The Journal of Laryngology & Otology

cambridge.org/jlo

Changes in vocal fold gene expression and histology after injection augmentation in a recurrent laryngeal nerve injury model

Ronit Malka[1], Alisa Isaac[2], Gabriela Gonzales[2], Solaleh Miar[3], Benjamin Walters[1], Amelia Baker[4], Teja Guda[2] and Gregory R Dion[1,5]

[1]Department of Otolaryngology – Head and Neck Surgery, Brooke Army Medical Center, Joint Base San Antonio, Fort Sam Houston, TX, USA, [2]Department of Biomedical Engineering and Chemical Engineering, University of Texas at San Antonio, San Antonio, TX, USA, [3]Department of Civil, Environmental, and Biomedical Engineering, University of Hartford, West Hartford, CT, USA, [4]Department of Anesthesiology, Brooke Army Medical Center, Joint Base San Antonio, Fort Sam Houston, TX, USA, and [5]Department of Otolaryngology – Head and Neck Surgery, University of Cincinnati, Cincinnati, OH, USA

Abstract

Objective. To investigate changes in neuroregenerative pathways with vocal fold denervation in response to vocal fold augmentation.

Methods. Eighteen Yorkshire crossbreed swine underwent left recurrent laryngeal nerve transection, followed by observation or augmentation with carboxymethylcellulose or calcium hydroxyapatite at two weeks. Polymerase chain reaction expression of genes regulating muscle growth (MyoD1, MyoG and FoxO1) and atrophy (FBXO32) were analysed at 4 and 12 weeks post-injection. Thyroarytenoid neuromuscular junction density was quantified using immunohistochemistry.

Results. Denervated vocal folds demonstrated reduced expression of MyoD1, MyoG, FoxO1 and FBXO32, but overexpression after augmentation. Healthy vocal folds showed increased early and late MyoD1, MyoG, FoxO1 and FBXO32 expression in all animals. Neuromuscular junction density had a slower decline in augmented compared to untreated denervated vocal folds, and was significantly reduced in healthy vocal folds contralateral to augmentation.

Conclusion. Injection augmentation may slow neuromuscular degeneration pathways in denervated vocal folds and reduce compensatory remodelling in contralateral healthy vocal folds.

Introduction

Unilateral vocal fold paralysis due to recurrent laryngeal nerve (RLN) denervation is a common pathology treated by laryngologists worldwide.[1–5] Temporary injection augmentation is typically employed within the first year after symptom onset while the possibility of spontaneous neural recovery still exists. Utilisation of more permanent procedures, such as medialisation thyroplasty or lipoaugmentation, is generally delayed until maximal nerve regeneration potential has been realised. While used primarily for providing improved short-term vocal outcomes, the long-term impact of injection augmentation, particularly early after initial injury, remains controversial. To date, injection augmentation has shown no demonstrated difference in long-term vocal outcomes after definitive framework surgery or in time taken to recover from idiopathic vocal fold paralysis, though this effect with iatrogenic vocal fold paralysis is less clear.[6–8] Multiple studies have demonstrated that patients treated with early injection augmentation undergo subsequent medialisation thyroplasty at significantly lower rates, suggesting a possible improvement in long-term function from this temporary intervention.[5,9–12] The possible relationship between early injection augmentation and improved long-term voice outcomes has been supported by early functional magnetic resonance imaging studies that suggest improved early brain stimulation of voice-related nuclei compared to untreated individuals.[13] However, these data have been mixed across studies.[4] While the clinical impact of early augmentation continues to be murky, the possible biological changes induced by injection augmentation remain unexplored.

Recent work evaluating the effect of injection augmentation on vocal fold stiffness in an RLN injury model demonstrated biomechanical changes in the injected fold and compensatory changes in the healthy contralateral side.[14] While the thyroarytenoid area was significantly decreased after RLN transection and unaffected by injection augmentation, vocal fold stiffness on the injected side was closer to native tissue properties than the untreated denervated vocal fold. Perhaps more interesting, however, was that healthy vocal folds contralateral to an injected vocal fold also showed increased stiffness, suggestive of compensatory changes separate from the biomechanical properties of the injectate itself.

Main Article

Ronit Malka takes responsibility for the integrity of the content of the paper

Presented at the Fall Voice Conference, 6–8 October 2022, San Francisco, CA, USA.

Cite this article: Malka R, Isaac A, Gonzales G, Miar S, Walters B, Baker A, Guda T, Dion GR. Changes in vocal fold gene expression and histology after injection augmentation in a recurrent laryngeal nerve injury model. *J Laryngol Otol* 2024;**138**:196–202. https://doi.org/10.1017/S0022215123001135

Received: 16 February 2023
Revised: 23 April 2023
Accepted: 31 May 2023
First published online: 21 June 2023

Keywords:
Recurrent laryngeal nerve injury; vocal fold paralysis; medialization laryngoplasty; vocal fold atrophy

Corresponding author:
Ronit Malka;
Email: ronit.e.malka@gmail.com

Evidence of altered neurotrophic factor expression has been described in unilateral vocal fold paralysis, but such changes have not been described after therapeutic intervention.[15–17] However, expression of muscle growth and atrophy genes has not been examined in vocal fold atrophy. While numerous gene targets are commercially available for rapid analysis from fresh or frozen specimens, myoblast determination protein 1 (MyoD1), myogenin (MyoG) and forkhead box O1 (FoxO1) are three commonly assessed proteins that are relatively over-expressed in muscle undergoing growth and differentiation.[18,19] Comparatively, F-box only protein 32 (FBXO32), a protein which helps facilitate apoptosis, is markedly overexpressed in skeletal muscle atrophy.[20] Furthermore, neuromuscular junction density is shown to increase in the first two weeks post-denervation, but subsequently decreases below baseline levels if reinnervation is not accomplished.[21,22] In the larynx, specifically, neuromuscular junction size and density in the thyroarytenoid have been demonstrated to decrease with age-related atrophy.[23,24] Thus, assessment of innervated neuromuscular junction density – specifically neuromuscular junction density loss associated with atrophy – aids in our understanding of neural regeneration patterns at distal targets after denervation injury. Specifically useful for this application is FluoroMyelin™ Red; this is a lipophilic immunohistochemical stain frequently used to assess peripheral nerve and neuromuscular recovery in numerous disease processes, but has not to date been utilised in vocal fold neuromuscular studies.[25–28]

This study set out to examine the changes observed in muscle growth and atrophy transcriptional activity, as well as innervated neuromuscular junction density, in denervated thyroarytenoid muscle both with and without injection augmentation. Based on prior clinical and *in vivo* data, we hypothesised that RLN transection and the resulting atrophy decreases vocal fold neuromuscular signalling and growth-related gene expression, and that augmentation alters these profiles closer to that of native tissue.

Materials and methods

After approval from the US Air Force 59th Medical Wing Institutional Animal Care and Use Committee (protocol FWH20190101AR), 18 Yorkshire crossbreed swine (*Sus scrofa*) underwent left RLN transection. The left side was chosen as it is more commonly injured iatrogenically.[29–33] After left vocal fold paralysis was confirmed endoscopically two weeks after injury, six animals each underwent observation, carboxymethylcellulose injection or calcium hydroxyapatite injection.

Three animals from each group were euthanised at four weeks post-operatively to assess for early post-injection results to simulate the optimised post-procedural voice. The remaining three animals from each group were euthanised at 12 weeks post-operatively to assess for waning post-injection results. All laryngeal specimens were harvested and underwent histological assessment of bilateral vocal folds.

Recurrent laryngeal nerve transection

Swine were anaesthetised via an intramuscular injection of Telazol® (tiletamine and zolazepam anaesthetic agent) (4.4 mg/kg) and ketamine (2.2 mg/kg), maintained with inhaled isoflurane titrated as needed for spontaneous ventilation, with vital signs continuously monitored. Intra-operative analgesia was provided with intramuscular buprenorphine (0.01–0.05 mg/kg).

Swine were positioned supine; the anterior cervical area was shaved, prepped with povidone-iodine solution, and the incision site injected with 1 per cent lidocaine with 1:100 000 adrenaline. Under sterile dissection, the left RLN was identified near the cricothyroid joint and a 2 cm nerve section was excised to prevent future potential re-anastomosis. For clarity, the terms 'healthy' (right-sided) and 'denervated' (left-sided) are used to distinguish sides throughout the manuscript.

Direct laryngoscopy and vocal fold augmentation

Fourteen days after RLN transection, swine were again anesthetised as described above. Direct laryngoscopy with spontaneous ventilation was performed to confirm left vocal fold immobility. Swine in the augmentation groups then underwent left vocal fold injection. Under direct visualisation with a zero-degree, 30 cm long telescope (Karl Storz, Culver City, California, USA), 0.5 cc of carboxymethylcellulose (Prolaryn Gel; Merz North America, Raleigh, North Carolina, USA) or calcium hydroxyapatite (Prolaryn Plus; Merz North America) was injected just lateral to the left vocal fold, bringing the injured vocal fold just past the midline. For clarity, animals undergoing observation are referred to as 'untreated' and animals undergoing injection augmentation are referred to as 'augmented' throughout the manuscript.

Euthanasia and specimen preservation

Animals were euthanised at the designated post-injection euthanasia date, or sooner if required because of animal distress or illness. Euthanasia was performed after general anaesthesia induction with intravenous pentobarbital (100 mg/kg) and confirmed. The larynx was extracted immediately after euthanasia and sectioned in the sagittal plane, with care taken to preserve the anterior commissure.[14,34,35] Specimens were then frozen at −80°C until histological analysis.

Gene expression profiling

Relative gene expression of MyoD1, MyoG, FoxO1 and FBXO32 was quantified using real-time quantitative polymerase chain reaction from posterior cricoarytenoid and lateral cricoarytenoid samples. RNA was extracted using Trizol lysing reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Qiagen RNeasy mini kit (Quiagen, Hilden, Germany) following manufacturer instructions, and the quantity of RNA in each sample was measured via a spectrophotometer (BioTek Synergy 2 with BioTek Take3 plate; BioTek, Winooski, Vermont, USA). RNA was converted to complementary DNA using Bio-Rad iScript Reverse Transcription Supermix for real-time quantitative polymerase chain reaction (Bio-Rad Laboratories, Hercules, California, USA) following manufacturer instructions. Quantitative polymerase chain reaction was performed by amplifying complementary DNA and collecting cycle threshold (Ct) values using a thermocycler (Bio-Rad C1000 Touch with iTaq Universal SYBR Green Supermix; Bio-Rad Laboratories, Hercules, California, USA). Fold changes were calculated relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels within the same sample. Expression ratios of the lateral cricoarytenoid relative to the posterior cricoarytenoid were calculated to isolate the effect of local injectate on the denervated lateral cricoarytenoid compared to isolated denervation

https://doi.org/10.1017/S0022215123001135 Published online by Cambridge University Press

experienced by the posterior cricoarytenoid. The primers used are listed in Supplementary Table 1.

Histological analysis

In order to quantify the thyroarytenoid muscle atrophy and evaluate the impact of the injectable materials on surrounding tissue, cross sections were collected from the anterior, middle and posterior vocal folds. As previously described, samples were cut perpendicularly to the vocal fold in 5 mm thick sections and fixed in 4 per cent formalin solution.[14] These samples were subsequently mounted in embedding moulds filled with optimum cutting temperature compound (Scigen Tissue Plus OCT Compound; Thermo Fisher Scientific). Moulds were stored at −80°C prior to sectioning. Samples were then cut to a tissue thickness of 14 µm using a cryostat (CryoStar NX70; Epredia, Kalamazoo, Michigan, USA) and thaw-mounted on glass slides. Slides were maintained at room temperature for 1 hour to dry, and subsequently kept in chilled acetone at −20°C for 10 minutes to improve the adhesion to the glass slides. Slides were hydrated with de-ionised water prior to staining. Samples were then stained with FluoroMyelin Red fluorescent myelin stain (Thermo Fisher Scientific).[36]

Neuromuscular junction quantification

Images of FluoroMyelin-stained tissue sections were collected with the PerkinElmer Operetta® CLS™ High Content Analysis system at 20× magnification. Thyroarytenoid muscle cross-sectional area was calculated by viewing the red channel of the image using ImageJ software (version 1.8.0; NIH Image, Bethesda, Maryland, USA) and adjusting the threshold (45–255) of the image. FluoroMyelin cross-sectional area was calculated by determining the area of FluoroMyelin staining by viewing the green channel of the image using ImageJ software and adjusting the threshold of the image (25–255). Percentage of FluoroMyelin expression was calculated as a ratio of the FluoroMyelin-stained area to the total thyroarytenoid muscle area.

Statistical analyses

Percentage of FluoroMyelin expression, and relative gene expression of MyoD1, MyoG, FoxO1 and FBXO32, were categorised based on treatment group (no treatment, carboxymethylcellulose, calcium hydroxyapatite) and study endpoint (4 weeks, 12 weeks). A two-way analysis of variance, followed by Tukey's post-hoc multiple comparisons testing, was conducted with GraphPad Prism statistical software (version 9.3.1 for Windows; GraphPad Software, San Diego, California, USA).

Results

Gene expression

As demonstrated in Figure 1, transcriptional activity associated with muscle growth (MyoD1, MyoG and FoxO1) was, overall, increased in the untreated denervated lateral cricoarytenoid compared to the ipsilateral posterior cricoarytenoid at four weeks post-injection (six weeks post-injury), with an expression ratio of 6.56 (standard deviation (SD) = 10.42) for MyoD1, 0.85 (0.98) for MyoG, and 4.48 (7.59) for FoxO1 (Figure 1). This trend was reversed at 12 weeks post-injection, with expression ratios (SDs) of 0.69 (0.48) for MyoD1, 0.69

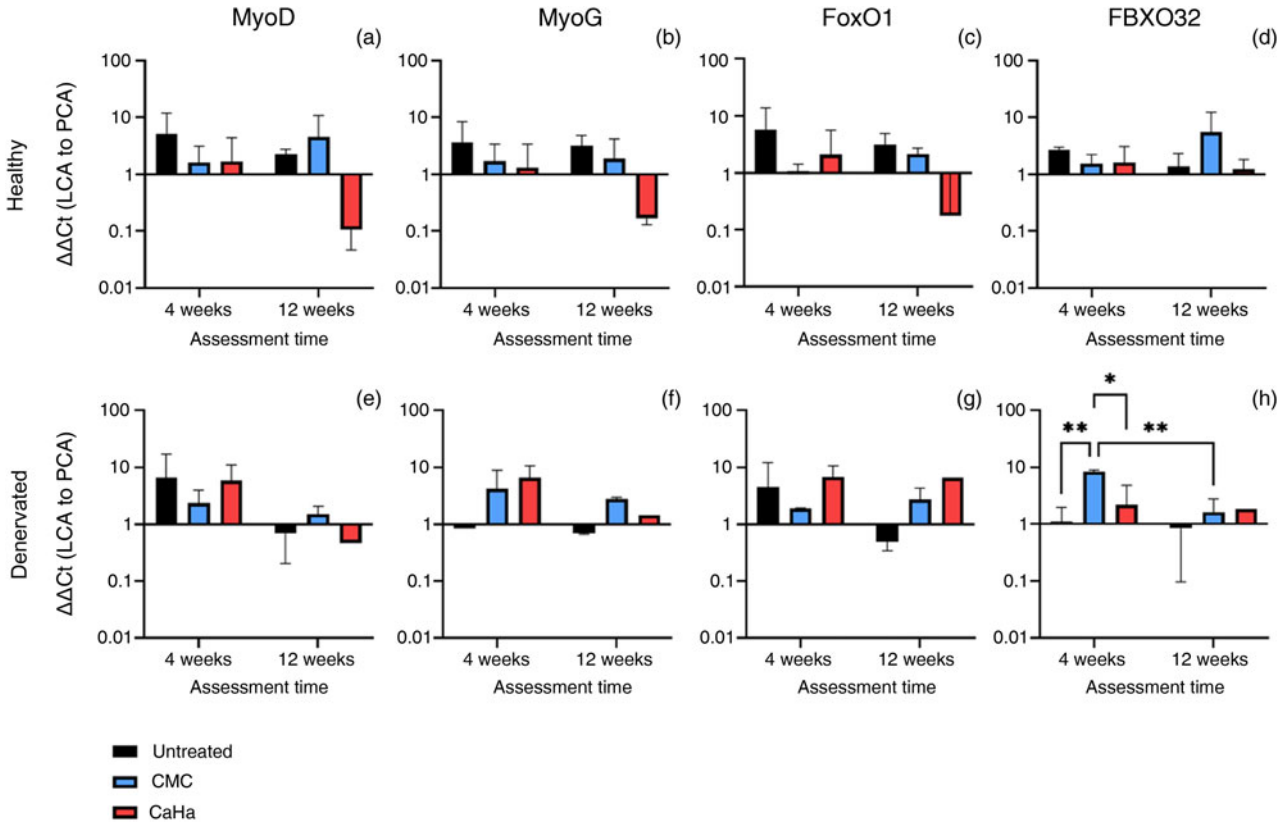

Figure 1. Transcriptional activity of myoblast determination protein 1 (MyoD1), myogenin (MyoG), forkhead box O1 (FoxO1) and F-box only protein 32 (FBXO32), represented as a ratio of lateral cricoarytenoid (LCA) transcriptional activity to posterior cricoarytenoid (PCA) transcriptional activity, for healthy (a–d) and denervated (e–h) vocal folds in untreated and augmented cohorts. *$p < 0.05$; **$p < 0.005$. CMC = carboxymethylcellulose; CaHa = calcium hydroxyapatite; Ct = cycle threshold

https://doi.org/10.1017/S0022215123001135 Published online by Cambridge University Press

(0.02) for MyoG, and 0.49 (0.63) for FoxO1. FBXO32 expression associated with muscle atrophy showed less pronounced overexpression moving toward underexpression in the untreated lateral cricoarytenoid relative to the posterior cricoarytenoid, with an expression ratio (SD) of 1.12 (0.85) at 4 weeks and 0.85 (0.75) at 12 weeks. On the healthy side, both muscle growth and atrophy gene expression were relatively increased in the lateral cricoarytenoid relative to the posterior cricoarytenoid at four weeks, with expression ratios (SDs) of 5.06 (6.82) for MyoD1, 3.65 (4.75) for MyoG, 5.79 (8.09) for FoxO1, and 2.67 (0.33) for FBXO32. This persisted to a lesser degree in augmented animals at 12 weeks post-injection, with expression ratios (SDs) of 2.28 (0.48) for MyoD1, 3.17 (1.62) for MyoG, 3.12 (1.78) for FoxO1, and 1.39 (0.94) for FBXO32. These differences did not achieve statistical significance.

In denervated vocal folds undergoing injection augmentation, FBXO32 showed significantly increased expression with augmentation compared to no treatment, with expression ratios of 8.41 (SD = 0.56, $p < 0.005$) for carboxymethylcellulose augmentation and 2.20 (SD = 2.61, $p < 0.05$) for calcium hydroxyapatite augmentation. At 12 weeks, this overexpression persisted but was significantly reduced in the carboxymethylcellulose-augmented group compared to at 4 weeks, with an expression ratio of 1.62 (SD = 1.15, $p < 0.05$). At 12 weeks, only one vocal fold injected with calcium hydroxyapatite could be analysed, additionally showing reduced expression compared to at 4 weeks, but increased expression relative to untreated vocal folds, with an expression ratio of 1.85. Relative overexpression of myogenic genes in the denervated lateral cricoarytenoid was seen at the four-week timepoint, with expression ratios (SDs) of 2.37 (1.63) for MyoD1, 4.15 (4.71) for MyoG, and 1.91 (0.03) for FoxO1 in the carboxymethylcellulose-injected group, and expression ratios of 5.87 (5.10) for MyoD1, 6.62 (4.11) for MyoG, and 6.82 (3.95) for FoxO1 in the calcium hydroxyapatite injected group. This persisted but was less pronounced at 12 weeks, with expression ratios (SDs) of 1.51 (0.56) for MyoD1, 2.80 (0.02) for MyoG, and 2.71 (1.61) for FoxO1 in the carboxymethylcellulose-augmented group, and expression ratios of 0.47 for MyoD1, 1.45 for MyoG, and 6.59 for FoxO1 in the calcium hydroxyapatite augmented group.

Healthy vocal folds in animals undergoing carboxymethylcellulose injection augmentation revealed relative overall increased growth and atrophy gene expression in the lateral cricoarytenoid relative to the posterior cricoarytenoid, with expression ratios (SDs) of 1.61 (1.48) for MyoD1, 1.70 (1.67) for MyoG, 1.07 (0.34) for FoxO1, and 1.53 (0.72) for FBXO32 at 4 weeks, and expression ratios of 4.54 (6.19) for MyoD1, 1.87 (2.30) for MyoG, 2.14 (0.59) for FoxO1, and 5.52 (6.76) for FBXO32 at 12 weeks. Healthy vocal folds contralateral to calcium hydroxyapatite injection showed increased muscle growth and atrophy gene expression at 4 weeks but decreased muscle growth expression at 12 weeks, with expression ratios (SDs) of 1.67 (2.67) for MyoD1, 1.32 (2.04) for MyoG, 2.10 (3.49) for FoxO1, and 1.61 (0.94) for FBXO32 at 4 weeks, compared to 0.11 (0.06) for MyoD1, 0.17 (0.04) for MyoG, 0.18 (0.20) for FoxO1, and 1.21 (0.61) for FBXO32 at 12 weeks. These differences did not meet statistical significance.

Neuromuscular junction density

Untreated denervated vocal folds demonstrated decreased neuromuscular junction density compared to the contralateral healthy side, with 7.95 ± 12.14 per cent FluoroMyelin Red staining on the denervated vocal folds compared to 31.93 ± 26.33 per cent in the healthy vocal folds at four weeks post-injection (Figure 2). This trend persisted at the 12-week post-injection timepoint, with 11.6 ± 8.65 per cent signal on the denervated side compared to 30.83 ± 15.71 per cent on the healthy side. This difference did not achieve statistical significance until 12 weeks post-augmentation ($p < 0.05$).

Augmented vocal folds demonstrated slightly increased neuromuscular junction density compared to untreated vocal folds on the denervated side at four weeks post-injection, with 16.80 ± 6.68 per cent signal with carboxymethylcellulose and 19.68 ± 14.56 per cent with calcium hydroxyapatite. At 12 weeks post-injection, however, augmented vocal folds demonstrated slightly decreased neuromuscular junction density compared to untreated denervated vocal folds, with 6.92 ± 6.44 per cent signal with carboxymethylcellulose and 1.81 ± 0.444 per cent with calcium hydroxyapatite. Differences between augmented and untreated denervated vocal folds did not achieve statistical significance at either timepoint.

Healthy vocal folds demonstrated decreased neuromuscular junction density in animals undergoing injection augmentation compared to untreated animals at both timepoints, with the carboxymethylcellulose group showing 3.19 ± 6.66 per cent signal and 4.77 ± 5.99 per cent signal, and the calcium hydroxyapatite group showing 20.31 ± 21.36 per cent and 10.52 ± 2.46 per cent signal, at 4 and 12 weeks post-injection respectively. These findings achieved statistical significance at the 12-week timepoint ($p < 0.05$).

Discussion

This is the first study, to the authors' knowledge, to analyse the effect of injection augmentation on laryngeal neuromuscular pathways after unilateral denervation injury. However, the authors would like to acknowledge that this study was limited in the number of test animals, and thus was inadequately powered to detect a difference in gene expression in particular. Staffing issues related to the coronavirus disease 2019 pandemic resulted in some sample degradation and loss, particularly among the calcium hydroxyapatite augmented samples, which further reduces study power. Additionally, several animals required early euthanasia because of the development of pneumonia. Though necropsy typically revealed bilateral lung infections in these cases, and the clinical suspicion was that these infections were due to underlying latent atypical lung infections which are common in this species of farm swine rather than aspiration secondary to the procedure, because of a lack of unoperated controls we were unable to rule out surgery as cause of pneumonia. It should also be noted that, while neuromuscular junction density is a validated method of histologically evaluating denervated muscle, other effects of denervation, such as morphological changes in the neuromuscular junction, were not analysed. Furthermore, we were unable to assess early denervation effects (i.e. within the first two weeks), given that the study design placed injection augmentation at the two-week post-transection timepoint; while this is the most clinically relevant timepoint, further analysis of the effects of ultra-early injection on neuromuscular junction density would contribute to our understanding of this topic. Despite these limitations, the authors feel these data contribute significantly to our understanding of neuromuscular pathways affected by injection augmentation.

Untreated denervated vocal folds demonstrated initial overexpression of myogenic genes at 4 weeks but relative

https://doi.org/10.1017/S0022215123001135 Published online by Cambridge University Press

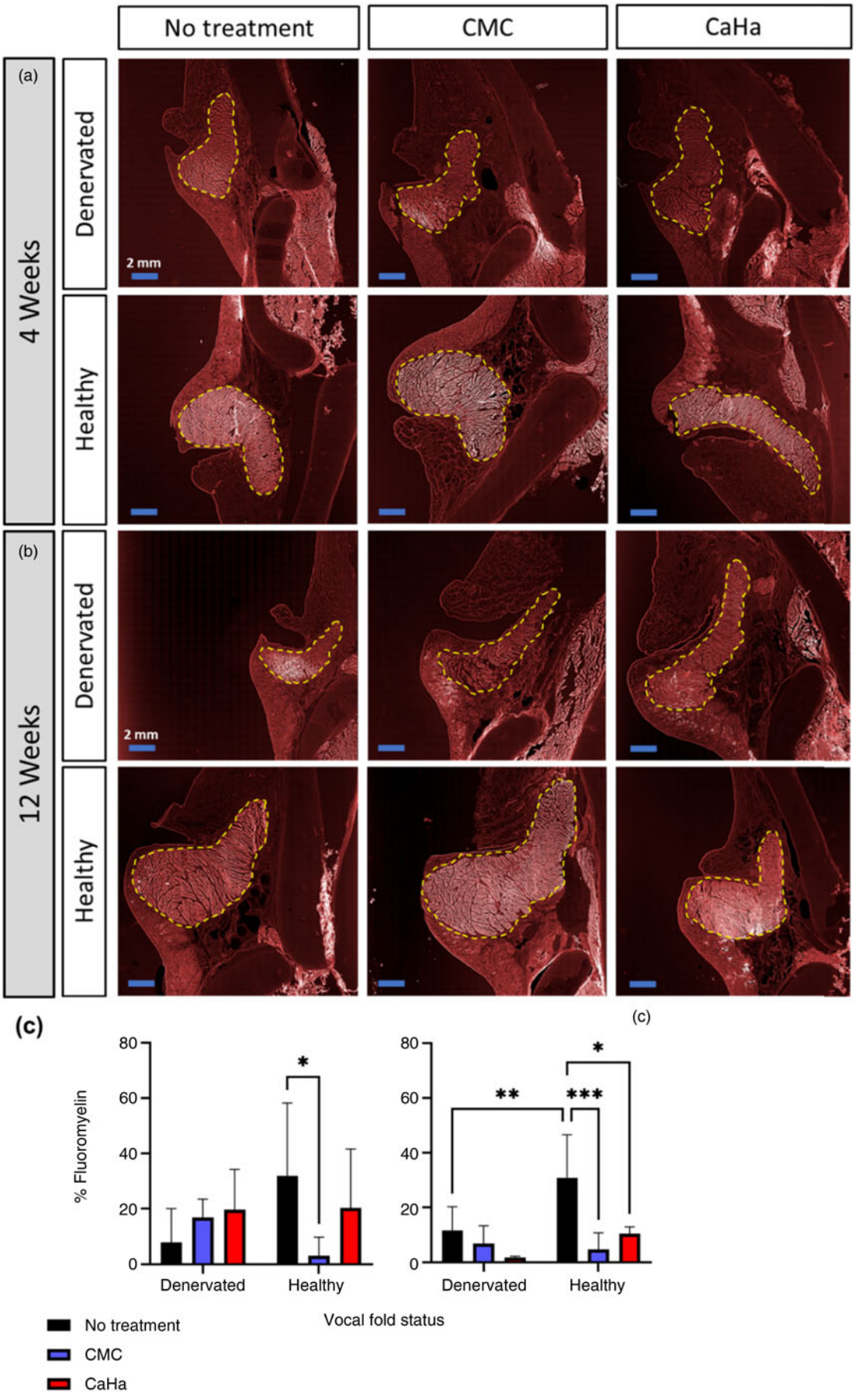

Figure 2. FluoroMyelin Red staining in healthy and denervated vocal fold specimens at 4-week (a) and 12-week (b) timepoints, with yellow dotted lines outlining thyroarytenoid muscle, white denoting increased staining saturation in neuromuscular junctions and red showing background lipid uptake. Overall neuromuscular junction saturation is higher in the healthy tissues, as demonstrated by increased white staining. (c) Neuromuscular junction density, as expressed by percentage of FluoroMyelin Red uptake relative to thyroarytenoid cross-sectional area at 4 and 12 weeks after injection augmentation. *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$. CMC = carboxymethylcellulose; CaHa = calcium hydroxyapatite

https://doi.org/10.1017/S0022215123001135 Published online by Cambridge University Press

underexpression of myogenic genes was noted by 12 weeks, while atrophy-related expression remained largely proportional to the ipsilateral denervated posterior cricoarytenoid. Augmented denervated vocal folds similarly demonstrated an initial upregulation of myogenic genes at four weeks. However, in contrast to untreated vocal folds, this effect appeared to persist at 12 weeks in augmented vocal folds. Similarly, muscle atrophy related gene expression demonstrated overexpression at both the 4- and 12-week timepoints. While not achieving statistical significance, this is thought to indicate increased muscle turnover in augmented vocal folds, as demonstrated with balanced atrophy- and growth-related gene expression, which persists as the injectate dissolves. Furthermore, the relative underexpression of muscle growth related genes at later timepoints not seen with augmentation may suggest that augmentation promotes a downregulation in muscle atrophy pathways. Though no significant differences have been seen in thyroarytenoid size with augmentation compared to no treatment in denervated vocal folds in prior studies, this effect may take longer than 12 weeks to manifest or may be too subtle to achieve significantly different changes in gross muscle size.[14]

Notably, a significant increase in atrophy-related gene expression was seen in carboxymethylcellulose augmentation relative to both calcium hydroxyapatite augmented and untreated denervated vocal folds at four weeks post-injection. However, there was a significant decrease in this atrophy-related gene expression to levels similar to that of calcium hydroxyapatite augmentation at 12 weeks, suggesting little to no lasting difference in gene expression pathways with the different injectate. On the contralateral side, healthy vocal folds demonstrated increased growth- and atrophy-related gene expression at both 4 weeks and 12 weeks, again suggestive of ongoing muscle remodelling associated with compensatory changes. One exception to this was in calcium hydroxyapatite augmented vocal folds, which demonstrated isolated decreased growth-related gene expression at 12 weeks. As only one sample was available for analysis at this timepoint for calcium hydroxyapatite augmented vocal folds, broad conclusions are challenging. Two potential explanations are: (1) that given the otherwise similar response in gene expression and neuromuscular junction density patterns between carboxymethylcellulose and calcium hydroxyapatite, this is an outlier; or (2) the improved stiffness provided by calcium hydroxyapatite augmentation was close to native properties and prevented remodelling.

Neuromuscular junction density was reduced in denervated vocal folds, both untreated and augmented, compared to contralateral healthy vocal folds, at both the 4- and 12-week timepoints, though this only achieved statistical significance at 12 weeks. This is consistent with prior data demonstrating decreased neuromuscular junction density with surgical denervation and atrophy.[21,23,24] Neuromuscular junction density increased in healthy vocal folds contralateral to untreated denervated vocal folds with time, suggestive of increased neural receptiveness, which may be compensatory in nature. Interestingly, neuromuscular junction density was also significantly decreased in healthy vocal folds contralateral to augmentation, compared to healthy vocal folds contralateral to untreated denervated vocal folds, at both the 4- and 12-week timepoints. Furthermore, while not achieving statistical significance, neuromuscular junction density in augmented vocal folds was higher at the 4-week timepoint and lower at the 12-week timepoint compared to untreated denervated vocal folds. This suggests that injection augmentation may ameliorate some of the neuromuscular junction loss seen with denervation at early timepoints when it is still maximally effective (e.g. the four-week timepoint), but this effect wanes with time as the injectate dissolves. This effect is additionally seen on the healthy vocal folds contralateral to the injection augmentation, and may be interpreted as decreased compensatory neuroresponsiveness after injury. It should also be noted that, while these findings appeared to be magnified in both the denervated vocal folds and contralateral healthy vocal folds with calcium hydroxyapatite augmentation, differences between calcium hydroxyapatite and carboxymethylcellulose augmented vocal folds did not achieve statistical significance.

Collectively, these data suggest that injection augmentation may decrease neuromuscular pathways, leading to muscle atrophy on the denervated side. Additionally, overall muscle remodelling of local adductors (lateral cricoarytenoid) appears to be of longer duration and possibly increased magnitude with injection augmentation, and compensatory muscle remodelling on the contralateral healthy side appears to be reduced. In conjunction with previously published data showing decreased vocal fold stiffness changes in injected relative to untreated denervated vocal folds, this suggests that injection augmentation reduces both the biomechanical effects of denervation and influences biochemical pathways associated with reinnervation and muscle remodelling on both the denervated side and contralateral healthy side.[14] These data additionally suggest that future studies analysing vocal fold alterations with therapy should not assume an untreated side may serve as a negative control, given apparently strong interactions between right and left vocal folds. Finally, whether these alterations in biochemical pathways can be further harnessed with bioactive rather than bioinert injectate is a question of great academic and clinical significance, and may yield very interesting results in future studies.

- Temporary injection augmentation is frequently employed for voice rehabilitation after unilateral vocal fold paralysis, particularly within the first year after symptom onset
- Possible long-term functional benefit from temporary injection augmentation exists, suggesting alteration in vocal fold structure persisting after injectate resorption
- Denervated vocal folds demonstrated reduced expression of muscle growth and atrophy-related genes, with overexpression of these genes after augmentation
- Healthy vocal folds showed increased early and late muscle growth- and atrophy-related gene expression in all animals, suggestive of compensatory remodelling
- Neuromuscular junction density decline was slower in augmented than untreated denervated vocal folds, and was significantly reduced in healthy vocal folds contralateral to augmentation
- Thus, injection augmentation may have decreased neuromuscular pathways, leading to muscle atrophy on the denervated side and reduced compensatory muscle remodelling on the contralateral healthy side

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022215123001135.

Competing interests. Financial support received from: United States Air Force 59th Clinical Research Division at Wilford Hall Ambulatory Surgery Center Graduate Medical Education Funding, University of Texas at San Antonio Office of the Vice President of Research Intramural Funding. The views expressed in this manuscript are those of the authors and do not reflect the official policy or position of the Department of the Army, Department of the Air Force, Department of Defense or the US Government. A Isaac was supported by K12 GM111726.

References

1 Courey MS, Naunheim MR. Injection laryngoplasty for management of neurological vocal fold immobility. *Adv Otorhinolaryngol* 2020;**85**:68–84

2 Kwon TK, Buckmire R. Injection laryngoplasty for management of unilateral vocal fold paralysis. *Curr Opin Otolaryngol Head Neck Surg* 2004;**12**:538–42

3 Bouhabel S, Hartnick CJ. Current trends in practices in the treatment of pediatric unilateral vocal fold immobility: a survey on injections, thyroplasty and nerve reinnervation. *Int J Pediatr Otorhinolaryngol* 2018;**109**:115–18

4 Francis DO, Williamson K, Hovis K, Gelbard A, Merati AL, Penson DF *et al.* Effect of injection augmentation on need for framework surgery in unilateral vocal fold paralysis. *Laryngoscope* 2016;**126**:128–34

5 Vila PM, Bhatt NK, Paniello RC. Early-injection laryngoplasty may lower risk of thyroplasty: a systematic review and meta-analysis. *Laryngoscope* 2018;**128**:935–40

6 Husain S, Sadoughi B, Mor N, Levin AM, Sulica L. Time course of recovery of idiopathic vocal fold paralysis. *Laryngoscope* 2018;**128**:148–52

7 Husain S, Sadoughi B, Mor N, Sulica L. Time course of recovery of iatrogenic vocal fold paralysis. *Laryngoscope* 2019;**129**:1159–63

8 Silva Merea V, Husain S, Sulica L. Medialization laryngoplasty after injection augmentation. *J Voice* 2018;**32**:249–55

9 Yung KC, Likhterov I, Courey MS. Effect of temporary vocal fold injection medialization on the rate of permanent medialization laryngoplasty in unilateral vocal fold paralysis patients. *Laryngoscope* 2011;**121**:2191–4

10 Alghonaim Y, Roskies M, Kost K, Young J. Evaluating the timing of injection laryngoplasty for vocal fold paralysis in an attempt to avoid future type 1 thyroplasty. *J Otolaryngol Head Neck Surg* 2013;**42**:24

11 Fang T-J. Intracordal hyaluronic acid injection during spontaneous reinnervation stage for unilateral recurrent laryngeal nerve paralysis. *Otolaryngol Head Neck Surg* 2013;**149**:P207–8

12 Friedman AD, Burns JA, Heaton JT, Zeitels SM. Early versus late injection medialization for unilateral vocal cord paralysis. *Laryngoscope* 2010;**120**:2042–6

13 Dedry M, Dricot L, Van Parys V, Boucquey D, Delinte N, van Lith-Bijl J *et al.* Brain adaptation following various unilateral vocal fold paralysis treatments: a magnetic resonance imaging based longitudinal case series. *Front Neurosci* 2022;**16**:947390

14 Miar S, Walters B, Gonzales G, Malka R, Baker A, Guda T *et al.* Augmentation and vocal fold biomechanics in a recurrent laryngeal nerve injury model. *Laryngoscope Investig Otolaryngol* 2022;**7**:1057–64

15 Hernandez-Morato I, Sharma S, Pitman MJ. Changes in neurotrophic factors of adult rat laryngeal muscles during nerve regeneration. *Neuroscience* 2016;**333**:44–53

16 Hernandez-Morato I, Tewari I, Sharma S, Pitman MJ. Blockade of glial-derived neurotrophic factor in laryngeal muscles promotes appropriate reinnervation. *Laryngoscope* 2016;**126**:E337–42

17 Wang B, Yuan J, Xu J, Xie J, Wang G, Dong P. Neurotrophin expression and laryngeal muscle pathophysiology following recurrent laryngeal nerve transection. *Mol Med Rep* 2016;**13**:1234–42

18 Zammit PS. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Semin Cell Dev Biol* 2017;**72**:19–32

19 Lee S, Dong HH. FoxO integration of insulin signaling with glucose and lipid metabolism. *J Endocrinol* 2017;**233**:R67–79

20 Bodine SC, Baehr LM. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1. *Am J Physiol Endocrinol Metab* 2014;**307**:E469–84

21 Yampolsky P, Pacifici PG, Witzemann V. Differential muscle-driven synaptic remodeling in the neuromuscular junction after denervation. *Eur J Neurosci* 2010;**31**:646–58

22 Huang X, Jiang J, Xu J. Denervation-related neuromuscular junction changes: from degeneration to regeneration. *Front Mol Neurosci* 2022;**14**:810919

23 McMullen CA, Andrade FH. Functional and morphological evidence of age-related denervation in rat laryngeal muscles. *J Gerontol A Biol Sci Med Sci* 2009;**64**:435–42

24 Connor NP, Suzuki T, Sewall GK, Lee K, Heisey DM. Neuromuscular junction changes in aged rat thyroarytenoid muscle. *Ann Otol Rhinol Laryngol* 2002;**111**:579–86

25 Ditsworth D, Maldonado M, McAlonis-Downes M, Sun S, Seelman A, Drenner K *et al.* Mutant TDP-43 within motor neurons drives disease onset but not progression in amyotrophic lateral sclerosis. *Acta Neuropathol* 2017;**133**:907–22

26 Ling S-C, Dastidar SG, Tokunaga S, Ho WY, Lim K, Ilieva H *et al.* Overriding FUS autoregulation in mice triggers gain-of-toxic dysfunctions in RNA metabolism and autophagy-lysosome axis. *eLife* 2019;**8**:e40811

27 Wang W, Kang S, Coto Hernandez I, Jowett N. A rapid protocol for intraoperative assessment of peripheral nerve myelinated axon count and its application to cross-facial nerve grafting. *Plast Reconstr Surg* 2019;**143**:771–8

28 Mohan S, Coto Hernández I, Selig MK, Shibata S, Jowett N. Stain-free resolution of unmyelinated axons in transgenic mice using fluorescence microscopy. *J Neuropathol Exp Neurol* 2019;**78**:1178–80

29 Weisberg NK, Spengler DM, Netterville JL. Stretch-induced nerve injury as a cause of paralysis secondary to the anterior cervical approach. *Otolaryngol Head Neck Surg* 1997;**116**:317–26

30 Beutler WJ, Sweeney CA, Connolly PJ. Recurrent laryngeal nerve injury with anterior cervical spine surgery risk with laterality of surgical approach. *Spine (Phila Pa 1976)* 2001;**26**:1337–42

31 Netterville JL, Koriwchak MJ, Winkle M, Courey MS, Ossoff RH. Vocal fold paralysis following the anterior approach to the cervical spine. *Ann Otol Rhinol Laryngol* 1996;**105**:85–91

32 Alimoglu O, Akdag M, Kaya B, Eryilmaz R, Okan I, Coskun A *et al.* Recurrent laryngeal nerve palsy after thyroid surgery. *Int Surg* 2008;**93**:257–60

33 Serpell JW, Lee JC, Yeung MJ, Grodski S, Johnson W, Bailey M. Differential recurrent laryngeal nerve palsy rates after thyroidectomy. *Surgery* 2014;**156**:1157–66

34 Dion GR, Benedict PA, Coelho PG, Amin MR, Branski RC. Impact of medialization laryngoplasty on dynamic nanomechanical vocal fold structure properties. *Laryngoscope* 2018;**128**:1163–9

35 Dion GR, Coelho PG, Teng S, Janal MN, Amin MR, Branski RC. Dynamic nanomechanical analysis of the vocal fold structure in excised larynges. *Laryngoscope* 2017;**127**:E225–30

36 García-García ÓD, Weiss T, Chato-Astrain J, Raimondo S, Carriel V. Staining methods for normal and regenerative myelin in the nervous system. *Methods Mol Biol* 2023;**2566**:187–203

https://doi.org/10.1017/S0022215123001135 Published online by Cambridge University Press