Functional Electrical Stimulation following nerve injury in a Large Animal Model

Marta Cercone[1], DVM, PhD, Jonathan Jarvis[2], PhD, Norm Ducharme[1], DVM, MS, Justin Perkins[3], BVet Med, MS, Richard Piercy[3], MA VetMB, PhD, Michael Willand[4], MASc, PhD, Lisa Mitchell[1], Michael Sledziona[1], Leo Soderholm[1], Jon Cheetham*[1], VetMB, PhD

1. Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

2. John Moores University, Liverpool, UK

3. Comparative Neuromuscular Diseases Laboratory, Department of Clinical Sciences and Services Royal Veterinary College, London, UK

4. Department of Surgery, Division of Plastic Reconstructive Surgery, The Hospital for Sick Children, Toronto, Canada

***Corresponding Author:** Jonathan Cheetham

Department of Clinical Sciences, Cornell University, Ithaca, NY 14853, USA

telephone: 607.253.3100; E-mail: jc485@cornell.edu

Running Title: FES during PCA Reinnervation

Acknowledgements: This study was funded by Med-El Elektromedizinische Geräte GmbH, Innsbruck, Austria.

Number of Words in Abstract: 150

Number of Words in Manuscript: 3992

Ethical Publication Statement: We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Disclosure of Conflicts of Interests: None of the authors has any conflict of interest to disclose.

Functional Electrical Stimulation following nerve injury in a Large Animal Model

ABSTRACT:

Introduction: Functional electrical stimulation (FES) effects on reinnervation of the posterior cricoarytenoid muscle (PCA) were evaluated following recurrent laryngeal nerve (RLn) axonotmesis.

Methods: Right RLn cryo-injury and intramuscular electrodes implantation in ipsilateral PCA were performed in 15 horses. PCA was stimulated for 20weeks in eight animals; seven were used as controls. Reinnervation was monitored through muscle response to hypercapnia, electrical stimulation and exercise. At the end of the study, muscle fiber type proportions and minimum fiber diameters (MFD), and RLn axon number and degree of myelination were determined.

Results: Laryngeal function returned normal in both groups within 22weeks. FES improved muscle strength and geometry, and induced an increased type I:II fiber proportion ($p=0.038$) in the stimulated PCA. FES showed no deleterious effects on reinnervation.

Discussion: Intramuscular electrical stimulation did not delay PCA reinnervation after axonotmesis. FES can represent a supportive treatment to promote laryngeal functional recovery after RLn injury.

Keywords: larynx; posterior cricoarytenoid muscle; functional electrical stimulation; cryo injury; reinnervation; equine

Introduction

Functional recovery of muscles after denervation caused by peripheral nerve injury can be compromised by several factors including axons failing to reach the muscle fibers and inability of muscle fibers to recover from denervation atrophy.[1] Following peripheral nerve injury, muscle mass decreases significantly even after immediate nerve repair, and significant muscle changes occur after 1 month of denervation precluding full recovery.[2] Functional electrical stimulation (FES) has proved to have beneficial effects in improving mass and ultrastructural characteristics in long term denervated muscles.[3,4] Moreover, evidence of the FES positive effect on muscle apoptosis modulation,[5] muscle receptivity to regenerating axons,[6] and recovery of size, functional and histochemical muscle properties during reinnervation,[7] have been recently described. However, despite the literature supporting the positive effect of FES, a number of studies describe detrimental effects of FES following peripheral nerve injury.[8–10] Additionally, it has been shown that increased neuromuscular activity of partially denervated muscle, prevents Schwann cells bridge formation resulting in a decrease of terminal axonal sprouting (though not nodal sprouting) which is often incorrectly associated with a supposed detrimental effect of FES on reinnervation.[11,12]

Currently, there is limited information regarding the appropriate timeline or the effects of stimulating a muscle prior and during the reinnervation period. Zealear et al. demonstrated that chronic stimulation of the canine *posterior cricoarytenoid* muscle (PCA) increases reinnervation magnitude and significantly promotes selective appropriate reinnervation after Recurrent Laryngeal nerve (RLn) section and

anastomosis.[13] Others have shown that laryngeal pacing based on neuroprosthetic devices restores effective ventilation in patients with vocal cord paralysis (VCP), both in humans and animals.[14–20]

The larynx is a critical component of the respiratory system, regulating airflow and preventing aspiration of pharyngeal contents during swallowing. Impairment of laryngeal functions following RLn injury can lead to devastating consequences including VCP, dyspnea and dysphagia. The PCA is the sole arytenoid abductor, responsible for maintaining glottic patency, and preservation or restoration of its function is essential to avoid the need for tracheostomy in the case of bilateral VCP.

In a previous study, we demonstrated that electrical stimulation can arrest and reverse the atrophic consequences of denervation on the equine PCA muscle after RLn transection.[21] The horse has been used as model for studying laryngeal paralysis as the neuroanatomy, physiology and outcome measures are well described.[22,23] Furthermore, PCA electrical stimulation maintains airway patency during strenuous exercise in horses with induced transient laryngeal paralysis.[24]

Given the number of contradictory studies in small animal models, our group decided to investigate the effects of daily FES on the PCA reinnervation following injury to the RLn. In this study, we hypothesized that intramuscular electrical stimulation of the PCA would not delay its rate of reinnervation after axonotmesis.

METHODS

This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, federal and state regulations, and was approved by the Cornell University Institutional Animal Care and Use Committee (IACUC).

Experimental design. Fifteen adult Thoroughbred horses (8 females, 7 castrated males; age 7.3 ± 2.5 years; weight 518 ± 34 kg) with normal laryngeal function (Havemayer grade ≤ II at rest, grade A during exercise)[25] were instrumented with two intramuscular electrodes in the right PCA, connected to a customized stimulator implanted subcutaneously in the cervical area. The system allowed daily electrical activation to the PCA. After right RLn freezing injury, muscle native function and response to stimulation were evaluated over 22 weeks to monitor the reinnervation process.

Surgical procedure. Horses were maintained under general inhalatory anesthesia in left lateral recumbency. A videoendoscope was placed through the right nostril into the nasopharynx to monitor arytenoid movement during surgery. The laryngeal area was clipped and prepared for aseptic surgery. A longitudinal 5cm incision was made ventral to the linguofacial vein. The caudo-lateral aspect of the cricoid cartilage was exposed through blunt dissection and electrodes with a spiraling cathode at their tip were positioned in the right PCA using an insertion cannula (Pajunk 18G needle, Pajunk GmbH, Germany). An insulated wire (passed through the cannula) was used to stimulate (1mA, 2Hz, 0.15ms pulses) the muscle with a portable device (Stiwell, MEDE-EL Elektromedizinische Geräte Gesellschaft m.b.H., Innsbruck, AustriaMed-El, Innsbruck,

Austria) to determine the location at which arytenoid abduction was achieved most effectively.

Two quadripolar electrodes (K5-P4, Osypka AG, Rheinfelden, Germany), each with two leads, were then inserted in the right PCA; one electrode was placed in the lateral neuro-muscular compartment and the other more rostrally in the medial compartment.[22] The PCA muscle is approximately 5cm long, 3cm wide and 1.2cm thick in adult horses.[26] Each electrode was secured to the closest laryngeal cartilage border with 2/0 nylon suture. Electrodes leads were tunneled subcutaneously to be connected to the implanted stimulation device. One lead of one electrode was connected to a second customized implant (MEDE-EL Elektromedizinische Geräte Gesellschaft m.b.H., Innsbruck, AustriaMed-El, Innsbruck, Austria) to monitor electrode impedance throughout the study. The right RLn was approached through a 10cm mid cervical incision dorsal to the jugular vein, and directly stimulated (1mA, 1Hz, 0.1ms) with an insulated needle (Stimuplex Insulated Needle; Braun Medical, Bethlehem, PA) to evoke arytenoid abduction twitches. The distal right RLn was then isolated about 2cm caudally to the cricoid cartilage and exposed to freezing injury (-60ºC for 2 minutes), using a non-traumatic stainless-steel probe precooled in liquid nitrogen, to produce axonotmesis.[27,28] Activity of the right PCA evoked by stimulating the most proximal exposed RLn was monitored endoscopically prior to freezing injury and 10 minutes after its completion to ensure denervation. As expected, no arytenoid twitches were recorded after the injury in all horses. Broad-spectrum antibiotics (trimethoprim-sulfamethoxazole 30mg/kg, PO, BID) and the non-steroidal anti-inflammatory phenylbutazone (1mg/kg, PO, BID), were administered for 7

days postoperatively; horses were examined daily for any signs of complications or illness.

Electrical Stimulation. FES began two weeks after nerve injury to allow resolution of tissue swelling and electrodes stabilization. One of the electrodes was used as cathode, the other as anode. The right PCA was stimulated in eight randomly selected horses (referred to as the FES+ group); seven horses were implanted, underwent axonotmesis, but were not stimulated (controls, referred to as the FES- group). FES+ horses were stimulated 60 minutes twice-daily for twenty weeks (22Hz, 20ms, 10mA, 3.5sec ON, 6.5sec OFF) to produce 55,440 impulses/day (daily frequency equivalent to 0.64Hz or 2.9% daily activation). These parameters were based on prior work on equine larynx and a pilot trial (Ducharme and Cheetham, 2011, unpublished material). The right *lateral cricoarytenoid* muscle (LCA), also denervated by the nerve injury, was not stimulated and served as internal control. Response to FES was monitored monthly through endoscopic and ultrasonographic exam.

Outcome measures- Overview

The effects of denervation, reinnervation and stimulation were determined immediately after surgery, two weeks from the nerve injury, and monthly thereafter through assessments of PCA contraction judged indirectly by arytenoid abduction response to hypercapnia, electrical stimulation and incremental exercise. PCA size was measured by ultrasound. After six months, quality of RLn reinnervation were estimated through response to hypercapnia before and after RLn anesthetic block. At the end of the study, immunohistochemistry of the right PCA and LCA muscles was performed to determine

fiber type proportions and minimum fiber diameters (MFD) distributions; axon number and degree of myelination of the right RLn were determined distally to the injury site.

Arytenoid abduction. With the horse standing and sedated (detomidine 0.01mg/kg, IV), a videoendoscope (Olympus GIF-140) was placed in the right ventral nasal meatus and laryngeal endoscopy was recorded for subsequent analysis as previously described.[23,24] Maximal arytenoid abduction was measured to assess PCA activation during hypercapnia and electrical stimulation.

Transient hypercapnia. Return of arytenoid function with reinnervation was assessed by inducing transient hypercapnia to stimulate ventilatory drive and PCA contraction.[29] The rebreathing test is routinely used in horses to induce hyperventilation: the horse muzzle is enclosed in a 30lt plastic bag, so the horse will re-breath expired air loaded with CO_2. The CO_2 inside the bag increases progressively at each breath, consequently causing blood CO_2 to rise, enhancing respiratory rate and depth. The test was terminated when the horse reached maximal glottic opening or demonstrated intolerance.

At the end of study, to confirm absence of synkinetic reinnervation from axonal misdirection at the site of nerve injury, the rebreathing test was performed before and after a right RLn anesthetic block. With the animal under sedation, a stimulating injectable 22G needle (UniPlex Nanoline cannula, PAJUNK® GmbH, Germany) was advanced dorsal to the jugular vein, perpendicular to the skin, in the caudal portion of the neck and its tip located near to the RLn. Supramaximal stimulation (10mA, 100usec) was applied to stimulate all axons within the RLn and the corresponding arytenoid twitch was assessed on endoscopy. After confirmation of right arytenoid response to the RLn

stimulation, 5ml lidocaine 2% was injected through the needle to anesthetize the RLn and the rebreathing test was repeated.

Acute stimulation. Muscle responsiveness and contraction force were determined by measuring arytenoid abduction evoked by brief PCA electrical stimulation. Stimuli were applied during expiratory hold (the short hesitation at the end of expiration), continuously for 10 seconds, using fixed pulse frequency (40Hz) and duration (10ms). These parameters were chosen as 40Hz is above the fusion frequency for the equine PCA.[24] The sequence of amplitudes used was randomized and varied from 0.5mA to a maximum of 10mA, with at least 30sec interval between each stimulation session to permit muscle recovery.

Standardized Exercise Protocol. Fatigue resistance under increasing inspiratory negative pressure loads was determined by incremental exercise on high-speed treadmill. The horses were trained 5 days/week on a high-speed treadmill. A standardized exercise test was performed monthly to evaluate horses at exercise intensities corresponding to 50, 80, 90 and 100% of their maximum heart rate.[30] During the exercise trial, each speed was maintained for 1minute while simultaneously recording heart rate, upper airway videoendoscopy, pharyngeal airway pressures, and accelerometer measurements. Laryngeal function was recorded using a wire-less videoendoscope (Optomed) placed into the nasopharynx via the right ventral nasal meatus. Nasopharyngeal pressure was measured using a Teflon catheter (1.3mm ID, Neoflon; Cole-Parmer, Chicago, IL) inserted through the left ventral nasal meatus and attached to differential pressure

transducers (Celesco LCVR; Celesco Transducers Products, Canoga Park, CA) referenced to atmospheric pressure and calibrated from -70 to 70 mmHg. [31,32]

Muscle size. PCA size was monitored by transesophageal ultrasound under sedation as previously described.[26] A pediatric transesophageal echocardiography probe (9T, pediatric TEE probe, diameter 7.5mm, 3.3–10.0MHz, Vivid 7) was placed transnasally into the oesophagus to image the right and left PCA and to measure the dorsal-ventral thickness of the midbody (60 mm caudal to the palatopharyngeal arch) and caudal portions (caudally to the cricoid sagittal ridge) of the muscles.

PCA volume was estimated immediately post-implantation, 13 and 22 weeks later, using a quantitative Computed Tomography (QCT) technique.[26] At the end of the study, the laryngeal muscles were excised and weighed.

Muscle immunohistochemistry. Following euthanasia, the right PCA and LCA muscles were isolated and mid-body transverse sections embedded in cutting medium (Tissue-Tek OCT Compound, Sakura Finetek, Netherlands) and frozen in melting isopentane precooled in liquid nitrogen (-156°C). Thereafter, 7µm cryosections were air-dried onto glass slides and stored at -80°C and processed as previously described.[21] All mature skeletal muscle fiber types (I, IIa and IIx), hybrid fibers and collagen V were identified in single cryosections using a multiple immunofluorescence labelling technique with 4 different primary antibodies.[33] Fibers ($n > 400$) were manually measured, to obtain the minimal fiber diameter (MFD) and assigned a fiber type or hybrid fiber designation by relative fluorescence.

Nerve histomorphometry. Sections of the right RLn taken 5mm distal to the site of freezing injury were fixed in 10% formaldehyde, embedded in epoxy resin, sectioned transversely and stained with azure II/methylene blue/safranin. Photomicrographs of fascicules were taken using a digital microscope (Carl Zeiss, Jena, Germany), magnified to 40X and viewed in Axiovision (Axiovision 4, Carl Zeiss, Jena, Germany). Automated analysis of the nerve fibers was performed with Volocity (version 6.1.1, PerkinElmer) using a customized bespoke modular program. Number of axons, diameter of the nerve fibers and axons, myelin sheath thickness and the g-ratio (minimum axon diameter divided by the minimum fiber diameter) were calculated.

***Data analysis*.** Endoscopic images of the *rima glottidis* were captured from the digital recordings using editing software (Video Wizard, Womble Multimedia, CA, USA) to measure arytenoid abduction. Image frames corresponding to the expiratory holding phase while the PCA was stimulated, and the inspiratory phase during rebreathing test and exercise were evaluated. Breathing phases during exercise were identified using synchronized airway pressure traces overlying the video recordings. The degree of arytenoid abduction was measured as previously described.[21,23,24] MFD obtained for each fiber type within each muscle were allocated into 5um bins and plotted as histogram envelopes. For continuous outcome measures (arytenoid abduction, PCA volume and thickness, muscle weight) a mixed effect model was fitted to the data to determine the relationship between the outcome variable and relevant fixed effects, using horse as a random effect. Morphometric data of muscle and nerve fibers were fitted in a mixed effect

model with muscle and nerve nested within horse identity. Tukey's *post hoc* tests and linear contrasts were used as appropriate. Statistical analysis was performed using JMP (SAS Institute, Cary, North Carolina, USA). Significance was set at $p<0.05$ throughout.

RESULTS

All horses recovered uneventfully from surgery. Minimal post-operative swelling resolved in few days. No evidence of discomfort or complications associated with the surgery, the electrical stimulation or the implants were detected throughout the study.

Impedance. A small increase in impedance was recorded starting 4 weeks post-implant (Suppl. Fig 1). Thereafter no significant changes and no difference between FES+ and FES- groups were observed. The increase represents minimal fibrosis around the electrodes during the first few weeks after implantation, without negative effects, as already reported.[34]

Function during transient hypercapnia. Progressive reinnervation of the PCA, determined by right arytenoid abduction in response to hypercapnia, began 14 weeks after the nerve injury, and full recovery was reached at 18 weeks in both FES+ and FES- horses, with no difference between groups ($p>0.05$, Fig. 1A). Full recovery also supported the absence of synkinetic reinnervation resulting from axonal misdirection at the site of nerve injury. All horses responded to RLn electrical stimulation with abduction, confirming right PCA reinnervation from the abductor branch fibers; then the RLn

anesthetic block induced complete transient right arytenoid paralysis in all horses with sole left arytenoid abduction in response to the rebreathing test.

Response to acute electrical stimulation. Baseline data were obtained within 48h from nerve injury, prior to degeneration of the remnant axons.[35] The response to stimulation decreased significantly within two weeks of nerve injury in both groups (Fig. 1B). This response returned more rapidly in the FES+ group beginning after 4 weeks of FES training (six weeks after nerve injury), indicating a greater excitability and strength of the stimulated PCA during the recovery phase. There was no difference between the responses at time 0 and after 22 weeks ($p>0.05$) suggesting full recovery in both groups following reinnervation.

Function under inspiratory load. PCA function under increasing levels of negative inspiratory pressure load was markedly reduced after denervation ($p<0.001$, Fig. 1C). Both groups presented a concurrent significant compensatory increase in left arytenoid abduction ($p=0.037$). A similar gradual but incomplete return to function on the right side was detected in both groups ($p=0.03$).

Muscle size. PCA thickness and the right:left (R:L) ratio of muscle thicknesses were significantly greater in the FES+ group from 6 weeks after injury indicating a positive training effect of electrical stimulation ($p<0.05$, Fig. 2A-B). This effect was most pronounced in the caudal (inferior) portion of the PCA muscle which undergoes more rapid atrophy during denervation.[26]

CT-determined PCA volume remained stable throughout the study, with no significant difference between the two groups (Fig. 2C). No significant difference was observed in right:left muscle mass ratio at the end of the study (Fig. 2D).

Muscle immunohistochemistry. Representative images of the PCA and LCA immunohistochemistry are shown in Figure 3. Fiber type I:II ratio was increased in stimulated PCA ($p=0.038$, one-tail t-test, Fig. 4). The opposite effect was found in the unstimulated LCA of FES+ animals (Figure 4, $p<0.01$). No significant difference between PCA and LCA fiber type proportion was found in the FES– group (Fig. 4, $p>0.15$).

MFD distributions were significantly altered by FES (Fig. 5) that induced a bimodal distribution in the PCA fiber type I (two-tailed Mann–Whitney rank sum test, $p=0.0014$), and a left shift of the fiber type II distribution (two-tailed Mann–Whitney rank sum test, $p<0.0001$). Fiber type I and IIa distributions were unaltered in the unstimulated LCA muscle.

Nerve histomorphometry. Reinnervation following injury was accompanied by reduced axon diameters ($p=0.0003$), decreased overall fiber diameter ($p<0.001$), myelin thickness ($p<0.0001$) and myelin/axon thickness ratio, and increased g-ratio ($p=0.0052$) ($p<0.0001$, Fig. 6). No significant differences were found between stimulated and unstimulated animals.

DISCUSSION

Our data demonstrate that intramuscular electrical stimulation improves PCA responsiveness and geometry after axonotmesis, and increases the proportion of type I:II fibers. Being type I fibers more sensitive to muscle wasting after denervation, leading to a slow-to-fast fiber type shift,[37] the higher type I:II ratio suggests that FES supports reinnervation. Improved muscle responsiveness during the course of reinnervation could be a consequence of improved preservation of fiber size and function during the period of denervation. When regenerating axons reconnect with stimulated muscle fibers, the excitability of the fibers is enhanced compared to fibers that did not receive stimulation during the denervation period. FES improved muscle strength as shown from the electrically-induced arytenoid abduction test, however, it did not ameliorate fatigue resistance as shown by arytenoid collapse during exercise.[38,39] As fatigue resistance mostly depends not only on fiber composition but also on oxidative capacity (number of mitochondria and oxidative enzymes, capillary density) it is possible that the overall oxidative capacity of the muscle was not yet back to normal level.

We were unable to detect any negative impact of FES on reinnervation following freezing nerve injury in this large animal model. The lack of deleterious effect on reinnervation confirms a previous report using a rat lower limb model.[40]

The effect of electrical stimulation on sprouting has been often quoted as one factor for limiting the use of FES during the reinnervation period. Neuromuscular activity of partially denervated muscles reduces terminal sprouting by preventing Schwann cell bridging forming between innervated and denervated end-plates (Tam et al, 2001; Tam and Gordon 2003), but no deleterious effect on nodal sprouting was recorded (Love et al,

2003). After RLn cryo injury, axonal sprouting starts within the first week near the injury site (Nahm et al, 90). Our outcome measures suggest that FES applied starting 2 weeks after injury has no negative effects on sprouting progression and myelination or that terminal sprouting contributed minimally to RLn reinnervation in our model.

Myosin heavy chain genes expression is influenced by innervation, with upregulation of type IIx genes in denervated muscles and complete loss of type I fiber from the PCA muscle after RLn transection.[37,41] This dependence of fiber type expression upon neural input is also evident in the equine PCA that shows an increase in type IIx fibers after complete denervation and a marked reduction in type I fiber after progressive denervation induced by idiopathic recurrent laryngeal neuropathy.[21,42] The specific morphometric changes of laryngeal muscles after denervation and reinnervation seem to be species-specific, with a reduction of type I fibers in rats and an increase in dogs.[43–45] The innervated equine PCA presents 30-40% type I fibers,[21] while in this study the reinnervated PCA after RLn axonotmesis presents a higher proportion of type I fibers (>50%), with the highest value in the stimulated group suggesting a positive effect of FES on these fibers. These findings confirm a preferential denervation atrophy of type II fibers and increase in type I fibers after reinnervation as reported after other peripheral nerve injuries in different species.[46,47]

The higher type I fiber proportion in the stimulated group confirms previous findings by Carraro et al,[48] of an upregulation of slow fibers after chronic electrical stimulation. Downregulation of type I fibers has been induced in the innervated PCA by non-use after joint fixation, suggesting that absence of mechanical stretch alters the trophic factors

locally in the muscle.[49] The high type I fiber content also in the unstimulated group could have resulted from a combination of reinnervation and muscle passive stretching during treadmill training.

In a similar population of horses,[21] the innervated PCA showed 37.4 ± 4.2μm MFD, that significantly reduced after RLn transection (23.6 ± 4.2μm). In the present study, 6 months after RLn axonotmesis, MFD resulted higher than normal in both FES+ and FES- horses (43.20 ± 0.23 μm) indicating that reinnervation occurred in both groups.[46,51] Interestingly, the right LCA showed a decreased type I fiber proportion and MFD (31.4 ± 0.14 vs 40.4 ± 4.2 μm), suggesting delayed or reduced reinnervation, so indicating a preferential reinnervation of the abductor muscle after RLn injury and ipsilateral FES.

Reinnervation after freezing injury induced an increased number of myelinated axons with smaller diameter compared to normal, as already reported.[52,53] Considering that axon diameter and degree of myelination depends on the axon maturation, the axonal growth rate seems lower than in other studies where the RLn morphologic appearance was comparable to control nerve 20 weeks after injury.[54] Rate of reinnervation is determined by many factors like species, age, type of nerves, nature of injury, and distance from target organ and neuronal cell body.[55] The horse is a domestic species with the longest RLn so the reinnervation rate could be delayed due to the long distance between the cell body and lesion site.[56] Considering the adequate return to function despite the differing axonal morphology triggers a question about the possibility that in the horse, axon size and myelination will never return to pre-damage values, or the need in this species of a much longer interval before completion of the reinnervation process.[55–57]

After RLn transection (without the possibility of nerve regrowth), a 30-40% decrease in PCA volume developed within 12 weeks,[21] while the RLn freezing injury, inducing transient denervation, caused only a 10% decrease in PCA volume. Axonotmesis, compared to the nerve transection, allowed gradual reinnervation and prevented marked atrophy. Beside reinnervation, training on high-speed treadmill possibly influenced PCA size, favoring anti-atrophic conditions. During incremental exercise, horses experience a nine-fold increase in breathing frequency and five-fold increase in respiratory myoelectrical activity, indicative of muscle recruitment.[59] Treadmill training as well as electrical stimulation create a permissive environment for nerve regeneration, enhancing brain-derived neurotrophic factor (BDNF), whose deprivation results in impaired myelination.[60,61]

Acute short-term electrical nerve stimulation accelerates nerve regeneration via enhancement of cell body response to nerve injury (Al-majed, 2000). The positive effect of electrical stimulation on axonal regrowth was recorded by Al-Majed after proximal nerve stump continuous stimulation for 1 hour to 2 weeks after acute repair. The FES protocol in our study was started later than two weeks after injury, and the earliest exposure of the muscle to electrical stimulation was 24-48h after injury, when the muscle was subjected to transient short burst of stimulation to record the baseline response of the muscle. The electrical field created by the intramuscular electrodes during FES can spread out of the muscle border, raising the question on the possible effect of FES directly on the nerve stump. We did not directly investigate the extension of the electrical field in

our study, but the response to FES was assessed on endoscopic and ultrasonographic exams monthly. During these examinations, we did not detect activation of tissues surrounding the PCA, such as the cricopharyngeous muscle laying directly over it, or activation of the contra-lateral PCA muscle, suggesting an insubstantial spread of current out of the PCA.

The main limitations of this study include the lack of histomorphometry data of the muscles before the RLn injury and of the regenerating nerve at an earlier timeline point. Those data would help in a more complete assessment of the denervation and reinnervation effect on the equine PCA muscle as well as in calculating the axonal re-growth rate, to establish if more than six months are required to complete the nerve maturation. However, this study gives support to the principle that stimulation of a muscle during the period of denervation improves the eventual neuromuscular recovery. Some authors have argued that such stimulation might inhibit the reconnection of the regenerating axons with the denervated muscle fibers. This objection is largely based on experiments in which intermittent high frequency stimulation, delivered 24 hours per day reduced the amount of terminal sprouting in denervated or partially denervated mouse muscle. In fact, those studies showed no significant effect on nodal sprouting which may be more closely relevant to the regrowth of whole axons after axonotmesis. This study shows that 60 minutes twice daily stimulation appears to achieve a useful preservation of muscle function without inhibition of reinnervation.

Abbreviations: BDNF, brain-derived neurotrophic factor; CT, computed tomography; FES, functional electrical stimulation; LCA, *lateral cricoarytenoid* muscle; MFD, minimum fiber diameter; PCA, *posterior cricoarytenoid* muscle; RLn, recurrent laryngeal nerve; VCP, vocal cord paralysis.

References

1. Fu SY, Gordon T. Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *J Neurosci*. 1995;**15**:3886-3895.

2. Kobayashi J, Mackinnon SE, Watanabe O, et al. The effect of duration of muscle denervation on functional recovery in the rat model. *Muscle Nerve*. 1997;**20**:858-866.

3. Kern H, Salmons S, Mayr W, Rossini K, Carraro U. Recovery of long-term denervated human muscles induced by electrical stimulation. *Muscle Nerve*. 2005;**31**:98-101.

4. Boncompagni S, Kern H, Rossini K, et al. Structural differentiation of skeletal muscle fibers in the absence of innervation in humans. *Proc Natl Acad Sci U S A*. 2007;**104**:19339-19344.

5. Lim J-Y, Han TR. Effect of electromyostimulation on apoptosis-related factors in denervation and reinnervation of rat skeletal muscles. *Muscle Nerve*. 2010;**42**:422-430.

6. Willand MP, Holmes M, Bain JR, Fahnestock M, De Bruin H. Electrical muscle stimulation after immediate nerve repair reduces muscle atrophy without affecting reinnervation. *Muscle Nerve*. 2013;**48**:219-225.

7. Marqueste T, Decherchi P, Desplanches D, Favier R, Grelot L, Jammes Y. Chronic electrostimulation after nerve repair by self-anastomosis: effects on the size, the mechanical, histochemical and biochemical muscle properties. *Acta Neuropathol*. 2006;**111**:589-600.

8. Russo TL, Peviani SM, Freria CM, Gigo-Benato D, Geuna S, Salvini TF. Electrical stimulation based on chronaxie reduces atrogin-1 and myoD gene expressions in denervated rat muscle. *Muscle Nerve*. 2007;**35**:87-97.

9. Gigo-Benato D, Russo TL, Geuna S, Domingues NR, Salvini TF, Parizotto NA. Electrical stimulation impairs early functional recovery and accentuates skeletal muscle atrophy after sciatic nerve crush injury in rats. *Muscle Nerve*. 2010;**41**:685-693.

10. Pinheiro-Dardis CM, Erbereli BT, Gigo-Benato D, Castro PATS, Russo TL. Electrical stimulation delays reinnervation in denervated rat muscle. *Muscle Nerve*. 2017;**56**:E108-E118.

11. Brown MC, Holland RL. A central role for denervated tissues in causing nerve sprouting. *Nature*. 1979;**282**:724-726.

12. Love FM, Son YJ, Thompson WJ. Activity alters muscle reinnervation and terminal sprouting by reducing the number of Schwann cell pathways that grow to link synaptic sites. *J Neurobiol*. 2003;**54**:566-576.

13. Zealear DL, Rodriguez RJ, Kenny T, et al. Electrical stimulation of a denervated muscle promotes selective reinnervation by native over foreign motoneurons. *J Neurophysiol*. 2002;**87**:2195-2199.

14. Otto RA, Templer J, Davis W, Homeyer D, Stroble M. Coordinated electrical pacing of vocal cord abductors in recurrent laryngeal nerve paralysis. *Otolaryngol Head Neck Surg*. 1985;**93**:634-638.

15. Bergmann K, Warzel H, Eckhardt HU, Hopstock U, Hermann V, Gerhardt HJ. Long-term implantation of a system of electrical stimulation of paralyzed laryngeal muscles in dogs. *Laryngoscope*. 1988;**98**:455-459.

16. Zrunek M, Bigenzahn W, Mayr W, Unger E, Feldner-Busztin H. A laryngeal pacemaker for inspiration-controlled, direct electrical stimulation of the denervated posterior cricoarytenoid muscle in sheep. *Eur Arch Otorhinolaryngol*. 1991;**248**:445-448.

17. Sanders I. Electrical Stimulation of Laryngeal Muscle. *Otolaryngolic Clin North Am*. 1991;**24**:1253-1274.

18. Zealear DL, Billante CR, Courey MS, et al. Reanimation of the paralyzed human larynx with an implantable electrical stimulation device. *The Laryngoscope;*. 2003;**113**:1149-1156.

19. Nomura K, Kunibe I, Katada A, et al. Bilateral motion restored to the paralyzed canine larynx with implantable stimulator. *Laryngoscope*. 2010;**120**:2399-2409.

20. Mueller AH, Hagen R, Foerster G, Grossmann W, Baumbusch K, Pototschnig C. Laryngeal pacing via an implantable stimulator for the rehabilitation of subjects

suffering from bilateral vocal fold paralysis: A prospective first-in-human study. *Laryngoscope*. 2016;**126**:1810-1816.

21. Cheetham J, Perkins JD, Jarvis JC, et al. Effects of Functional Electrical Stimulation on Denervated Laryngeal Muscle in a Large Animal Model. *Artif Organs*. 2015;**39**:876-885.

22. Cheetham J, Radcliffe CR, Ducharme NG, Sanders I, Mu L, Hermanson JW. Neuroanatomy of the equine dorsal cricoarytenoid muscle: Surgical implications. *Equine Vet J*. 2008;**40**:70-75.

23. Ducharme NG, Cheetham J, Sanders I, et al. Considerations for pacing of the cricoarytenoid dorsalis muscle by neuroprosthesis in horses. *Equine Vet J*. 2010;**42**:534-540.

24. Cheetham J, Regner A, Jarvis JC, et al. Functional electrical stimulation of intrinsic laryngeal muscles under varying loads in exercising horses. *PLoS One*. 2011;**6**:e24258.

25. Dixon P, Robinson E, Wade JF. Workshop summary. In: Dixon P, Robinson E, Wade JF, eds. *Proceedings of a Workshop on Equine Recurrent Laryngeal Neuropathy, 7-10 September, 2003.* **Vol 11**. Havemeyer Foundation Monograph Series 11. Stratford-upon-Avon, UK: R & W Publications (Newmarket) Ltd; 2004:93-97.

26. Kenny M, Cercone M, Rawlinson JJ, et al. Transoesophageal ultrasound and computer tomographic assessment of the equine cricoarytenoid dorsalis muscle: Relationship between muscle geometry and exercising laryngeal function. *Equine*

Vet J. 2017;**49**:395-400.

27. Mira J. Maintien de la continuité de la lame basale des fibres nerveuses périphériques après “section” des axons par congélation localisée. *CR Acad Sc Paris*. 1971;**273**:1836-1839.

28. Fasano VA, Peirone SM, Zeme S, et al. Cryoanalgesia. Ultrastructural study on cryolytic lesion of sciatic nerve in rat and rabbit. *Acta Neurochir*. 1987;**39**:177-180.

29. Adachi T, Umezaki T, Matsuse T, Shin T. Changes in laryngeal muscle activities during hypercapnia in the cat. *Otolaryngol Neck Surg*. 1998;**118**:537-544.

30. Rose RJ, Hendrickson DK, Knight PK. Clinical exercise testing in the normal thoroughbred racehorse. *Aust Vet J*. 1990;**67**:345-348.

31. Nielan GJ, Rehder RS, Ducharme NG, Hackett RP. Measurement of tracheal static pressure in exercising horses. *Vet Surg*. 1992;**21**:423-428.

32. Ducharme NG, Hackett RP, Ainsworth DM, Erb HN, Shannon KJ. Repeatability and normal values for measurement of pharyngeal and tracheal pressures in exercising horses. *Am J Vet Res*. 1994;**55**:368-374.

33. Tulloch LK, Perkins JD, Piercy RJ. Multiple immunofluorescence labelling enables simultaneous identification of all mature fibre types in a single equine skeletal muscle cryosection. *Equine Vet J*. 2011;**43**:500-503.

34. Charlet de Sauvage R, Lima da Costa D, Erre JP, Aran JM. Electrical and physiological changes during short-term and chronic electrical stimulation of the normal cochlea. *Hear Res*. 1997;**110**:119-134.

35. Gutmann E, Zelena J. *Morphological Changes in the Denervated Muscle*. The

Denerv. (Gutmann E, ed.). Prague: Czechoslovak Academy of Sciences; 1962.

36. Nahm I, Shin T, Watanabe H, Maeyama T. Misdirected regeneration of injured recurrent laryngeal nerve in the cat. *Am J Otolaryngol*. 1993;**14**:43-48.

37. Ciciliot S, Rossi AC, Dyar KA, Blaauw B, Schiaffino S. Muscle type and fiber type specificity in muscle wasting. *Int J Biochem Cell Biol*. 2013;**45**:2191-2199.

38. Ashley Z, Sutherland H, Russold MF, et al. Therapeutic stimulation of denervated muscles: The influence of pattern. *Muscle and Nerve*. 2008;**38**:875-886.

39. Salmons S, Jarvis JC. Functional electrical stimulation of denervated muscles: an experimental evaluation. *Artif Organs*. 2008;**32**:597-603.

40. Willand MP, Chiang CD, Zhang JJ, Kemp SWP, Borschel GH, Gordon T. Daily Electrical Muscle Stimulation Enhances Functional Recovery Following Nerve Transection and Repair in Rats. *Neurorehabil Neural Repair*. 2015;**29**:690-700.

41. DelGaudio JM, Sciote JJ. Changes in myosin expression in denervated laryngeal muscle. *Ann Otol Rhinol Laryngol*. 1997;**106**:1076-1081.

42. Adreani CM, Li ZB, Lehar M, et al. Myosin heavy chain composition in normal and atrophic equine laryngeal muscle. *Vet Pathol*. 2006;**43**:881-889.

43. Shiotani A, Flint PW. Myosin heavy chain composition in rat laryngeal muscles after denervation. *Laryngoscope*. 1998;**108**:1225-1229.

44. Wu YZ, Baker MJ, Marie JP, Crumley R, Caiozzo VJ. The plasticity of denervated and reinnervated laryngeal muscle: focus on single-fiber myosin heavy-chain isoform expression. *Arch Otolaryngol Head Neck Surg*. 2004;**130**:1070-1082.

45. Li J, Liu S, Cheng Q, et al. Changes in electrical response function and myosin

heavy chain isoforms following denervation and reinnervation of bilateral posterior cricoarytenoid muscles in dogs. *Acta Otolaryngol*. 2014;134:318-325.

46. Shindo ML, Herzon GD, Hanson DG, Cain DJ, Sahgal V. Effects of denervation on laryngeal muscles: a canine model. *Laryngoscope*. 1992;**102**:663-669.

47. Borisov AB, Dedkov EI, Carlson BM. Interrelations of myogenic response, progressive atrophy of muscle fibers, and cell death in denervated skeletal muscle. *Anat Rec*. 2001;**264**:203-218.

48. Carraro U, Catani C, Saggin L, et al. Isomyosin changes after functional electrostimulation of denervated sheep muscle. *Muscle Nerve*. 1988;**11**:1016-1028.

49. Shiotani A, Nakagawa H, Flint PW. Modulation of Myosin Heavy Chains in Rat Laryngeal Muscle. *Laryngoscope*. 2001;**111**:472-477.

50. Kletzien H, Russell JA, Connor NP. The effects of treadmill running on aging laryngeal muscle structure. *Laryngoscope*. 2016;**126**:672-677.

51. Birchall M, Idowu B, Murison P, et al. Laryngeal abductor muscle reinnervation in a pig model. *Acta Otolaryngol*. 2004;**124**:839-846.

52. Mira J. Variations du nombre et du caliber des fibres nerveuses myélinisées régénérées aprés une “section” des axons par congélation localisée. *CR Acad Sc Paris*. 1972;**275**:979-982.

53. Kerns JM, Braverman B, Mathew A, Lucchinetti C, Ivankovich AD. A comparison of cryoprobe and crush lesions in the rat sciatic nerve. *Pain*. 1991;**47**:31-39.

54. Pitman MJ, Weissbrod P, Roark R, Sharma S, Schaefer SD. Electromyographic

and histologic evolution of the recurrent laryngeal nerve from transection and anastomosis to mature reinnervation. *Laryngoscope*. 2011;**121**:325-331.

55. Nahm I, Shin T, Chiba T. Regeneration of the recurrent laryngeal nerve in the guinea pig: reorganization of motoneurons after freezing injury. *Am J Otolaryngol*. 1990;**11**:90-98.

56. Verdú E, Ceballos D, Vilches JJ, Navarro X. Influence of aging on peripheral nerve function and regeneration. *J Peripher Nerv Syst*. 2000;**5**:191-208.

57. Muratori L, Ronchi G, Raimondo S, Giacobini-Robecchi MG, Fornaro M, Geuna S. Can regenerated nerve fibers return to normal size? A long-term post-traumatic study of the rat median nerve crush injury model. *Microsurgery*. 2012;**32**:383-387.

58. Marqueste T, Alliez J-R, Alluin O, Jammes Y, Decherchi P. Neuromuscular rehabilitation by treadmill running or electrical stimulation after peripheral nerve injury and repair. *J Appl Physiol*. 2004;**96**:1988-1995.

59. Ainsworth DM, Eicker SW, Nalevanko ME, Ducharme NG, Hackett RP, Snedden K. The effect of exercise on diaphragmatic activation in horses. *Respir Physiol*. 1996;**106**:35-46.

60. Wilhelm JC, Xu M, Cucoranu D, et al. Cooperative roles of BDNF expression in neurons and Schwann cells are modulated by exercise to facilitate nerve regeneration. *J Neurosci*. 2012;**32**:5002-5009.

61. Willand MP, Rosa E, Michalski B, et al. Electrical muscle stimulation elevates intramuscular BDNF and GDNF mRNA following peripheral nerve injury and repair in rats. *Neuroscience*. 2016;**334**:93-104.

62. Sakakima H, Yoshida Y. Effects of short duration static stretching on the denervated and reinnervated soleus muscle morphology in the rat. *Arch Phys Med Rehabil*. 2003;**84**:1339-1342.

Figure 1. Arytenoid response to transient hypercapnia, acute *posterior cricoarytenoid* muscle (PCA) electrical stimulation and incremental exercise. In (A), nerve injury caused right arytenoid collapse below resting angle within the first 6 weeks. Reinnervation induced progressive return of the right PCA response and consequent increased abduction. At 22 weeks, complete reinnervation is indicated by a similar response of right and left arytenoids to hypercapnia. No significant difference between stimulated (FES+) and control (FES-) subjects was detected at any timepoint. Resting angles are cumulative of right and left arytenoids before the test from both groups. In (B), FES promoted an earlier response to acute maximal electrical stimulation of the muscle. The significant difference between the two groups (asterisks), observed 6 weeks after injury, persisted to 18 weeks and it was lost by 22 weeks. Resting angles are cumulative of the right arytenoid before the test from both groups. In (C), nerve injury produced ipsilateral marked arytenoid collapse at exercise with recovery over 22 weeks. Compensatory hyperabduction of the contralateral arytenoid cartilage reduced during the recovery period. Left arytenoid angles are combined from both groups. Asterisks indicate significant difference between pre-injury and post-injury arytenoid abduction. Data are mean ± SEM. * ($p<0.05$), **($P<0.01$), ***($p<0.001$).

Figure 2. *Posterior cricoarytenoid* (PCA) muscle geometry and volume. Functional electrical stimulation (FES+) increased PCA muscle mid-body (6,10,18,22 weeks) (A) and caudal (6,10,22 weeks) (B) right to left muscle thickness ratio. Minimal changes in PCA muscle volume (C) or mass (D) right:left ratio were identified. Muscle volumes were determined using computed tomography reconstruction. Cricoid cartilage volume was used as an internal control and showed no changes in volume over time ($p=0.82$). Data are mean ± SEM. Asterisks indicate significant difference between FES+ and FES- subjects. * ($p<0.05$), ** ($p<0.01$).

Figure 3. Representative immunohistochemistry images of laryngeal muscles. *Posterior cricoarytenoid* (PCA) and *lateral cricoarytenoid* (LCA) muscles labeled simultaneously with antibodies specific for type I fibers (blue), type IIa fibers (red) and collagen V (green) (Tulloch et al, 2011)[33]. (+, muscle that received FES)

Figure 4. Fiber type proportions of the laryngeal muscles. Functional electrical stimulation (FES+) induced an increase in type I to type II ratio in stimulated *posterior cricoarytenoid* (PCA) muscle but a reduction in type I:II ratio in unstimulated *lateral cricoarytenoid* (LCA) from the same animals. Immunohistochemistry was performed on mid-body muscle sections (7 um) and fibers were identified with different mouse monoclonal antibodies

labelled with fluorescent fragments designed for different emitting wavelengths (Tulloch et al, 2011)[33]. Data are mean ± SEM. * ($p<0.05$), ** ($p<0.01$)

Figure 5. Fiber diameter distribution of the laryngeal muscles. Functional electrical stimulation (FES+) increased the proportion of small diameter type I and type II muscle fibers in the stimulated right *posterior cricoarytenoid* (PCA) muscle (upper panels). No changes were observed in the right *lateral cricoarytenoid* (LCA) muscle (internal control, lower panels). FES was only applied to the right PCA of the FES+ group. Dotted lines represent animal in which FES was not applied (FES-). Bin size is 5um. Asterisks indicate significant difference in diameter distribution between the FES+ and FES- groups: * ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)

Figure 6. Distal Recurrent Laryngeal nerve (RLn) morphometry in uninjured condition and after freezing injury and reinnervation. The mean axon count is higher after injury and reinnervation, but not statistically significant. The other morphometric characteristics of the RLn were significantly different from control after injury and reinnervation, but no significative difference was found between FES+ and FES- group regarding axon count and diameter, myelin thickness and myelin/axon ratio, fiber diameter and g ratio indicating a similar degree of reinnervation between control horse and horses underwent PCA electrical stimulation. Data are mean ± SEM. * ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)