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Improved Adductor Function After Canine Recurrent Laryngeal Nerve Injury and Repair Using Muscle Progenitor Cells

Randal C. Paniello, MD PhD¹, Sarah Brookes, DVM², Neel K. Bhatt, MD¹, Khadijeh Bijangi-Vishehsaraei, PhD³, Hongji Zhang, PhD², Stacey Halum, MD²

¹Department of Otolaryngology – Head and Neck Surgery, Washington University School of Medicine, St. Louis, MO

²Department of Otolaryngology – Head and Neck Surgery, Purdue University, Indianapolis, IN

³Department of Otolaryngology – Head and Neck Surgery, Indiana University, Indianapolis, IN

Abstract

Objective—Muscle progenitor cells (MPCs) can be isolated from muscle samples and grown to a critical mass in culture. They have been shown to survive and integrate when implanted into rat laryngeal muscles. In this study, the ability of MPC implants to enhance adductor function of reinnervated thyroarytenoid muscles was tested in a canine model.

Study Design—Animal study.

Methods—Sternocleidomastoid muscle samples were harvested from three canines. MPCs were isolated and cultured to 10⁷ cells over 4–5 weeks, then implanted into right thyroarytenoid muscles after ipsilateral recurrent laryngeal nerve transection and repair. The left sides underwent the same nerve injury, but no cells were implanted. Laryngeal adductor force was measured pre-treatment and again six months later, and the muscles were harvested for histology.

Results—MPCs were successfully cultured from all dogs. Laryngeal adductor force measurements averaged 60% of their baseline pre-treatment values in non-implanted controls, 98% after implantation with MPCs, and 128% after implantation with motor endplate-enhanced MPCs. Histology confirmed the implanted MPCs survived, became integrated into thyroarytenoid muscle fibers, and were in close contact with nerve endings, suggesting functional innervation.

Conclusion—MPCs were shown to significantly enhance adductor function in this pilot canine study. Patient-specific MPC implantation could potentially be used to improve laryngeal function in patients with vocal fold paresis/paralysis, atrophy, and other conditions. Further experiments are planned.

Level of Evidence—N/A (animal study)

Correspondence: Randal C. Paniello, MD PhD, Department of Otolaryngology – Head & Neck Surgery, Washington University School of Medicine, 660 South Euclid Avenue, Box 8115, St. Louis MO 63110, Telephone: (314) 362-7252, Fax: (314) 362-7522, paniellor@ent.wustl.edu.

Conflicts of Interest: None

Keywords

larynx; recurrent laryngeal nerve; stem cells; paralysis; innervation

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INTRODUCTION

Restoration of movement of the paralyzed vocal fold has long been a goal of laryngologists treating vocal fold paralysis, but reports of successful restoration of vocal fold mobility have been quite limited. Purposeful vocal fold abduction¹ and adduction² have been achieved with reinnervation methods, but these procedures have not gained wide acceptance due to technical difficulty or donor site morbidity. The ideal procedure would restore mobility and laryngeal muscle mass in a high percentage of cases while being technically within the skillset of most otolaryngologists. Restoration of abductor movement would be particularly valuable for patients with bilateral vocal fold immobility, who often need a tracheostomy or other procedure for adequate airway.

Muscle progenitor cells (MPCs) (also called muscle stem cells) consist of satellite cells and myoblasts, and have the potential to increase muscle mass and to provide the stimulus for functional reinnervation when implanted into denervated muscles.^{3–5} MPCs can be derived from small samples of a patient's own tissue, and, thus, are not rejected by the immune system. They can be implanted into the laryngeal muscle by a simple injection, making this approach an attractive option for treating laryngeal paralysis.

Several investigators have found that implanted MPCs improve cardiac function and muscle mass following myocardial infarction.^{6–8} Plowman et al. reported attenuation of atrophy, increased myofiber diameter, and increased maximal contraction force after implantation of MPCs into partially denervated sheep tongues.⁹ Halum et al. have successfully cultured MPCs from rat skeletal muscle and implanted them into denervated thyroarytenoid muscles. They found the injected MPCs survived in their new muscle, attenuated atrophy, fused with the native myofibers, and enhanced reinnervation when programmed to express neurotrophic factor.^{10–12} The strength of the MPC-treated thyroarytenoid muscles could not be assessed in this rat model, however.

We wished to extend this study to a larger animal model, one more similar to the human larynx, so that functional measures of motor strength could be assessed after MPCs are therapeutically introduced into denervated adductor muscles. In this study, the Paniello lab, with experience in measuring laryngeal function in the canine model,^{13,14} collaborated with the Halum lab, with their extensive experience in cultivating MPCs as outlined above. We hypothesized that, in a canine model of vocal fold paralysis, MPCs implanted into the denervated thyroarytenoid muscle would enhance adductor function as measured by laryngeal adductor pressure (LAP) and glottic closing force (GCF).¹³

MATERIALS & METHODS

Three purpose-bred mongrel hounds weighing about 20 kg were obtained and housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care.

The study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.); the animal-use protocol was approved by the Institutional Animal Care and Use Committee of Washington University School of Medicine.

Initial Procedure – Baseline Data and Muscle Harvest

Under general anesthesia, a midline incision exposed the larynx and trachea. A tracheostomy was made between rings 8–12 as previously described.¹⁵ Both recurrent laryngeal nerves (RLNs) were dissected, fitted with Harvard electrodes, and connected to a custom constant-current laryngeal nerve stimulator.

Pretreatment baseline laryngeal adductor function was measured in two ways.¹³ First, LAPs were determined as previously described. Briefly, the cuff of an endotracheal tube is connected to a pressure transducer, and the tube is passed between the vocal folds while the RLN is stimulated at supramaximal current. Pressure measurements are made at each frequency from 20–100 Hz at 10 Hz intervals, and the unstimulated baseline pressure is subtracted. Laryngeal adductor muscles reach tetany at higher frequencies (70–100 Hz). Second, GCF was measured as previously described. Briefly, a suture is passed through a lateral minithyrotomy, through the ventricle, around the vocal process and back, forming a loop that is hooked onto a force gauge. The RLN is stimulated as described above and the force is recorded. The GCF and LAP have been shown to be highly correlated.¹³

Under an operating microscope, each RLN was transected 5 cm inferior to the cricothyroid joint and then immediately repaired using 9–0 nylon sutures for epineural anastomosis. A 3–4 gm portion of sternocleidomastoid muscle was harvested and placed in initial myogenic culture medium [F-10 medium (Gibco, Grand Island, New York; 11550–043), 20% fetal bovine serum (HyClone Laboratories, Thermo Fisher Scientific, Waltham, Massachusetts; SH30070.03), 1% penicillin/streptomycin/amphotericin B (Cellgro; Mediatech, Inc, Manassas, Virginia; 30–004-CI), and 1% chicken embryo extract (SeraLab, Haywards Heath, England; CE-650-J)]. The stoma was matured, the wound was closed and the dog recovered. The muscle sample was same-day shipped on ice to the Halum cell culture laboratory for derivation of MPCs.

MPC Cultures

The MPC culture techniques were followed as previously described.^{10,11} Briefly, the muscle sample was minced into small pieces and incubated in 0.2% collagenase type I (Worthington Biochemical Corp, Lakewood, New Jersey; LS004214) in a shaker at 37°C for 2 hours. Digested tissue was subjected to rigorous pipetting to dissociate fibers, and then filtered through a 100 nm pore-size strainer. The pellet was suspended in initial myogenic culture medium and seeded into a gelatin coated T25 flask. Fresh medium was added every other day. When primary cultures reached 70% confluency, they were passaged to prevent myotube formation. After the second passage the growth medium was changed to a myogenic culture medium (F-10 medium Gibco, Grand Island, New York; 11550–043), 10%

fetal bovine serum (HyClone Laboratories; SH30070.03) and 1% penicillin/streptomycin (HyClone Laboratories; J110381)).

Cells were labeled for subsequent identification in one of two ways. For dog 1, MPCs were transduced with green fluorescent protein (GFP)- expressing lentiviral vector at passage 2 in the presence of 8 µg/mL protamine sulfate (Sigma-Aldrich, p4020). For dogs 2 and 3, the MPCs were incubated with the fluorescent marker QTracker 565 (Molecular Probes; Q25001MP) for 60 minutes at 37°C. After incubation, the cells were washed twice with complete growth medium. Label uptake was confirmed with fluorescent microscopy.

To induce motor endplate expression (dog 3), acetylcholine chloride (40 nmol/L; Tocris Bioscience, Bristol, England; 2809), agrin (10 nmol/L; R&D Systems, Minneapolis, Minnesota; 550-AG), and neuregulin (2 nmol/L; R&D Systems; 378-SM), was added to culture medium and the culture continued for 7 days. These MPCs are referred to as motor endplate enhanced cells (MEEs).

When the cultures reached approximately 10^7 cells (within 4–5 weeks) they were shipped on ice back to the canine laryngeal physiology lab at Washington U.

Second Procedure - MPC Implantation

The MPCs were washed several times in PBS, then spun gently into a pellet with a volume of 0.5cc. The dog was placed under general anesthesia, intubated using the permanent stoma. Direct laryngoscopy was performed and the scope was suspended. An 18G angiocatheter was passed through the skin, through the cricothyroid membrane, and into the thyroarytenoid and possibly lateral cricoarytenoid muscles. The MPC syringe was attached and the cells were implanted, followed by a 0.5cc flush of normal saline. The dog was awakened and recovered.

Third Procedure – Final Data Collection

The first experiment (dog 1) was carried out only to confirm success of the process and viability of the transferred MPCs; the dog was euthanized 2 weeks following MPC implantation and the larynx was harvested for histologic study. Functional data was not collected.

Long term functional experiments were carried out for dogs 2 and 3. Six months after nerve transection and repair (5 months post- MPC implantation), the awake dog was examined for spontaneous vocal fold motion by inserting a scope through the tracheostomy and visualizing the vocal folds from below (“infraglottic exam”). Vocal fold movement was induced by introducing a few cc’s of water into the mouth from a syringe, causing the dog to swallow. Movement was scored on a scale of 0 (no movement) to 4 (complete adduction).

Next, the dog was anesthetized and the neck opened in the midline. Each RLN was dissected and an electrode placed 10cm inferior to the cricothyroid joint (dogs 2 and 3). Direct laryngoscopy was performed and the stimulated motion of the vocal folds was observed, videorecorded and scored on the same 0–4 scale. LAP and GCF were measured as described

above. The larynx was then harvested, placed in 4% paraformaldehyde and shipped to the Halum lab.

Histological and Immunohistochemistry(IHC) analysis

Larynges were fixed with 4% paraformaldehyde in PBS for 24 hours, then changed to 30% sucrose in PBS solution until tissues sunk to the bottom. Cryo-embedded sections were cut at a thickness of 12–14µm with the cryotome. Standard hematoxylin and eosin (H&E) staining was performed. For GFP analysis, unstained frozen sections were evaluated under fluorescent microscopy to evaluate for areas of green fluorescence, and IHC was performed with anti-GFP antibody to ensure the green fluorescence represented GFP (not nonspecific fluorescence).

For additional analysis of the motor endplates (staining with β III tubulin) with neuronal contact (staining with α -bungarotoxin) sections were permeabilized with Triton X-100 for 20 minutes at room temperature and then blocked with 1% BSA for 1 hour. Sections were then incubated with AlexaFluor 493 conjugated β III tubulin antibody (1:10) and AlexaFluor 647 conjugated α -bungarotoxin (1:1000) overnight at 4°C, then examined by fluorescent microscopy.

RESULTS

MPCs were successfully isolated and cultured from all 3 dogs. Dogs 1 and 2 were implanted with 10×10^6 MPCs; dog 3 received 12×10^6 MEEs.

Histology and Immunohistochemistry

The GFP and QTracker 545 fluorescent markers labels were present throughout the cultured cells, as seen on fluorescent microscopy (Fig. 1), suggesting effective cell labelling. In the harvested thyroarytenoid muscles, the fluorescent labels showed good survival of the implanted MPCs, with no label seen in the non-injected control muscles (Figs. 2–3). The muscle fibers that had MPCs incorporated tended to have larger diameters than the non-labeled fibers in all three dogs. The animal receiving the MEEs demonstrated areas of dense motor endplate expression with complexes of motor endplates which were fully innervated based on neuronal contact (Fig. 3). These areas of dense motor endplate expression corresponded well with the areas of fluorescent label (cell tracker) suggesting the changes occurred secondary to survival of the MEEs in those areas. Because of the large size of the canine adductor complex, targeted sectioning was performed through the anterior and mid body of the TA muscle (the site of MPC injection). While most of the sections demonstrated areas of fluorescence (cell survival), the size of the muscles prohibited accurate quantification.

Functional Measures (dogs 2 and 3)

Spontaneous adduction during swallow was seen in the right (MPC injected) vocal fold of both dogs, but not on the left (control) side. When the RLNs were stimulated with supramaximal constant current, movement was seen in both vocal folds, but with a significantly more normal range of motion on the MPC side (Table 1).

The measures of vocal fold adductor strength, LAP and GCF, both showed significantly more recovery in the MPC injected side than the control side (Figure 4). The MPCs implanted in dog 2 led to a recovery of adductor strength to normal (pre-treatment) levels by both measures; the saline control had typical recovery for the transection-repair nerve injury model. Dog 3 received motor endplate-enhanced cells, and recovered adductor strength to 28% higher than normal (by LAP); the GCF measure was even higher. These results are summarized in Table 1.

DISCUSSION

In this limited pilot study, implantation of autologous-derived muscle progenitor cells into denervated thyroarytenoid and possibly lateral cricoarytenoid muscles resulted in greater functional reinnervation than similar experiments without these cells. More significantly, purposeful adduction of the vocal fold was observed with a glottic closure reflex on swallow. This effect appears to be due to both increased muscle mass, as evidenced by increased myofiber diameter, as well as increased innervation (based on motor endplate-to-nerve contact), with a further increase when motor endplate expression was enhanced in dog 3. These data support a potential role for MPC implantation in the treatment of patients with RLN injury.

Kodama et al found significant increases in muscle transcription factors MyoD and M-cadherin in denervated rat thyroarytenoid muscle, which return to baseline after nerve recovery.¹⁶ These factors activate native MPCs and stimulate their proliferation. Cultured MPCs added to the denervated muscle would be expected to respond similarly to these transcription factors, as apparently occurred in these experiments. While we did not specifically study the relationship between innervation status and MPC survival, there was a mosaic pattern of uptake throughout the muscle rather than uniform uptake, supporting the concept that the innervation status may have played a role in the MPC-myofiber fusion process.

The RLN has a strong tendency to regenerate after injury, but the adductor and abductor fibers reinnervate the laryngeal muscles randomly, resulting in a range of potential outcomes.¹⁷ By implanting MPCs into only one muscle, we have the potential to “tip the scale” in favor of adduction for unilateral cases, or abduction in bilateral cases. The scale could be further tipped by inhibiting the antagonistic muscle from reinnervating, for example by early injection of vincristine. We have previously reported that adductor function improved when reinnervation of the posterior cricoarytenoid was inhibited by injecting it with vincristine.^{12,18}

Further experiments examining variables such as timing, number of cells to implant, the roles of other nerve and muscle growth factors, the magnitude of effect with other reinnervation methods such as ansa cervicalis, the effect on voice production, and the effect of MPC implantation into the posterior cricoarytenoid muscle, are planned. The procedure to implant these cells is fairly simple and should be within the skillset of any otolaryngologist that performs vocal fold injections. Commercial entities have already begun offering a

service to extract and culture MPCs from muscle samples. Thus, this approach may be closer to the ideal treatment for vocal paralysis.

CONCLUSION

Implantation of autologous-derived muscle progenitor cells was found to significantly increase adductor strength in a canine model of RLN transection and repair. Further work is warranted to evaluate this approach as a potential new therapy for vocal fold paralysis.

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Figure 1.
Dog 1 MPCs in culture under fluorescent microscopy. Left, 20x; right, 40x. The green fluorescence indicates successful GFP expression within the cells.

Figure 2.

Dog 1 muscle fiber specimen (14μm section thickness) imaged at 10x magnification (left) with magnified immunohistochemistry (IHC) views (right). (A) Fluorescent microscopy cross sectional image of negative myofiber control for GFP protein (B) IHC of negative control (longitudinal sectioning) demonstrates no anti-GFP staining. (C) Canine thyroarytenoid muscle under fluorescent microscopy in the area of injection demonstrates multiple GFP+ areas (bracket), with the GFP+ fibers [those that had fused with MPCs] demonstrating visibly larger myofiber diameters than adjacent areas, suggesting that the MPC fusion with myofibers enlarges the myofiber diameter. (D) Thyroarytenoid muscle injected with GFP+ MPCs on bright field mode microscopy demonstrates peripheral myofiber staining with anti-GFP (arrows; dark brown), with no GFP detected at the center of the myofiber (*), confirming peripheral fusion of the MPCs with the thyroarytenoid myofibers.

Figure 3.

Dog 3 muscle fiber specimen (12 μ m section thickness) imaged at low magnification with immunohistochemical analysis demonstrating motor endplates stained with bungarotoxin in red (depicted with white arrows) and neuron specific beta3-tubulin in green. Left, control side with rare motor endplates. Center, MEE side with densely distributed motor endplates (arrows) suggesting the MEE injection group resulted in a stable increased motor endplate expression. Right, a magnified (40x) view of the MEE injection group demonstrates a dense complex of innervated motor endplates (bracket) with the neuronal supply depicted with an arrow.

Figure 4.

Laryngeal adductor pressure (LAP) curves for dogs 2 and 3. Control sides (left) were implanted with normal saline; experimental sides (right) were implanted with MPCs (dog 2, top) or MEEs (dog 3, bottom). Pre-treatment measurements (open diamonds) have typical curves with plateau at 70–100 Hz. Six-month post-treatment measurements (solid circles) averaged about 60% of initial LAP plateau values for controls, but reached 98% for MPC-implanted dog 2 and 128% for MEE-implanted dog 3.

Table 1.

Functional measurements from dogs 2 and 3. Laryngeal adductor pressure (LAP) and glottic closing force (GCF) results expressed as proportion of pre-treatment measures. Dogs were implanted with autologous cultured muscle progenitor cells (MPCs) or MPCs with enhanced motor endplate expression (MEEs). Movement scores on scale of 0 (no movement) to 4 (normal movement).

			Injected	LAP	GCF	Vocal Fold Movement	
						Spont.	Induced
Dog 2	Rt	MPCs		<i>0.98</i>	<i>1.00</i>	3	4
	Lt	saline		0.63	0.67	0	2
Dog 3	Rt	MEEs		<i>1.28</i>	<i>1.44</i>	4	4
	Lt	saline		0.60	0.55	1	2