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Abstract

Bilateral Vocal Fold Paralysis (BVCP) is a life threatening condition that follows injury to the Recurrent Laryngeal nerve (RLn) and denervation of the intrinsic laryngeal musculature. Functional electrical stimulation (FES) enables restoration and control of a wide variety of motor functions impaired by lower motor neuron lesions. Here we evaluate the effects of FES on the sole arytenoid abductor, the posterior cricoarytenoid muscle (PCA) in a large animal model of RLn injury. Ten horse were instrumented with two quadripolar intra-muscular electrodes in the left PCA muscle. Following a twelve week denervation period, the PCA was stimulated using a once daily training session for eight weeks in seven animals. Three animals were used as unstimulated controls. Denervation produced a significant increase in rheobase ($p<0.001$). Electrical stimulation produced a 30% increase in fiber diameter in comparison with the unstimulated control group (33.9 ±2.6um FES+ , 23.6 ±4.2um FES -, $p=0.04$). A trend towards a decrease in the proportion of type 1 (slow) fibers and an increase in type 2a (fast) fibers was also observed. Despite these changes improvement in PCA function at rest was not observed. These data suggest that electrical stimulation using a relatively conservative set of stimulation parameters can reverse the muscle fiber atrophy produced by complete denervation while avoiding a shift to a slow (type 1) fiber type.

Introduction

Bilateral Vocal Fold Paralysis (BVCP) is a life threatening condition that follows injury to the Recurrent Laryngeal nerve (RLn), most commonly from trauma during thyroid surgery or anterior cervical approaches to the spine [1-4]. In the case of complete RLn transection without spontaneous recovery, the intrinsic laryngeal musculature is chronically denervated and the vocal folds remain immobile in a closed position producing dysphonia, stridor, aspiration and dyspnoea [5]. Functional electrical stimulation (FES) is a well-established treatment option for lower motor neuron deficits that enables restoration and control of a wide variety of motor functions impaired by lower motor neuron lesions, from locomotion to breathing. Despite the technical limitation represented by low excitability of muscle undergoing atrophy and fibrotic degeneration following permanent motor neuron damage, FES has also been used clinically to provide direct electrical activation of long term denervated muscle [6,7]. Animal models of RLn injury have been previously used to evaluate the use of FES to stimulate a denervated laryngeal musculature. These models have predominantly focused on the dog [8-10], sheep [11,12], cat [13], pig [14-16], and more recently, horse [17,18]. Confounding features of these animal studies have been variation in the parameters used for stimulation including number of pulses, daily activation rate and rest periods between stimulation, in addition to varying rates of denervation across species and both positive and negative effects of stimulation on muscle integrity and the process of reinnervation have been reported [19-22]. In this study we evaluate the effects on muscle size and function of direct intramuscular stimulation in denervated laryngeal muscle using relatively long stimulation pulses at a low daily activation frequency. We use an equine model as the large size of the posterior

cricoarytenoid muscle (PCA) reduces some of the technical difficulties associated with electrode placement in other species [23]. We focus on the PCA as the sole arytenoid abductor and because restored or returning PCA function is a requirement for tracheostomy removal in patients with BVCP [9]

Material and 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 local Institutional Animal Care and Use Committee (IACUC). Animals were brought into the research unit and given a 7-day acclimatization period prior to any procedure. Daily record logs of medical procedures were maintained.

Experimental design: Ten adult horses (Age range 5-7years, weight range 440-550kg) with normal arytenoid function (Havemeyer grade 1,[24]) were instrumented with two quadripolar intra-muscular electrodes (5 French) in the left posterior cricoarytenoid (PCA) muscle. Implanted electrodes were connected to a custom connector placed subcutaneously on the left side of the neck that facilitated FES using an external multichannel stimulating system (Master-8, AMPI, Israel).

Surgical procedure: Electrodes were implanted as previously described under general anesthesia [18]. Briefly, an open approach to the lateral compartment of the left PCA muscle was made and electrodes (quadripolar, K5-P4, Osypka AG, Rheinfelden, Germany, figure 1) with a spiraling cathode at their tip were inserted using an insertion cannula (Pajunk 18G needle, Pajunk GmbH, Germany). The lateral neuromuscular

compartment was selected as stimulation in this location produces the greatest arytenoid abduction [25]. Following a three week recovery period, and collection of baseline data, each horse was anesthetized again and the left RLn was approached through a 10cm mid cervical incision ventral to the jugular vein. Correct identification of the RLn was confirmed using direct stimulation with an insulated needle (Stimuplex Insulated Needle; Braun Medical, Bethlehem, PA) and 1 mA, 2 Hz, 0.15 ms duration pulses (Innervator 232; Fisher & Paykel Healthcare, Auckland, New Zealand): arytenoid abduction twitches were verified endoscopically. The RLn was transected and the proximal and distal ends ligated with 2/0 Ethilon. All horses received broad-spectrum antibiotics (trimethoprim-sulfadiazine 30mg/kg, PO, BID) and Phenylbutazone (1mg/kg, PO, BID) for 5 to 7 days. The effects of denervation were allowed to proceed without further intervention for twelve weeks and then the left PCA muscle was stimulated using a once daily training session for eight weeks (50Hz, 2ms pulse width, 2 s on 2s off 10 volts, for 48 min/day) to produce a total of 72,000 impulses/day (daily frequency equivalent 0.83 Hz). Daily activation below 1 Hz was used to minimize any shift to type I (slow) fibers [26]. All stimulation pulses used were biphasic with the negative (cathodic) phase occurring before the positive phase. Seven animals were stimulated using these parameters (referred to as the FES+ group) and three were implanted but not stimulated (controls, referred to as the FES- group)). The other intrinsic laryngeal muscles were not stimulated and served as internal controls.

Outcome measures:

Overview: The effects of denervation and stimulation were determined using quantitative assessments of muscle excitation (strength-duration curve), muscle contraction judged by arytenoid abduction response to increasing stimulation, computed-tomography based reconstruction of PCA volume and, standardized exercise tests to determine laryngeal function under increasing inspiratory loads. These observations were made immediately after surgery, after the 12 week period of denervation and after a subsequent eight weeks of FES. At the end of the study, immunohistochemistry of the left and right PCA and Lateral Cricoarytenoid muscles (LCA) was performed to determine fiber type distributions and minimum fiber (Feret's) diameters (MFD).

Strength-duration trials: To assess the excitation characteristics of the PCA muscle following denervation, rheobase - the minimum amplitude needed to elicit a threshold response at infinitely long pulse duration of the electrical stimulation and chronaxie -the pulse duration at twice the rheobase were determined [27]. The procedure was performed under light sedation with videoendoscopic observation of the left arytenoid cartilage [18]. Single biphasic balanced pulses were applied to the left PCA muscle with pulse durations from 0.5 ms to 8ms. Pulse amplitude was increased from zero in 0.1mA steps until a perceptible abduction twitch of the left arytenoid cartilage was observed via the endoscope (threshold). The test stimuli were applied during expiration hold (the short hesitation at the end of expiration) and the sequence of pulse durations was randomized. Threshold was readily observed endoscopically.

The PCA response to increasing levels of electrical stimulation was determined at rest by increasing the stimulation voltage (0, 0.5,2,..10 volts) of a short 10 second burst using fixed pulse duration (2ms) and frequency (50Hz). Thirty seconds rest was allowed

between bursts and the sequence of stimulation was randomized. These parameters were chosen as 50Hz is above the fusion frequency for the equine PCA [18]. The PCA response, measured by the degree of arytenoid abduction, was determined at each stimulation level using a flexible videoendoscope (Olympus GIF-140) passed into the nasopharynx via the right ventral nasal meatus (figure 2). Briefly, a line was drawn to connect the dorsal and ventral-most points of the rima glottidis and extending dorsally for a distance of one third of the dorsoventral height of the rima glottidis. Lines were then drawn from the dorsal most end of this line, tangential to each of the arytenoid cartilages. Finally, the angles between the dorsoventral line and the tangents were measured[28,29]

Determination of PCA volume using Computed Tomography: PCA volume was estimated in vivo using a validated quantitative Computed Tomography (QCT) technique [*WM, this paper is currently in review how would you like me to reference it?]*. Briefly, under general anesthesia, QCT (Aquilion LB16, Toshiba) with reconstruction was used to determine volume of the left and right PCA muscles. The cricoid cartilage was used as a volume standard throughout the study. To improve spatial resolution, scan spacing and thickness were set to minimum levels to minimize in-plane pixel size (0.25-0.50 mm), scan spacing (1 mm), and scan thickness (1 mm). DICOM sets were imported into CT reconstructive software (Mimics ™, Materialise, Belgium) using Lossless compression. A semi-automated segmentation tool, 3D LiveWire™, was used to outline the muscle of interest [30]. In-plane segmentation was repeated along the entire length of each PCA muscle, and the tool was used to segment each portion. A gradient magnitude of 60% with 0 attraction was used to best outline the muscle body. Mimics settings were further

adjusted to optimize smoothness without shrinkage (100 iterations, 1.00 smooth factor, shrinkage compensation *on*). Triangle reduction using an advanced edge reducing mode (0.0370mm tolerance, 15^{o} edge angle, and 100 iterations) was also applied. Other parameters remained at their default settings. From these operations, a custom three-dimensional (3D) reconstruction of each muscle was generated with associated geometrical properties. The volume of the cricoid cartilage was obtained at each time point to provide a known standard.

Exercise Protocol and Instrumentation: All horses were exercised on a treadmill (0^{o} incline) under control conditions to verify normal upper respiratory tract at exercise (laryngeal grade IA) [24] prior to denervation. PCA function under increasing levels of inspiratory load was determined using a standardized exercise test [18,31]. Each horse was subsequently evaluated at an exercise intensity corresponding to 50,80,90 and 100% of their maximum heart rate (HRmax) [32] to standardize the load experienced by the PCA muscle at increasing levels of exercise intensity. After a warm up period, one minute was spent at each level before increasing to the next. After the period of denervation, during each one minute interval, the PCA was unstimulated in the first 30 seconds to determine native function and the PCA was transiently stimulated in the second 30 seconds (50Hz, 2ms, 10V) to determine the response to stimulation. Horses were evaluated under these conditions before RLn transection (baseline), after the period of denervation and after the period of stimulation. No stimulation was applied at the baseline timepoint. Data recorded during exercise trials included heart rate, electrocardiogram and accelerometer measurements. Nasopharyngeal and laryngeal movements were recorded using a flexible

videoendoscope (Olympus GIF-140) passed into the nasopharynx via the right ventral nasal meatus and secured. Heart rate was measured by an on-board monitor (Hippocard Systems, Lexington, KY). Data were recorded onto DVD disks for subsequent analysis.

Immunohistochemistry:

Following euthanasia, the left and right PCA and LCA muscles were isolated and mid-body samples (6mm^3 approximately) embedded in cutting medium (Tissue-Tek OCT Compound, Sakura Finetek, Netherlands) so that the fibers were orientated vertically and frozen in melting isopentane precooled in liquid nitrogen (-156°C). Thereafter, 7µm cryosections were air-dried onto glass slides (Superfrost plus microscope slides, VWR International Ltd, Lutterworth, UK) and stored at -80°C. Routine Hematoxylin and Eosin staining was performed on two sections form each muscle.

All mature skeletal muscle fiber types (1, 2a and 2x), hybrid fibers and collagen V were identified in single equine muscle cryosections using a multiple immunofluorescence labelling technique with 4 different primary antibodies. [33]

Images were obtained using a Leica DMRA2, or DM4000 florescent microscope (Leica Microsystems (UK) Ltd, Buckinghamshire, U.K.) via the x10 objective and LEICA A4, L5, TX2, Y5 filters. The fluorescent camera used was a Zeiss MRm camera (ZEISS microscopy), with a dedicated software program (Axiovision 4.8, www.zeiss.co.uk). Background correction was performed in Volocity (Version 6, PerkinElmer, Massachusetts 02451, USA) and was performed by imaging the even field in all filters using Fluor-Ref (www. MicroscopyEducation.com). For determination of MFD by individual fiber type or hybrid fiber designations, merged images were imported into

Image J (www.imagej.nih.gov) and fibers (n >400) were manually measured and subjectively assigned a fiber type or hybrid fiber designation by relative fluorescence.

Separate 7 µm muscle sections were used to determine the percentage collagen V (severity of fibrosis) per unit area. A goat anti-collagen V antibody (1:20 diluted in phosphate buffered saline) (Goat anti-type V collagen, Southern Biotechnology, Birmingham, Alabama, USA) was used with a fluorescent secondary antibody (1:1000 diluted in phosphate buffered saline, Alexafluor 488 rabbit antigoat IgG, www.invitrogen.com). Two images were obtained from each muscle sample using the x10 objective and background corrected as described. Images were thresholded and analysed in Volocity (Version 6, PerkinElmer, Massachusetts 02451, USA) for objective quantification of collagen V expression per unit area.

Data Analysis

Strength duration data were subjected to curve fitting with the exponential function $V = a * e^{b+c*\tau} + d$ whereas V=Threshold voltage, τ = Stimulus pulse width and a, b, c, d being constants that are evaluated for every SDC Curve, using custom software (Matlab Curve-Fitting toolbox). Robust bisquare fitting and the trust region algorithm were used to optimize curve fit with a maximum of 500 iterations and 750 evaluations. Lower boundaries were 0.1, -0.1, -100 and 0, upper boundaries were 100, 100, -0.15 and 100 and starting points were 1.5, 1, -1.7 and 1 for a, b, c and d respectively. A logarithmically spaced weighing vector was used to assign the first and last measured values the weight 1. The minimum weight was 0.3 for the middle value.

Endoscopic images of the *rima glottidis* were captured from the DVD recordings using editing software (Video Wizard, Womble Multimedia, CA, USA). The degree of arytenoid abduction was measured using a previously validated technique [34] with commercially available software (Able Image Analyser). Briefly a line is drawn connecting the dorsal- and ventral- most points of the *rima glottidis.* This line was then extended dorsally for a distance of one third of the dorsoventral height of the *rima glottidis*. A tangential line to the arytenoid cartilages was drawn, and the angle between the dorsoventral line and the tangential line measured [18]. Minimum fiber diameters obtained for each fiber type within each muscle were allocated into 5um bins and plotted as histogram envelopes.

For continuous outcome measures (arytenoid abduction response, rheobase, chronaxie, PCA volume, fiber type proportion) 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; interaction terms were included as appropriate. Minimum fiber diameter data was fitted with a mixed effect model with muscle nested within horse identity. To determine PCA response at increasing levels of inspiratory load, separate models were fitted to the data for each time period (baseline, post denervation, post FES) and each condition during testing under load (native function or FES applied (stimulated)). 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. Postoperative swelling and discomfort were minimal and resolved within 10-14 days. Appropriate healing was confirmed in all horses at suture removal. Horses remained comfortable throughout the study.

Muscle Excitation characteristics: The algorithm used to fit the strength duration curves fit the raw data well with an R^2 (mean ± sd) of 0.97 ± 0.022. Evaluating changes in rheobase, there was no significant interaction between time point and animal group (FES + or control, p=0.11). Controlling for animal group (FES+ or FES-) there was a significant increase in rheobase following denervation (baseline 0.91± 0.44 mA, post denervation 4.96± 0.54 mA and post FES 3.64 ± 0.79 mA, p<0.001). There were no differences between the FES + and FES – groups. Controlling for animal group, mean chronaxie also slightly increased following denervation (1.01 ±0.54ms, 1.42 ±0.44ms, 1.32 ±0.60ms), although these changes were not significant (p=0.24).

Response to increasing stimulation: Prior to denervation, the PCA response to increasing stimulation, measured using left arytenoid abduction reached a plateau, with maximal arytenoid abduction of 50 degrees, above 3 volts of stimulation (figure 3). The response to increasing stimulation following denervation and FES was markedly damped. No significant differences were observed between FES+ and control horses.

Volumetric changes: Denervation produced a profound and significant 30-40% decrease in left PCA volume in both FES+ and control (FES-) groups over the twelve week denervation period (figure 4). In the FES + group, this atrophy did not proceed after the onset of stimulation, in contrast atrophy did proceed in the control group during this period. Left PCA volume was not significantly different between control and FES + groups at the post FES time point (linear contrast, $p=0.22$). CT determination of cricoid volume remained very stable throughout the time course with estimated volumes of 31.9 ± 3.5 cm^3, 31.6 ± 4.0 cm^3 , 29.6 ± 3.4 cm^3 at each time point and no significant difference between FES + and FES- groups.

Function under load: PCA function under increasing levels of inspiratory load (negative inspiratory pressure) was markedly reduced after denervation (figure 5). No significant differences were observed between FES + and control (FES -) groups either pre-denervation (baseline) or post-denervation in native function or function during transient electrical stimulation (stimulated). However, after the period during which the FES+ group received daily stimulation, native function was significantly improved at high levels of inspiratory load (90 and 100 HRmax) in FES+ animals compared to controls ($p=0.05$ and $p<0.01$ respectively, linear contrasts). No significant differences were observed between FES + and control animals during transient stimulation at exercise at this time point, although the means for function during transient stimulation showed the same trend as the means for the native function.

Immunohistochemistry:

Controlling for other factors in the model, denervation of the left PCA produced a 37% reduction in minimum diameter (mean ± se) for all fibers (23.6 ± 4.2 um) compared to the innervated right PCA muscle (37.4 ± 4.2 um, $p<0.05$). Denervation produced a similar decrease in the mean minimum fiber diameter of the left LCA (23.9 ± 4.2 um) compared to the innervated right LCA (40.4 ± 4.2 um, $p<0.05$).

Electrical stimulation of the left PCA muscle produced a 30% increase in fiber diameter in comparison with the unstimulated control group (33.9 ±2.6um FES+ , 23.6 ±4.2um FES -, $p=0.042$, linear contrast). The modal fiber diameter remained at approximately 25um, but a much higher proportion of fibers had hypertrophied to between 40-60um in the stimulated left PCA compared to the control left PCA (figure 6).

Electrical stimulation in the left PCA muscle produced a trend towards a decrease in the proportion of type 1 (slow) fibers (linear contrast, $p=0.09$) and a trend towards an increase in type 2a (fast) fibers (linear contrast, $p=0.052$, figure 7). No other significant differences in type 1 or type 2a fibers were observed. Overall there was a significant increase in the proportion of hybrid fibers in the denervated (left PCA and LCA) muscles compared to the innervated (right PCA and LCA) muscles (linear contrast, $p=0.0004$) with no hybrid fibers detected in the innervated muscles. There was also a significant decrease in the proportion of hybrid fibers in the stimulated PCA compared with the unstimulated PCA ($p=0.003$). There were no significant differences in any fiber type proportion between horses in the stimulated and unstimulated groups in any of the muscles to which FES was not applied – for example left LCA (L LCA stimulated) in the horses which received FES in the left PCA and the left LCA in the horses which did not receive FES in the left PCA (L LCA control). Overall model fits were good for each

fiber type (Model: Proportion ~ $Horse_{random}$ + muscle+ group (stimulated or control) + muscle*group; adjusted R^2 =0.63, 0.54, 0.98, 0.56 for types 1, 2a, 2x and hybrid fibers respectively). Unlabeled fibers summed to 0.54% across all animals and muscles and were excluded from subsequent analysis.

Denervation produced an increase in the proportion of collagen V expression per unit area (figure 8). Controlling for other factors in the model the denervated left PCA and LCA (56.4 ±3.6%, 59.9 ±5.6%) had significantly more collagen than the innervated right PCA and LCA (23.6 ±3.6%, 21.6 ± 3.6%, p=0.001). There was no effect of FES in the left PCA muscle (p=0.72, linear contrast) and no significant difference between other unstimulated muscles in the FES + or FES – groups (p=0.73).

Discussion

These data demonstrate arrest of, and partial recovery from the consequences of complete denervation using electrical stimulation in a large animal model. We identify increased muscle volume and fiber cross-sectional area within the the posterior cricoarytenoid muscle with stimulation We also identify a 30% increase in the muscle fiber diameter with electrical stimulation and a trend towards a shift in an increasing proportion of type 2a fibers with electrical stimulation. These changes were produced using a relatively conservative set of stimulation parameters [72,000 pulses/day, a daily activation rate of 1.83% (0.83 Hz) and a daily rest period of 23 hours]. These parameters were selected to avoid a shift to increasing proportions of type 1 fibers seen at daily stimulation frequencies above 1Hz [26]and achieved this goal.

The decrease in PCA volume of 30-40% over a 20 week denervation period, suggests that slow rather than rapid atrophy occurs after denervation in this model. This is consistent with slow atrophy and absence of necrosis found after denervation in other large animal [14,22,35] rather than rodent models of denervation. [36,37] The equine model may represent a good system in which to study the effects of electrical stimulation on laryngeal muscle as the larger size of the equine PCA reduces some of the technical considerations for electrode implantation, and endoscopic and volumetric reconstruction techniques allow longitudinal assessment of structure and function.

Denervation produced a 37% reduction in fiber diameter as anticipated. The relative proportion of collagen V also increased with denervation as fiber diameter decreased. This increase in the proportion of collagen V is a consequence of both the smaller fibers (producing an increase in circumference relative to cross-sectional area) and an increased amount. The changes in fiber diameter were partially reversed with electrical stimulation with a 30% increase in the shortest cross-sectional diameter (minimum fiber diameter, MFD) when FES was applied to the left PCA muscle. These changes are similar the 25% increase in MFD identified in other large animal models evaluating the effects of FES [38] in completely denervated laryngeal muscle. One limitation of this study is that no assessment of muscle recruitment by FES was made and additional optimization of electrode placement may improve the observed response. In addition, longer pulses [22] or a longer period of stimulation [39] may be required to optimize direct intramuscular stimulation of the denervated PCA muscle

The trend towards a shift in an increasing proportion of type 2a fibers with electrical stimulation suggests that the parameters used met the required goal of avoiding

a shift towards type 1 (slow) fibers which are characterized by reduced contraction force, and time to peak contraction, characteristics that are particularly important in the PCA which maintains airway patency during inspiration [11]

The increase in the proportion of type 1 fibers with denervation is anticipated as these fibers are more resistant to denervation [36,40-42]

The response of the PCA muscle under load, imposed by increasing levels of exercise intensity and so increasing the inspiratory pressure against which the PCA must contract to maintain airway patency, is interesting. FES has been shown to improve resting muscle tone even in the presence of complete denervation [43] and increased tonicity in the PCA muscle will prevent complete collapse of the arytenoid cartilage into the airway. This increase in tonicity is particularly important under high inspiratory loads (90 and 100% HRmax) and may explain the significant differences in native function at these levels, despite the absence of a detectable difference at lower levels of exercise intensity (50 and 80% HRmax). Testing of intramuscular stimulation during exercise subjects the arytenoid to high inspiratory negative pressures and may be a more strenuous requirement than restoration of abduction in humans and dogs [10,38,44] .

These data also demonstrate prevention of progression of the effects of denervation in the stimulated animals (figure 5) with equivalent function in the stimulated group after the denervation period and after FES. In contrast, the control group demonstrated considerable worsening in native function after the additional denervation period of eight weeks during which the FES+ animals were stimulated. This may be due

to increased resting muscle tone or shortened resting length in the stimulated CAD muscles. An alternative explanation would be additional innervation provided by the cranial laryngeal nerve. Function during stimulation was improved compared to native function and so although a similar trend to prevent progression during stimulated function was observed, this was less marked than the trend in changes in native function.

The prevention of progression identified by evaluating native function was reflected in volumetric changes of the PCA muscle suggesting that FES at these relatively low stimulation levels may reverse the ongoing atrophy produced by denervation. The use of cricoid cartilage volume provided a useful known standard over time. This technique has not been used in other animal models of laryngeal denervation will be used in future experiments evaluating the effects of FES on the rate of reinnervation after nerve injury. These techniques could also be applied in human patients to track the response to electrical stimulation or reinnervation.

The increase in rheobase following denervation is consistent with ongoing denervation and loss of neuromuscular junctions within the first 72 hours after denervation. This algorithm was practical to fit to the data and provides an unbiased method to obtain both chronaxie and rheobase without subjective curve fitting. It is therefore recommended for future studies evaluating the effects of electrical stimulation on reinnervation after nerve injury. The excitability of the PCA muscle improved only slightly with once-daily activation. This demonstrates that there was no evidence of reinnervation of the PCA.

In summary, these data suggest that electrical stimulation using a relatively conservative set of stimulation parameters can reverse the muscle fiber atrophy produced

406 by complete denervation while avoiding a shift to a slow (type 1) fiber type, and
407 improving function under load. This study also describes the use of an effective
408 algorithm to evaluate muscle excitation and the use of muscle reconstruction using
409 computed tomography data sets to longitudinally track the response to muscle
410 stimulation. These approaches will be used in future work to evaluate the effects of more
411 aggressive stimulation parameters, with a shorter interval between training, on denervated
412 muscle and to evaluate the effects of electrical stimulation on reinnervation in a large
413 animal model of nerve injury.

414 Figure Legends

415 Figure 1. Illustration of K5-P4 electrode. Note: 3 anodes each 1.5mm long, screw length
416 2.5 mm with 2.5 turns; screw diameter 1.7 mm; screw surface area 8 mm^2. Screw
417 material is Platinum-Iridium (80%-20%). Total electrode length 300 mm. Insertion tool
418 shown in place (left)

419

420 Figure 2. Quantification of right PCA muscle function using videoendoscopic assessment
421 of arytenoid abduction. Right arytenoid abduction at rest (A); and during stimulation at
422 rest (B). Images are obtained from the same horse at the same time point prior to
423 denervation.

424 Figure 3. PCA response to increasing levels of stimulation at rest. Response determined
425 as abduction of the left arytenoid in response to biphasic pulses with duration 2ms, 50Hz.
426 Data shown are mean and standard deviation.

427

428 Figure 4. Changes in left PCA volume muscle in response to denervation and subsequent
429 stimulation. Data shown are mean and standard deviation. Different letters denote
430 significant differences between groups (Tukey's post hoc test, $p<0.05$).

431

432 Figure 5. PCA response to increasing levels of inspiratory load and increasing exercise
433 intensity (%HRmax). Native function significantly improved at high levels of inspiratory
434 load (90 and 100 HRmax) after FES+ animals compared to controls (*, $p=0.05$ and **,
435 $p<0.01$, linear contrasts).

436

Figure 6. Fiber diameter distribution (%) in the denervated left PCA and LCA muscles compared to contralateral innervated controls (right PCA and LCA). Bin size is 5um. FES was only applied to the left PCA in stimulated animals (solid line). Other muscles served as internal controls and FES was not applied. Dotted lines describe animals in which FES was not applied. Denervation produced a left shift in fiber diameter distribution ($p<0.001$). Within the left PCA muscle, electrical stimulation produced an increase in fiber diameter (*, $p=0.042$).

Figure 7. Proportion of fiber types 1, 2a and 2x within each PCA and LCA muscles.

Figure 8. Representative immunohistochemistry images of instrinsic laryngeal musculature labeled simulataneously for type 1, 2a and 2x fibers. Note increasing collagen V proportion with decreasing fiber diameter.

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