysis and dysphagia, mimicking iatrogenic RLN injury in human patients. In additional pilot studies, we investigated intraoperative vagal nerve stimulation (iVNS) as a potential therapeutic option when injury does occur. Preliminary results look promising, as iVNS-treated mice had markedly improved recovery of VF mobility compared to untreated mice (Fig 1). In addition, corresponding regenerative improvement was suggested by our preliminary transmission electron microscopy (TEM) results (Fig 2). However, results have been variable in subsequent studies. This is likely due to inconsistency in our crush force over time, leading to variable injury severity between individual mice. Thus, we sought to identify a new method to create consistent compression injuries to the RLN of mice.

We hypothesized that an aneurysm clip with a known closing force would provide a reliable crush injury in our model, similar to a previous study conducted in rats [2]. For this study, we performed surgery on ten B6 mice (4 months old) of either sex to create a right RLN compression injury. Mice were randomly allocated to one of two study end-points to investigate critical phases of nerve regeneration: 3 days post-injury to capture degenerative changes, and 2 weeks post-injury to capture regenerative changes. Mice were anesthetized and placed on a surgical platform beneath a microscope. Prior to incision, a miniaturized endoscope was inserted transorally to visualize the VFs and ensure normal bilateral movement. Next, a ventral neck incision was made; the right RLN was isolated near the 5th tracheal ring and crushed with an aneurysm clip (1.30 N of force) for 30 seconds. The aneurysm clip was coated with carbon powder to mark the injury site. Immediately after injury, endoscopy was performed again to confirm ipsilateral VF paralysis. The incision was sutured closed and postsurgical analgesics were administered post-surgery. Mice were re-anesthetized for repeat endoscopy either at 3 days (n=5) or 2 weeks (n=5) post-surgery. After VF movement was video recorded, mice were euthanized for transmission electron microscopy (TEM) to quantify nerve degeneration and regeneration at each time point, as described below.
Mice were euthanized using an overdose of pentobarbital, followed by transcardial perfusion with saline then 4% paraformaldehyde (PFA). Beneath a dissecting microscope, the strap muscles overlying the larynx and trachea were gently removed to expose the RLN bilaterally. To avoid mechanical damage to the RLN during dissection, we collected a single block dissection spanning from the larynx to the 7th tracheal ring for each mouse. The specimen block was bisected immediately distal to the right RLN crush site, which was readily identified by the carbon marking. Specimens were post-fixed in 2% PFA/2% glutaraldehyde in 100mM sodium cacodylate buffer and then sent to the MU Electron Microscopy Core Facility for processing using their standard protocol. During processing, specimens were cut into right and left sides, with the left side serving as the uninjured control. Cross-sections (85 nm) were cut immediately distal to the RLN injury site using an ultramicrotome and a diamond knife. Images were acquired with a JEOL JEM 1400 transmission electron microscope at 80 kV on a Gatan Ultrascan 1000 CCD. Digital images were stored as TIF files for subsequent quantitative analysis.

Our preliminary results demonstrate that our TEM protocol produces excellent morphology of RLN samples. We are currently in the process of collecting images from all 10 samples. Automated and semi-automated image analysis will be used to quantify total axon count, axon caliber, myelin thickness, and density of myelin debris. For this purpose, we will extend and adapt our earlier histopathology and TEM image analysis works [3]. Our goal is to demonstrate the reliability of an aneurysm clip for use in our future studies with this model. Moreover, results will identify a narrow set of morphometric variables that readily distinguish between control and RLN-injured samples to include in our ongoing and future work.

References:

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Figure 1: VF mobility improves in a stair step pattern after iVNS treatment in RLN-injured mice. Untreated mice fluctuate in mobility, with minimal recovery after 12 weeks. Error bars: ±1 standard error of the mean.

Figure 2. Representative cross-sectional TEM images of the RLN 12 weeks post-surgery. Compared to controls (uninjured RLN, LEFT), marked RLN degeneration (arrows) was apparent in RLN injury samples collected distal to the injury site (MIDDLE). After iVNS, ultrastructural morphology of the injured RLN was visibly improved, though not to control levels (RIGHT).